BORRELIA BURGDORFERI (STRAIN *B. AFZELII*) ANTIBODIES AMONG MALAYSIAN BLOOD DONORS AND PATIENTS

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Abstract. In this study, the presence of IgG and IgM antibodies against *Borrelia burgdorferi* (strain *B. afzelii*) among Malaysian blood donors and patients admitted to hospital with various infectious diseases was determined. Sera were screened using enzyme-linked immunosorbent assay (ELISA); positive sera were then subjected to Western blot testing. All but one of the blood donors were negative for borrelial antibodies. Of 121 patients' sera, IgM antibodies were detected in 24 (19.8%) and IgG antibodies were detected in 5 (4.1%) sera. Only one of two patients with skin manifestations suggestive of Lyme disease had IgM antibody against *B. afzelii*. Of 30 patients with exposure to tick typhus, 4 (13.3%) were IgM positive and 1 (3.3%) was IgG positive. Based on the detection of antigenic bands by Western blot, 6 patients' sera showed positive reactions. Antigenic bands of p39, p41 and p59/62 kDa were the commonest findings of Western blotting. This study provides serological evidence of *B. afzelii* infections in Malaysia; further investigation is needed to correlate serological and clinical findings.

INTRODUCTION

Lyme borreliosis (LB) disease, is a tick-borne spirochetal disease endemic to regions of the world's temperate zones (Bennett, 1995). Lyme disease is often said to be associated with 'protean' manifestations (Nadelman and Wormser, 1998). The clinical spectrum of LB varies from cutaneous erythema migrans to severe arthritis, acrodermatitis chronica, and cardiac and neurological manifestations (Steere, 1989). *Borrelia burgdorferi* isolates are divided into three different genospecies of the *B. burgdorferi* sensu lato group: *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii*; on the basis of DNA sequence identity (Welsh *et al*, 1992). The diagnosis of LB is primarily based on clinical findings and may be assisted by the results of serological tests. The use of enzyme-linked immunosorbent assay (ELISA) and Western blot in a two-step testing strategy has gained wide acceptance for Lyme diagnosis. In addition, Western blot is both sensitive and specific and has been recommended as a confirmatory test for the serodiagnosis of LB in the United States (CDC, 1995).

Lyme disease is endemic in Japan (Kawabata *et al*, 1987), and had been reported in other parts of Asia, including China (Chengxu *et al*, 1988), Taiwan (Shih *et al*, 1998) and Korea (Kim *et al*, 1999). However, no seropostive cases were reported in patients presenting with annular erythema in Singapore (Goh *et al*, 1996) and patients presenting with mono/oligoarticular disease of unknown etiology in India (Handa *et al*, 1999). The knowledge of the epidemiological features of borreliosis is limited in the tropics. The reports of 2 patients that presented with the symptoms and seropositivity for *B. burgdorferi* after tick bites in South Africa and Southeast Asia suggested the presence of LB in tropical and subtropical areas (Stanek *et al*, 1986). It is not known whether LB exists in Malaysia. There are no published reports of any likely symptoms or the severity, incidence, or prevalence of any suspected cases. The aim of this study was to determine the serological status of *B. burgdorferi* among Malaysian blood donors

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and patients.

MATERIALS AND METHODS

Human sera

A total of 183 human sera were screened for borrelial antibodies using ELISA. These sera include: (a) those of 30 blood donors (obtained from the Blood Bank, Kuala Lumpur Hospital in 1999; (b) those of 121 patients from various regions of Malaysia (1999-2001), obtained from the Institute for Medical Research, Kuala Lumpur. Patients were clinically diagnosed as having either leptospirosis or melioidosis or rickettsial infections at the time of admission; (c) those of 2 patients with erythema migrans; (d) those of 30 patients with exposure to tick typhus. IgM antibodies against TT118 spotted fever group (SFG) rickettsiae of ≥ 1:50 were detected by indirect immunoperoxidase assay.

ELISA

The IgG and IgM ELISAs were performed according to the manufacturer's instructions (Biotest, Germany). Complete antigens were prepared using a strain of *B. burgdorferi* sensu lato group (*B. afzelii*), which was grown in Kelly medium and purified by ultrasonication and centrifugation. The ELISA cut-off value was calculated from the absorbance of negative control and the absorbance of the cut-off control. Patients' sera were interpreted and quantitated by means of the calculation of the OD ratio (OD value of sample/cut-off value). Sera with ratios of >1.100 were considered as positive whereas sera with ratios of 0.900-1.100 were deemed inconclusive.

Western blot

The IgG and IgM BIOTEST-BLOTs (Biotest, Germany) were used according to the manufacturer's instructions. Complete antigens of *B. afzelii* were treated with sodium dodecyl sulphate and electrophoretically separated in polyacrylamide gel according to the molecular weight of proteins. With the aid of a kit-specific evaluation diagram, the band pattern was assessed. At least one clear antigen band from a group of highly specific borrelial proteins (p100, p59/62, p41, p39, p35, p25, p20 kDa) is required on the Western blot strip for an IgM positive reaction. At least 3 clear antigen bands from a group of highly specific borrelial proteins (p100, p59/62, p41, p39, p35, p25, p20 kDa) are required on the Western blot strip for a IgG positive reaction.

RESULTS

All blood donors were negative for borrelial IgG and IgM except one, in whom IgM was detected. Table 1 shows the detection of IgM and IgG antibodies among patients with vari-

Table 1
Seroreactivities of Malaysian patients against *B. burgdorferi* (strain *B. afzelii*) as determined by IgM and IgG ELISA.

Clinical diagnosis	No. tested	No. (%) sera with IgM		No. (%) sera with IgG	
		Positive	Inconclusive	Positive	Inconclusive
Leptospirosis	50	12(24.0)	4(8.0)	2(4.0)	0(0.0)
Melioidosis	40	4(10.0)	2(5.0)	3(7.5)	2(5.0)
Rickettsial infections	31	7(25.8)	2(6.5)	0(0.0)	1(3.2)
Tick typhus	30	4(13.3)	3(10.0)	1(3.3)	1(3.3)
Lyme disease	2	1(50.0)	1(50.0)	0(0.0)	0(0.0)
Total	153	28(16.3)	12(6.5)	6(3.3)	4(2.2)

Inconclusive: ratios of 0.900-1.100; Positive: ratios of >1.100

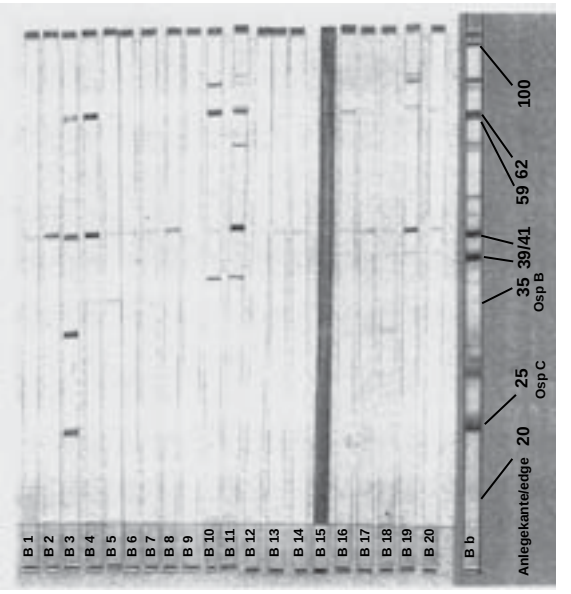

Fig 1–Western blot analysis of patients' sera with positive IgM ELISA. With the aid of a kit-specific evaluation diagram (Bb) the band pattern was read. B1-B19: patients' sera; B20: serum from a blood donor. B3, B4, B10, B11, B15 and B19 were interpreted as positive based on the guidelines provided by the manufacturer.

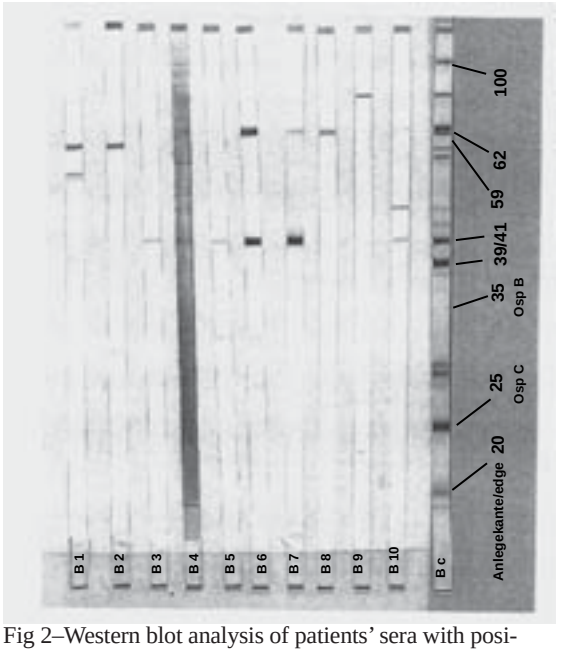

Fig 2–Western blot analysis of patients' sera with positive IgG ELISA. With the aid of a kit-specific evaluation diagram (Bc) the band pattern was read. None of the sera was interpreted as positive based on the guidelines provided by the manufacturer.

Table 2
B. afzelii proteins that were reactive to the human sera upon testing by Western blot.

Borrelial proteins	No. serum with IgM	No. serum with IgG
P20	1	1
P25	5	1
P35	7	1
P39	10	4
P41	20	8
P59/62	14	6
P100	1	0
No. sera tested	20	10

ous clinical presentations. Of the 121 patients' sera, IgM antibodies were detected in 24 (19.8%) and IgG antibodies were detected in 5 (4.1%). The presence of both IgM and IgG antibodies against *B. afzelii* was only detected in a patient's serum. Only one of the two patients with erythema migrans had IgM antibodies against *B. afzelii*. Of the 30 patients with exposure to tick typhus, 4 (13.3%) were IgM positive and 1 (3.3%) was IgG positive. IgM and IgG antibodies with ELISA ratios of 0.900-1.100 were detected in 12 and 4 patients' sera respectively.

Sera with positive IgM and IgG ELISA findings were tested by Western blot. Figs 1 and 2 show the reactivities of some patients' sera against borrelial antigens and the results are summarized in Table 2. The three most common antigen bands observed were p39kDa, p41 kDa and p59/62 double bands, for both IgG and IgM Western blots. Other antigenic bands included p20, p25, p35 and p100 kDa. Based on the guidelines provided by the manufacturer, the sera of 6 patients were interpreted as IgM positive by the Western blot. The clinical presentations of these patients were shown in Table 3. Sera of the blood donor and patient with erythema migrans although positive by IgM ELISA, were negative upon testing with Western blot (not shown). None of the IgG sera tested was positive by Western blot (Fig 2).

Table 3
Clinical summary of patients with positive Western blot reactions.

No.	Age	Race	Sex	ELISA ratio		Clinical summary	Other laboratory findings
				IgM	IgG		
1 (B3)	33	Indian	Female	2.924	0.452	Fever for 9 days, headache, arthralgia, myalgia	[a]Leptospiral IgM antibody negative
2 (B4)	22	Indonesian	Male	5.737	0.421	Febrile illness	Leptospiral IgM antibody positive
3 (B10)	23	Malay	Male	2.937	0.454	Fever, arthralgia, myalgia for 1 month	Leptospiral IgM antibody negative
4 (B11)	31	Indonesian	Male	3.878	0.239	Fever and chills	Leptospiral IgM antibody positive
5 (B15)	42	Aborigine	Male	4.119	0.411	Remittent fever for 10 days	[b]Antibody (IgG and IgM) against *Orientia tsutsugamushi, Rickettsia typhi* and TT118 SFG rickettsiae: < 1:50
6 (B19)	Adult	Malay	Male	1.300	0.471	Febrile illness	Antibody (IgG and IgM) against *O. tsutsugamushi* and *R. typhi*: <1:50. Antibody against TT118 SFG rickettsiae: IgG < 1:50, IgM 1:400

Notes: Parentheses indicate the label on the Western blot strips (Fig 1)
[a]The presence of leptospiral IgM antibodies was determined by a commercial IgM ELISA assay (PanBio, Australia).
[b]The presence of IgM and IgG antibodies against *O. tsutsugamushi, R. typhi* and TT118 SFG rickettsiae were determined by indirect immunoperoxidase assay. An antibody titer of ≥ 1:50 was considered as positive.

DISCUSSION

As with other spirochetal illnesses, LB occurs in stages, with remissions, relapses, and different clinical manifestations, during each stage. Stage 1 generally lasts for several weeks and consists of erythema migrans and symptoms generally suggestive of infection. The diagnosis of early illness is difficult as only 41-50% patients may develop erythema migrans and the dermatological manifestations may pass unnoticed (Guy *et al*, 1989). Stage 1 is also associated with severe headache, fever, and fatigue, which usually resolve spontaneously within 5-21 days (Feder *et al*, 1993). Other than a typical erythema migrans and a nonspecific flu-like illness of early infections, patients may present without any signs and symptoms (Guy *et al*, 1989). Stage 2 occurs during the following several months. During stage 2, meningitis, carditis, or migratory musculoskeletal pains are the predominant symptoms. Stage 3 follows months to years after the onset of infection and includes chronic joint, neurologic or skin involvement. This stage of the illness may mimic several immune-mediated diseases of unknown etiology, including rheumatoid arthritis and multiple sclerosis (Steere *et al*, 1986). The prevalence of LB in Malaysia has not been established and there are no clinical reports of LB in Malaysia.

The antibody responses to three genomic groups of *B. burgdorferi* (*B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*) by ELISA are similar (Dressler *et al*, 1993). As the infecting strain of LB is not known in the local population, *B. afzelii* was used as the antigen for detection of borrelial antibodies in this study. ELISA screening of human sera in this study showed that a high number of patients had IgM antibodies against *B. afzelii* compared with blood donors. As studies have shown that ELISA tests may show cross-reactions with antibodies produced in response to other organisms, including oral spirochetes, and *Treponema pallidum*, and to heat-shock proteins, which are widely conserved in many species (Baker-Zander and Lukehart, 1984; Magnarelli *et al*, 1990), Western blot was used to increase the specificity of the diagnosis. Five patients with IgM antibodies showed positive reactions with the highly specific proteins of *B. afzelii* (Fig 1). Patients with exposure to tick typhus and thus experience of tick bites were also examined for their exposure to borrelial infection. Of 30 patients with exposure to tick typhus, 4 (13.3%) were IgM positive and 1 (3.3%) was IgG positive. IgM positive reactions were demonstrated in a patient's serum upon testing by Western blot B19 (Fig 1). All these findings provide serological evidence that borrelial infections are present in Malaysia. The detection of borrelial antibodies among those patients with exposure to tick typhus suggests that co-infections and mixed infections of tick typhus and LB may occur.

Of the 6 patients found to be positive by Western blots, none had IgG antibodies detected (ELISA ratio <0.500, Table 3): all had fever at the time of diagnosis and two suffered from arthralgia and myalgia (Table 3). Erythema migrans was not reported in any of the cases. Four patients were suspected of having leptospirosis and two were suspected of having rickettsial infections. Two patients had IgM antibodies to leptospirosis and one had IgM antibodies to SFG rickettsiae. The correlation of clinical and serological findings is important when making an accurate diagnosis of LB; however, it seems impossible to draw any conclusions based on the limited clinical history of the patients in this study (Table 3).

As cross-reaction among species of *Borrelia* cannot be distinguished serologically (Banerjee *et al*, 1992), there is a possibility that patients may have been infected with species other than *B. burgdorferi*. Tick-borne relapsing fever (caused by spirochetes of the genus *Borrelia*) usually presents with the sudden onset of a fever that lasts for about three days, followed by an afebrile period of variable duration and then a sudden return of fever, often with arthralgia (Barbour *et al*, 1982). The infection also causes aseptic meningitis, myocarditis, peripheral neuritis, ophthalmic involvement, coagulopathy, and neonatal death (Banerjee *et al*, 1992). The disease is most probably con-

fined to parts of Africa, Asia, and the Americas (Stanek, 1995). The incidence of tick-borne relapsing fever in Malaysia is unknown.

In this study, immunogenic proteins of *B. afzelii* reactive to sera of local patients were identified. The flagellin protein (41kDa) has been shown to be one of the first proteins to which antibodies are directed after infection with *B. burgdorferi* and is significant for the IgM immunoblot (Engstrom *et al*, 1995). All the sera tested were seroreactive with the 41 kDa protein, although only six showed an intense reaction (Fig 1). The p59/62 kDa protein is the heat-shock protein of *B. afzelii:* this protein reacted with various intensities with 14 sera. The 39 kDa protein, which reacted with 10 IgM positive sera in this study, was found to be the most significant marker for LB: approximately 50% of serum samples from patients with early disease reacted with this protein (Ma *et al*, 1992).

The interpretation of Western blot has been difficult, due to the subjective reading of band strength and differences in the immune response to the various clinical presentations of LB (Dressler *et al*, 1993; Engstrom *et al*, 1995). In a retrospective analysis of 225 cases and control subjects in the United States, Dressler *et al* (1993) suggested that optimum discrimination using Western blot could be obtained by finding at least 2 of the 8 most common IgM bands in early disease (18, 21, 28, 37, 41, 45, 58 and 93 kDa) and by finding at least 5 of the 10 most common IgG bands after the first weeks of infection (18, 21, 28, 30, 39, 41, 45, 58, 66 and 93 kDa). However, this criterion may be different in various geographical regions, due to different species and strains of *B. burgdorferi* and heterogeneity within these strains (Dressler *et al*, 1993; Engstrom *et al*, 1995). Variations in antigen composition among these spirochetes may affect the assay of serum antibodies (Magnarelli *et al*, 1994).

It has been suggested that early antibiotic treatment may abort the antibody response in patients treated for *B. burgdorferi* (Shrestha *et al*, 1985). Only six of 20 sera were positive by Western blot in this study. It may be necessary to look at sequential specimens, especially for the sera that were positive by ELISA. As different species of pathogenic *B. burgdorferi* produce different symptoms (Baranton *et al*, 2001), further work is required to identify the local species and strains of *B. burgdorferi* and to determine their prevalence and presentation among local populations.

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