ANTIGENIC TYPES OF *ORIENTIA TSUTSUGAMUSHI* IN MALAYSIA

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Abstract. The seroprevalence of various *Orientia tsutsugamushi* (OT) strains among Malaysian patients with suspected scrub typhus infections was determined using an IIP assay. IgG against a single OT strain were detected in six sera (3 Karp, 1 Gilliam and 2 TC586-2), whereas IgM antibodies against a single OT strain were noted in 3 sera (3 Gilliam). IgG reactive to all OT strains were present in 33 (47.1%) of the 70 sera and IgM reactive to all OT strains were present in 22 (78.6%) of the 28 sera. The fact that most sera were reactive to multiple OT strains suggests that group-specific antigens are involved in scrub typhus infections, whereas very few were due to strain-specific epitopes present on these strains. Peak IgG and IgM titers were noted more frequently against Gilliam, Karp, and TA763 strains: this suggests that these strains may be the commonest infecting strains among Malaysian patients. Two predominant OT polypeptides consistently reacted with patients' sera: the 70 kDa and 56 kDa proteins.

INTRODUCTION

Karp, Kato, and Gilliam strains have been used as a set of standard strains for the clinical diagnosis and experimental study of scrub typhus. However, many isolated *Orientia tsutsugamushi* (OT) strains, which are distinguishable from prototype strains both in antigenicity and in virulence, have been reported in various parts of Asia (Shirai *et al*, 1982; Tamura *et al*, 1997; Seong *et al*, 1997). In Southeast Asia, Elisberg *et al* (1978) reported the isolation of five OT strains, *ie*, TA 678, TA686, TA716, TA763, and TH1817. Using various Southeast Asian OT strains for serological classification, Shirai *et al*. (1979) reported the presence of 29 antigenic types in 114 isolates of OT strains recovered from febrile patients in rural areas. However, detailed characteristics of the antigenic heterogeneity and the relationship between antigenic diversity and the degree of virulence of the organism remain to be determined.

The variability of human responses to OT strains and their geographical distribution in Malaysia is not well understood. A particular serotype from one region may be rare or nonexistant in another area. As the isolation of a significant number of strains from human cases is cumbersome (Brown *et al*, 1983; Chouriyagune *et al*, 1992), it was decided to investigate the seroreactivity of local patients' sera to various OT strains. In those cases where the infecting strain was known, the homologous antibody appeared more rapidly, attained a higher titer, and persisted for much longer (Robinson *et al*, 1976). The findings from this study may reflect the seroprevalence of the various OT strains that infect the local population.

The objective of this study was to determine the prevalence of the various OT strains commonly found in Southeast Asia and among Malaysian patients with suspected scrub typhus infection. The constitutional proteins of these OT strains were compared by sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting techniques.

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MATERIALS AND METHODS

Preparation of OT antigens for indirect immunoperoxidase assay

Individual whole cell OT antigens, *ie*, Karp, Kato, Gilliam, TA678, TA686, TA716, TA763, TH1817, and TC586 strains, were grown in embryonated hen eggs; infected yolk sacs were diluted to a 20% (w/v) suspension with 0.01 M phosphate buffered saline (PBS), pH 7.3, to yield approximately 1,000 organisms per 400x microscopic field. Individual antigens were dotted onto teflon-coated slides using a pen-nib filled with antigen. The slides were then air-dried at room temperature for at least 30 minutes before being fixed in acetone at 4ºC for 10 minutes. The slides were then allowed to air-dry at room temperature and stored at -20ºC prior to use.

Indirect immunoperoxidase (IIP) assay

The IIP ssays were performed as described by Kelly *et al* (1988) in order to determine the specific IgG and IgM antibody levels against OT strains. Ten microliters of serially diluted patients' sera (from 1:50 to 1:1,600) were first placed on the antigen spots and the slides were then incubated at 37ºC for 30 minutes in a moist incubator chamber. After washing with PBS (3 changes of 5 minutes each), peroxidase conjugated anti-human IgG (HRP-IgG, Dakopatt) or anti-human IgM (HRP-IgM, Dakopatt), diluted 1:100, was allowed to react with the antigen spots at 37ºC for 30 minutes. After a second washing, the slides were treated with the substrate solution [3,3-diaminobenzidine tetrahydrochloride (DAB)] in the dark at room temperature for 10 minutes. Finally, the slides were faintly stained with dilute methylene blue solution for 5 seconds. After the final washing, air-dried slides were mounted with Permount mounting medium (Fisher, USA) for examination by ordinary light microscopy at 400x magnification. Positive reactions in the IIP assay were indicated by the finding of brownish-stained rickettsiae in the respective antigenic smear, whereas no organisms could be seen in negative reaction. The reciprocal of the highest serum dilution with positive reaction is expressed as the IIP antibody titer.

Comparison of constitutional proteins by SDS-PAGE and immunoblotting

For preparation of antigenic components for SDS-PAGE and immunoblotting analysis, various OT strains, *ie*, Karp, Kato, Gilliam, TA678, TA686, TA716, TA763, TH1817, and TC586 strains, were proliferated in embryonated hen eggs and purified as described by Eisemann and Osterman (1976). Briefly, 20% (w/v) of infected yolk sac suspension in PBS was subjected to centrifugation at 40,000*g* for 1 hour in a discontinuous 50% (w/v) sucrose gradient using a SW28 rotor (Beckman, USA). The pellet was resuspended in brain heart infusion broth and recentrifuged at 12,000*g* for another hour. The pellet was then resuspended in PBS, pH 7.3. SDS-PAGE was performed as described by Laemmli (1970) using 10% polyacrylamide gels in a vertical slab gel apparatus. Immunoblotting was accomplished by the techniques of Towbin *et al* (1979) using a EC140 Mini Blot Module according to the manufacturer's instruction.

Patients' sera

For determining the prevalence of each OT strain by IIP assay, the sera of 84 patients admitted to Kuala Lumpur Hospital (1998-1999) with a clinical history suggestive of scrub typhus infection were used. Seventy of the sera were reactive to a mixture of Karp, Kato and Gilliam antigens (IIP antibody titers of ≥ 1:400) and 14 were negative (IIP antibody titers of (1:50). For immunoblotting studies, individual patients' sera with IIP titers (both IgG and IgM) of ≥ 1:1,600 were used. A pooled positive serum was prepared by mixing several patients' sera with high IIP antibody titers (≥ 1:1,600). A pooled negative serum was prepared by mixing several patients' sera with negative IIP antibody titers.

RESULTS

There was no seroreactivity with any of the OT strains when 14 negative sera were used

Table 1
Seroreactivity of various OT strains with sera of Malaysian patients: IIP assay.

O. tsutsugamushi strain	No. (%) positive sera	
	IgG	IgM
Karp	3(4.3)	
Gilliam	1(1.4)	3(10.7)
TC586	2(2.9)	
Karp, Gilliam	7(10.0)	2(7.1)
Karp, Kato	1(1.4)	
Gilliam, TA763	1(1.4)	
Gillliam, TC586	3(4.3)	
Karp, Gilliam, TC586	5(7.1)	
Karp, Kato, Gilliam	5(7.1)	
Karp, Kato, Gilliam, TC586	4(5.7)	
Karp, Kato, Gilliam, TA763, TA716	1(1.4)	
Karp, Kato, TA763, TA678, TA716	1(1.4)	
Karp, Kato, Gilliam, TA763, TA716	1(1.4)	
Karp, Gilliam, TA763, TA678, TH1817	1(1.4)	
Karp, Kato, TA763, TA686, TA716, TH1817	1(1.4)	
Karp, Kato, Gilliam, TA763, TA678, TA716, TH1817	1(1.4)	
Karp, Kato, Gilliam, TA763, TA716, TA678, TA686, TH1817, TC586	33(47.1)	22(78.6)
Total number of sera tested	70	28

Table 2
IgG and IgM seroreactivities of various OT strains with sera from Malaysian patients.

O. tsutsugamushi strain	No. (%) positive sera		No. positive sera with highest IIP antibody titer	
	IgG	IgM	IgG	IgM
Karp	63 (90.0)	25 (89.3)	23	11
Gilliam	62 (88.6)	28 (100)	36	23
TA763	38 (54.3)	23 (82.1)	19	13
TC586	47 (67.1)	22 (78.6)	9	0
TH1817	35 (50.0)	23 (82.1)	8	4
Kato	48 (68.6)	22 (78.6)	4	3
TA716	38 (54.3)	22 (78.6)	4	0
TA686	34 (48.6)	22 (78.6)	3	1
TA678	35 (50.0)	23 (82.1)	3	1
Total	70	28		

in the IIP assay. Table 1 shows the seroreactivity of OT strains to the positive sera. All had IgG antibodies, of which 33 (47.1%) sera had IgG reactive to all the OT strains (range of antiboy titer: 1:50 to 1:25,600). Twenty-eight sera had IgM against mixtures of OT strains (Karp, Kato and Gilliam), of which 22 (78.6%) had IgM antibodies reactive to all the OT strains (range of titer: 1:50 to 1:25,600). IgG against a single OT strain were noted in 3 (Karp), 1 (Gilliam), and 2 (TC586) sera. IgM against a single OT strain (Gilliam) was detected in 3 patients' sera.

The IgG and IgM seroreactivities of various OT strains with positive patients' sera are shown in Table 2. IgG antibodies to Karp, Gilliam,

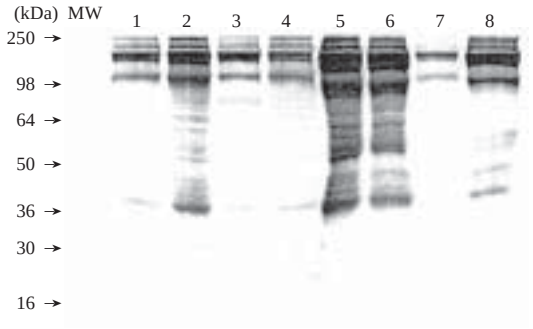

Fig 1–Protein profiles of whole cell lysates of OT strains on 10% polyacrylamide gel stained with Coomassie blue. Lanes: 1, Karp; 2, Kato; 3, Gilliam; 4, TH1817; 5, TA763; 6, TA716; 7, TA686; 8, TA678. The positions of molecular weight standards (in kilodaltons) are indicated on the left.

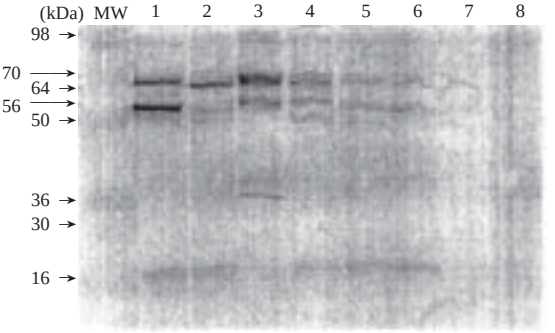

Fig 2b–Immunoblotting profiles of whole cell lysates of OT strains with IgM of a pooled positive patient serum. Lanes: 1, Karp; 2, Kato; 3, Gilliam; 4, TH1817; 5, TA763; 6, TA716; 7, TA686; 8, TA678. The positions of molecular weight standards (in kilodaltons) are indicated on the left.

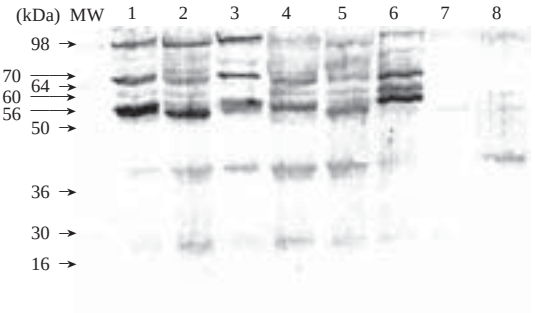

Fig 2a–Immunoblotting profiles of whole cell lysates of OT strains with IgG of a pooled positive serum. Lanes: 1, Karp; 2, Kato; 3, Gilliam; 4, TH1817; 5, TA763; 6, TA716; 7, TA686; 8, TA678. The positions of molecular weight standards (in kilodaltons) are indicated on the left.

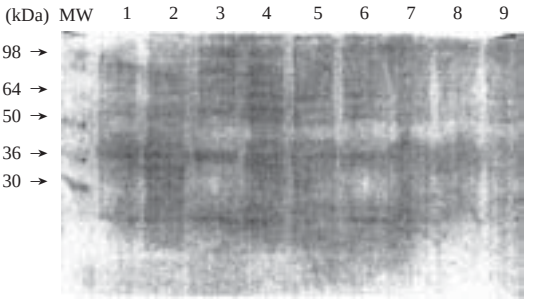

Fig 3–Immunoblotting profiles with IgG and IgM antibodies of a pooled negative serum. Lanes: 1, Karp; 2, Kato; 3, Gilliam; 4, TH1817; 5, TA763; 6, TA716; 7, TA686; 8, TA678; 9, TC586. The positions of molecular weight standards (in kilodaltons) are indicated on the left.

and Kato strains were the most prevalent, whereas IgM antibodies were most prevalent to Gilliam, Karp, TH1817, TA763 and TA678 strains. Peak IgG titers were noted to be reactive to Gilliam (36 sera), Karp (23 sera), and TA763 (19 sera) strains, while peak IgM antibody titers were reactive to Gilliam (23 sera), TA763 (13 sera), and Karp (11 sera) strains.

SDS-PAGE analysis (Fig 1) showed that all the OT strains had very similar protein profiles. However, some difference in the content of polypeptides was observed. The major proteins of the OT strains were polypeptides with a molecular weight > 100 kDa and 98 kDa. The antigenic properties of the polypeptides were analyzed by immunoblotting using pooled and individual patients' sera. The use of the pooled positive and negative sera in this study was to overcome the variation of immune response among individuals, thereby giving a more authentic picture of the humoral response of patients suffering from scrub typhus. Figs 2a and 2b show the intense immune responses

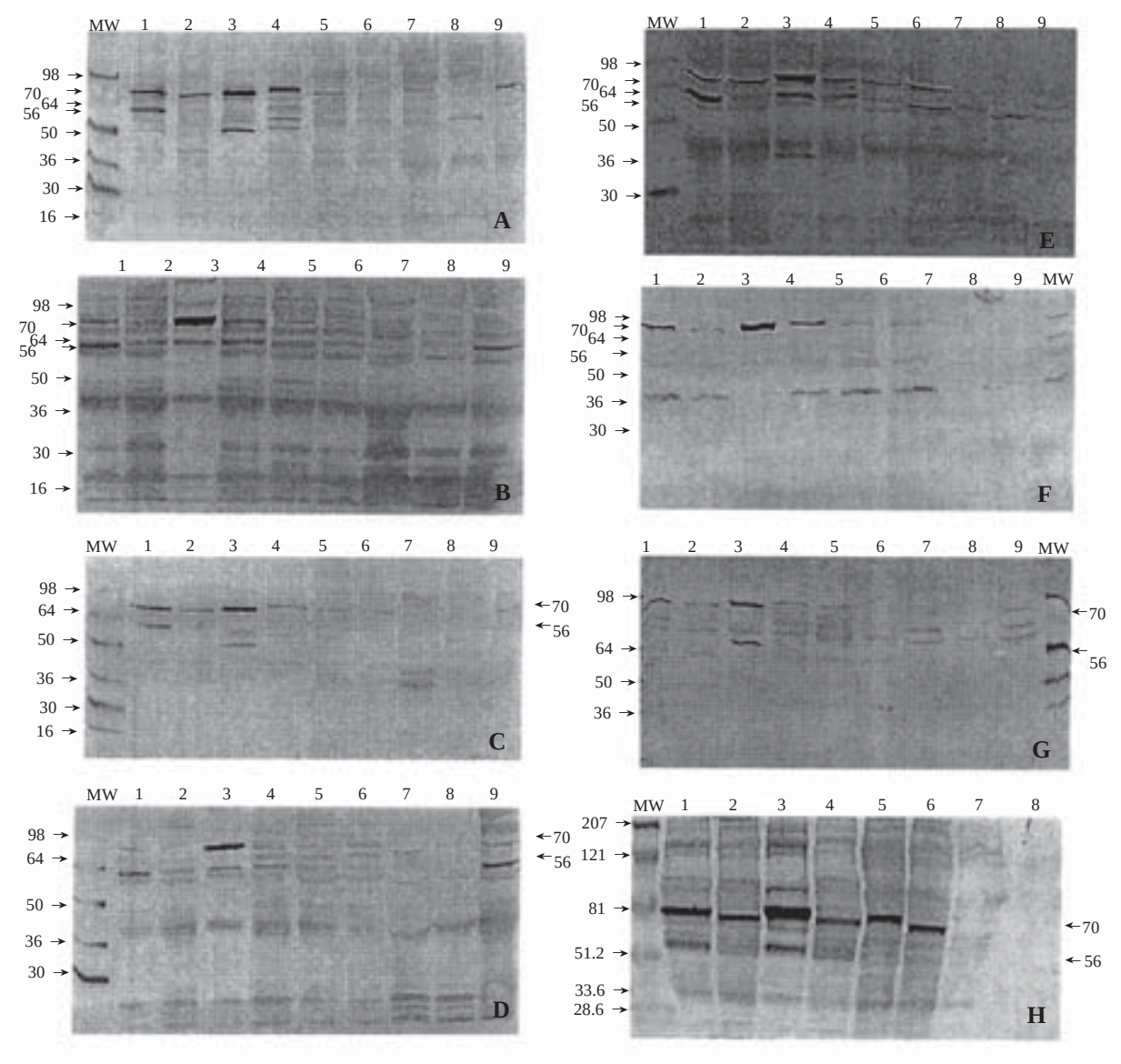

Fig 4–Immunoblotting profiles of whole cell lysates of OT strains with IgG and IgM of 4 patients' sera A, B, C, D are IgG profiles whereas E, F, G, H are IgM profiles. Lanes: 1, Karp; 2, Kato; 3, Gilliam; 4, TH1817; 5, TA763; 6, TA716; 7, TA686; 8, TA678; 9, TC586 . The positions of molecular weight standards (in kilodaltons) are indicated on the left.

of the IgG and IgM antibodies in a pooled positive serum to polypeptides of the OT strain. A clear reaction with the 56 kDa and the 70 kDa polypeptides was noted in both IgG and IgM immunoblots. The IgG and IgM immunoblotting profiles of various OT strains were almost identical, but the intensities of TA686 and TA678 strains were weaker. There was no seroreactivity observed with these polypeptides when the negative pooled serum was used (Fig 3). IgG and IgM immunoblot analysis with individual patients' sera revealed multiple polypeptides that were immunogenic to patients' sera. Polypeptides of the Karp, Kato, and Gilliam strains demonstrated stronger immune responses than the other OT strains.

All the 10 patients' sera reacted with the 56 and 70 kDa OT polypeptides with various intensities; other polypeptides (46, 50 and 60 kDa) were reactive with some but not all of the sera. Fig 4 shows the IgG and IgM immunoblots of 4 individual patients' sera.

DISCUSSION

In this study, most of the sera reacted with more than one, if not all, of the OT strains. The fact that most sera were reactive to multiple strains, suggests that group-specific antigens are involved in scrub typhus infections; very few were due to strain-specific epitopes present on the strains. This finding was also demonstrated by the immunoblotting of the OT polypeptides.

As the presence of IgM is indicative of recent or current infection, and given the fact that the infecting strain induces homologous antibody which appears more rapidly and attains a higher titer, the OT strains that showed the highest IgM antibody titers among others in serum specimens may be regarded as the current infecting strain. In this study, peak IgM titers were noted more frequently against Gilliam, Karp, and TA763 strains (Table 2), suggesting that these strains may be the commonest OT strains infecting the local population.

Multiple strains of OT and the antigenic heterogeneity of scrub typhus are of major concern in the serodiagnosis of, and development of a vaccine for, this disease (Traub and Weisseman, 1968). One of the requirements of an effective serodiagnostic reagent is the identification of the most prominent antigenic and immunogenic strains associated with the relevant human disease. In addition, knowledge of the antigenic types of organisms present in an endemic area is a better guide to the selection of OT strains for use in a vaccine: this is because the protection produced in animals or man either by infection or vaccination appears to be short lived (Eisenberg and Osterman, 1978). The findings in this study suggest that Gilliam, Karp, and TA763 strains should be given a higher priority for inclusion in any candidate scrub typhus vaccine or serodiagnostic reagent that is to be used in this region.

Shirai *et al* (1979, 1981) demonstrated that Karp and its related strains (TA763, TA716, TA686) were the predominant strains in humans and in chiggers in Peninsular Malaysia, whereas Gilliam strain was not common and tended to exist alone. The prevalence of OT strains among patients with scrub typhus was dependent on the endemicity of the vectors and rodents. It is not known whether the high seroprevalence of Gilliam strain noted in this study is a recent development or due to other factors. Similarly, although previous studies (Shirai *et al*, 1979; 1980; 1981) showed no cross-reactivity of the local OT strains with TA678 strain and little cross-reactivity with the Kato strains, in this study, seroreactivities of patients' sera with TA678 and Kato strains were demonstrated by IIP assay (Table 1 and Table 2) for the first time. As there might be different epidemiological characteristics, such as vector species and locality, the type of chiggers harboring these rickettsiae should be identified in order to prevent and control scrub typhus.

Eisemann and Osterman (1981) were the first to demonstrate that several components of the rickettsial surface are antigenically reactive with antibodies against homologous or heterologous strains of OT. Comparative immunoblotting analyses of OT variants from Japan with guinea pig hyperimmune sera (Tamura *et al*, 1985) and strain-specific monoclonal antibodies (Murata *et al*, 1986) demonstrated that the 56 kDa major polypeptide on the rickettsial surface reacts strongly with homologous antiserum, but faintly or moderately with heterologous antiserum. The findings suggested that this polypeptide has strain- or type-specific antigenicity. However, some of the monoclonal antibodies that were reactive with the 56 kDa polypeptide also showed cross-reactivity with heterologous strains of OT (Hanson, 1985), indicating that the 56 kDa polypeptide epitopes are both strain-specific and common to several different strains. In this study, using individual patients' serum, a considerable degree of cross-reactivity of various OT strains was noted with the 56 kDa polypeptide during immunoblotting.

However, due to the unavailability of monoclonal antibodies specific to each OT strain, the strain specificity of the 56 kDa of OT strain was not determined. All sera also reacted with the 70 kDa polypeptide; and some reacted with the 46 kDa polypeptide, suggesting that both these polypeptides have the characteristics of group- or species-specific antigenicity, as reported by others (Tamura *et al*, 1985; Murata *et al*, 1986).

In the past, it was not known whether the antigenic combinations that reacted with two or more OT strains comprised a single organism with a mosaic of antigenic determinants or a mixture of organisms (Shirai *et al*, 1979). The findings in this study showed that this could be due to a close antigenic relationship among OT strains. However, the possibility of patients being infected by a mixture of scrub typhus strains in an endemic area, such as Malaysia, can not be denied. Shirai *et al* (1981) reported that individual chiggers can be simultaneously infected with more than one antigenic strain of OT, with as few as one and as many as six antigens detected in individual chiggers; these infected chiggers can transmit their multiple infections to a host. Simultaneous infection with multiple OT strains therefore can occur in humans (Shirai *et al*, 1979; 1980) in highly endemic areas. Exposures to more than one episode of scrub typhus infection in some patients may also complicate the immunological responses of people from endemic areas. This study identified two major OT proteins, which may be the main epitopes that elicit humoral responses in the local population. It has yet to be determined whether the major antigenic proteins identified in this study have any role in protection against scrub typhus infections.

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REFERENCES

Brown GW, Shirai A, Rogers C, Groves MG. Diagnostic criteria for scrub typhus : probability values for immunofluorescent antibody and Proteus OX-K agglutinin titers. *Am J Trop Med Hyg* 1983; 23: 1101-7.

Chouriyagune C, Watt G, Strickman D, Jinasen R. The Weil-Felix test for the diagnosis of scrub typhus in Thailand. *Intern Med* (Thailand) 1992; 8: 29-33.

Eisemann CS, Osterman JV. Proteins of typhus and spotted fever group rickettsiae. *Infect Immun* 1976; 14: 155-62.

Eisemann CS, Osterman JV. Antigens of scrub typhus rickettsiae: separation by polyacrylamide gel electrophoresis and identification by enzyme-linked immunosorbent assay. *Infect Immun* 1981; 32: 525-33.

Eisenberg GH Jr, Osterman JV. Gamma-irradiated scrub typhus immunogens: development and duration of immunity. *Infect Immun* 1978; 22: 80-6.

Elisberg BL, Needy CF, Bozeman FM. Antigenic inter-relationships among strains of *Rickettsia tsutsugamushi.* In: Kazar J, Ormsbee RA, Tarasevich IN, eds. Rickettsiae and rickettsial diseases. Bratislava: VEDA Publ House, 1978: 253-62.

Hanson B. Identification and partial characterization of *Rickettsia tsutsugamushi* major protein immunogens. *Infect Immun* 1985; 50: 603-9.

Kelly DJ, Wong PW, Gan E, Lewis GE Jr. Comparative evaluation of the indirect immunoperoxidase test for the serodiagnosis of rickettsial disease. *Am J Trop Med Hyg* 1988; 38: 400-6.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227: 680-5.

Murata M, Yoshida Y, *et al*. Production and characterization of monoclonal strain-specific antibodies against prototype strains of *Rickettsia tsutsugamushi*. *Microbiol Immunol* 1986; 30: 599-610.

Robinson DM, Brown G, Gan E, Huxsoll DL. Adaptation of a microimmunofluorescence test to the study of human *Rickettsia tsutsugamushi* antibody. *Am J Trop Med Hyg* 1976; 25: 900-5.

Seong SY, Park SG, Kim HR, *et al*. Isolation of a new *Orientia tsutsugamushi* serotype. *Microbiol Immunol* 1997; 41: 437-43.

Shirai A, Robinson DM, Brown GW, Gan E, Huxsoll DL. Antigenic analysis by direct immunofluorecence of 114 isolates of *Rickettsia tsutsugamushi* recovered from febrile patients in rural Malaysia. *Jpn J Med Sci Biol* 1979; 32: 337-44.

Shirai A, Dohany AL, Gan E, Chan TC, Huxsoll DL. Antigenic classification of *Rickettsia tsutsugamushi* isolates from small mammals trapped in developing oil palm complex in Peninsular Malaysia. *Jpn J Med Sci Biol* 1980; 33: 231-4.

Shirai A, Dohany AL, Ram S, Chiang GL, Huxsoll DL. Serological classification of *Rickettsia tsutsugamushi* organisms found in chiggers (Acarina: Trombiculidae) collected in Peninsular Malaysia. *Trans R Soc Trop Med Hyg* 1981; 75: 580-2.

Shirai A, Coolbaugh JC, Gan E, Chan TC, Huxsoll DL, Groves MG. Serological analysis of scrub typhus isolated from the Pescadores and Philippine Islands. *Jpn J Med Sci Biol* 1982; 35: 255-9.

Tamura A, Ohashi N, Urakami H, Takahashi K, Oyanagi M. Analysis of polypeptide composition and antigenic components of *Rickettsia tsutsugamushi* by polyacrylamide gel electrophoresis and immunoblotting. *Infect Immun* 1985; 48: 671-5.

Tamura A, Ohashi N, Koyama Y, *et al.* Characterization of *Orientia tsutsugamushi* isolated in Taiwan by immunofluorescence and restriction fragment length polymorphism analyses. *FEMS Microbiol Lett* 1997; 150: 225-31.

Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979; 76: 4350-4.

Traub R, Weisseman CL Jr. Ecological considerations in scrub typhus. 2. Vector species. *Bull WHO* 1968; 39: 219-30.