

RESEARCH NOTE

THE USE OF THE INDIRECT IMMUNOPEROXIDASE TEST FOR THE SERODIAGNOSIS OF RICKETTSIAL DISEASES IN MALAYSIA

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Abstract. The indirect immunoperoxidase (IIP) test has been used extensively in most government hospitals in Malaysia for the serodiagnosis of scrub typhus, murine typhus and tick typhus during the 1990s. The test was used to determine the IgG and IgM antibody titers in patients' sera for three rickettsial species, *ie* *Orientia tsutsugamushi* OT; the causative agent of scrub typhus), *Rickettsia typhi* (RT; the causative agent of murine typhus), and TT118 spotted fever group rickettsiae (TT; the causative agent of tick typhus). The serological findings obtained from Malaysian hospitals using the IIP test (1994-1999) were analyzed. During the six-year period, a total of 61,501 patients' sera were tested, of which 9.6%, 10.5%, and 12.9% had antibody (IgG and/or IgM of $\geq 1:50$) for OT, RT and TT respectively. A total of 8.6%, 9.8%, and 9.7% of sera had IgG antibody of $\geq 1:50$ for OT, RT, and TT respectively, indicating past infection. A total of 3.4%, 3.8%, and 6.4 % of sera had IgM antibody of $\geq 1:50$ for OT, RT, and TT respectively, indicating recent infection. A total of 2,986 (4.9%), 1,882 (3.1%), and 1,574 (2.6%) of sera had IgG and/or IgM antibody titers of $\geq 1:400$ for OT, RT, and TT respectively, suggesting active rickettsial infection. The seropositivity rates of OT, RT and TT varied according to geographical locations. While the seropositivity of OT remained constant during the six-year period, a reduction in the seropositivity of both RT and TT was noted during recent years. The serological findings reflect the endemicity of rickettsial diseases, including tick typhus, and endemic typhus in various parts of Malaysia. Awareness of these diseases by health and medical staff and by the general public is important if the mortality and morbidity associated with scrub typhus, tick typhus, and murine typhus in Malaysia, are to be reduced.

INTRODUCTION

Rickettsial infections are a major cause of febrile illness throughout the Asia-Pacific region (WHO, 1993). These short, rod-shaped or coccobacillary, obligate intracellular bacteria comprise three antigenically defined groups: those causing scrub typhus; those causing typhus; those causing spotted fever. The organisms are transmitted through the bite of mites, fleas and ticks. The organisms multiply locally at the site of the bites and spread to the endothelial cells of blood vessels. An inflammatory response develops, causing vasculitis; the triad of fever, headache, and rash may follow. The disease often affects multiple organs: the skin, skeletal muscles, central nervous system,

myocardium, lungs, liver and kidneys may be involved (Berman and Kundin, 1973; Rapmund, 1984). Recognition of scrub typhus on the basis of clinical signs and symptoms is often difficult due to the variety of manifestations of the disease, which in many instances mimic those of other infectious diseases. The rickettsioses are treatable; they can be quite serious if misdiagnosed.

Several tests have been developed for the serodiagnosis of rickettsial infections: the indirect immunofluorescence test, the latex test, indirect hemagglutination assay, enzyme-linked immunosorbent assay, and dipstick enzyme-immunoassay. The indirect immunoperoxidase (IIP) test, used for the detection of IgM and IgG to rickettsial agents (scrub typhus, en-

demic typhus and tick typhus), was developed at the Institute for Medical Research (Kelly *et al.*, 1988). The IIP test is generally accepted as the 'standard' assay and was recommended by the World Health Organization (WHO) as the routine serological diagnostic method for scrub typhus [WHO meeting of the Task Force on the Serological Diagnosis of Tsutsugamushi Disease (Scrub Typhus), Nishiharashino, Okinawa, Japan, 24 November 1986]. The test was introduced into clinical laboratories throughout Malaysia in the early 1990s.

Rickettsial test kits are prepared by the Rickettsial Laboratory of the Institute for Medical Research (IMR), Malaysia and supplied to government hospitals for the serodiagnosis of rickettsial infections. There are 3 types of rickettsial antigens fixed to each antigen slide, *ie* *Orientia tsutsugamushi*, (OT; a mixture of serotypes Karp, Kato and Gilliam), *Rickettsia typhi* (RT; Wilmington strain) and TT118 (TT), an unclassified spotted fever group (SFG) rickettsiae. Training is provided by the IMR for hospital medical laboratory technologists prior to their routine use of the IIP technique. To ensure that the quality of the test is not compromised, the IMR conducts an ongoing corroboration program for laboratories that use IIP test kits.

The IIP test is performed as described by Kelly *et al.* (1988): ten microliters of serially diluted (from 1:50 to 1:1,600) patients' sera are first placed on the antigen spots and incubated in a moist chamber at 37°C for 30 minutes. After washing with phosphate buffer saline (3 changes of 5 minutes each), peroxidase-conjugated anti-human IgG (HRP-IgG, Dakopatt) or anti-human IgM (HRP-IgM, Dakopatt) diluted 1:100 is added to react with the antigen spots at 37°C for 30 minutes. After a second washing, the antigen spots are treated with a substrate solution (3, 3-diaminobenzidine tetrahydrochloride) in the dark at room temperature for 10 minutes. Finally, the antigen spots are faintly stained with dilute methylene blue solution for 5 seconds. After final washing, air-dried slides are mounted with Permout mounting medium (Fisher, USA) for observa-

tion with an ordinary light microscope at 400x magnification. Positive reactions are indicated by the observation of brownish-stained rickettsiae in the respective antigenic smear, whereas no organisms can be seen in negative reactions. The reciprocal of the highest serum dilution that produces a positive reaction is expressed the IIP antibody titer.

With the IIP technique, specific IgM antibody is first detected on the third or fourth day after the onset of illness; the IgM titer subsequently rises rapidly to 5,120 or more between the tenth and fifteenth day; a few months to one year after the onset of the disease, IgM is undetectable. Specific IgG antibody can be detected after the seventh day; the titer of IgG antibody rises more slowly than the titer of IgM antibody, reaching 5,120 or more after about three weeks (Kawamura *et al.*, 1995). A four-fold or greater rise in antibody titer in paired sera collected a minimum of three days apart should be demonstrated in order to diagnose rickettsial infections. An IgG and/or IgM titer of $\geq 1:50$ indicates a past or recent exposure whereas a titer of $\geq 1:400$ for IgG and/or IgM antibodies is strongly suggestive of an active infection.

This paper gives an analysis of the serological findings obtained from eight Malaysian hospitals (Table 1) using the IIP test (1994-1999). Sera were obtained from patients presenting with febrile illness, as requested by hospital clinicians. Only very few paired sera were reported by each hospital and, therefore, the analysis was mainly based on single serum samples. Tables 1, 2 and 3 show the seropositivity (IgG and/or IgM titers of at least 1:50) of patients to each of the groups of rickettsiae in various regions of Malaysia. A total of 61,501 sera were tested during the six-year period, of which 9.6% had antibodies against OT (Table 1), 10.5% had antibodies against RT (Table 2), and 12.9% had antibodies against TT (Table 3). The results indicate a higher prevalence of exposure to TT in the Malaysian population.

The seropositivity of OT, RT and TT differs

Table 1

Seropositivity of patients' sera (IgG and/or IgM antibody titer $\geq 1:50$) as determined by the indirect immunoperoxidase test for *Orientia tsutsugamushi* in eight Malaysian hospitals during a six-year period (1994-1999).

Hospital	No. sera	% sera reactive to <i>Orientia tsutsugamushi</i>						Overall seropositivity
		1994	1995	1996	1997	1998	1999	
Pulau Pinang	6,538	2.9	4.6	3.6	3.5	2.0	2.7	3.3
Ipoh, Perak	20,385	14.6	13.7	13.4	10.7	15.9	13.7	13.7
Sultanah Aminah, Johor	1,367	6.4	0.0	3.8	3.8	7.7	11.0	5.3
Alor Setar, Kedah	11,455	8.1	7.3	12.3	10.9	16.1	3.7	9.7
Kuala Terengganu, Terengganu	8,222	16.6	15.1	8.2	7.0	5.5	NA	11.9
Kota Baru, Kelantan	1,447	NA	21.7	NA	20.4	9.9	21.4	19.4
Umum Kuching, Sarawak	1,050	9.3	17.9	19.1	17.1	NA	NA	15.7
Queen Elizabeth, Sabah	11,037	3.8	2.2	3.0	0.7	0.0	NA	2.6
Total	61,501	9.7	9.7	9.0	8.6	12.2	9.4	9.6

NA=Not available for analysis.

Table 2

Seropositivity of patients' sera (IgG and/or IgM antibody titer $\geq 1:50$) as determined by the indirect immunoperoxidase test for *Rickettsia typhi* in eight Malaysian hospitals during a six-year period (1994-1999).

Hospital	No. sera	% sera reactive to <i>Rickettsia typhi</i>						Overall seropositivity
		1994	1995	1996	1997	1998	1999	
Pulau Pinang	6,538	5.1	12.3	5.9	5.6	1.4	2.4	5.6
Ipoh, Perak	20,385	7.7	14.8	9.1	8.5	5.9	5.4	9.1
Sultanah Aminah, Johor	1,367	3.5	0.0	0.5	0.5	0.0	1.2	1.5
Alor Setar, Kedah	11,455	35.0	40.7	37.7	22.4	23.4	14.1	29.8
Kuala Terengganu, Terengganu	8,222	6.1	8.4	3.9	3.5	0.5	NA	5.6
Kota Baru, Kelantan	1,447	NA	17.4	NA	9.0	13.5	6.6	9.3
Umum Kuching, Sarawak	1,050	4.8	3.3	7.5	0.0	NA	NA	3.7
Queen Elizabeth, Sabah	11,037	2.2	1.1	1.1	0.2	0.0	NA	1.3
Total	61,501	10.0	13.8	11.1	8.5	8.8	7.6	10.5

NA=Not available for analysis.

among regions; the overall seropositivity of OT ranged from 2.6 (Queen Elizabeth Hospital, Sabah) to 19.4% (Kota Baru Hospital, Kelantan). The seropositivity of OT in each hospital during the 6 year period was quite constant (Table 1). The overall seropositivity of RT ranged from 1.3 (Queen Elizabeth Hos-

pital, Sabah) to 29.8% (Alor Setar Hospital, Kedah). A decline on the seropositivity of RT was however noted in recent years (Table 2). The overall seropositivity for TT ranged from 1.1 (Sultan Aminah Hospital, Johor) to 29.4% (Alor Setar Hospital, Kedah). A decline in the seropositivity was noted in more recent years

Table 3

Seropositivity of patients' sera (IgG and/or IgM antibody titer $\geq 1:50$) as determined by the indirect immunoperoxidase test for TT 118 SFG rickettsiae in eight Malaysian hospitals during a six-year period (1994-1999).

Hospital	No. sera	% sera reactive to TT 118 SFG rickettsia						Overall seropositivity
		1994	1995	1996	1997	1998	1999	
Pulau Pinang	6,538	1.3	7.6	2.7	3.4	0.3	0.3	2.8
Ipoh, Perak	20,385	16.5	23.4	19.0	12.6	4.6	3.3	14.6
Sultanah Aminah, Johor	1,367	1.8	0.0	2.8	0.0	0.9	0.0	1.1
Alor Setar, Kedah	11,455	25.7	38.3	41.1	25.4	22.7	17.4	29.4
Kuala Terengganu, Terengganu	8,222	11.4	14.7	15.1	5.4	3.3	NA	12.2
Kota Baru, Kelantan	1,447	NA	26.1	NA	9.2	10.5	2.1	8.4
Umum Kuching, Sarawak	1,050	11.1	6.7	14.5	0.5	NA	NA	7.8
Queen Elizabeth, Sabah	11,037	4.6	0.5	0.3	0.0	0.0	NA	1.6
Total	61,501	12.3	17.0	16.3	10.2	7.8	7.1	12.9

NA=Not available for analysis.

Table 4

Antibody titers of patients' sera reactive to *Orientia tsutsugamushi*, *Rickettsia typhi* and TT118 SFG rickettsiae as determined by the indirect immunoperoxidase test.

Antibody titer		% sera reactive to		
IgG	IgM	<i>Orientia tsutsugamushi</i>	<i>Rickettsia typhi</i>	TT 118 SFG rickettsiae
< 1:50	< 1:50	90.3	89.5	87.1
< 1:50	50-200	0.8	0.6	2.9
< 1:50	≥ 400	0.2	0.1	0.3
50-200	< 1:50	3.2	5.3	5.1
50-200	50-200	0.1	0.1	0.2
50-200	≥ 400	0.8	1.5	2.3
≥ 400	< 1:50	3.0	1.4	1.3
≥ 400	50-200	0.7	0.6	0.5
≥ 400	≥ 400	0.9	0.9	0.3

(Table 3). The decrease in the seropositivity of RT and TT was obvious in those sera tested in 4 hospitals, *ie* Pulau Pinang Hospital, Ipoh Hospital, Alor Setar Hospital and Kuala Terengganu Hospital (Tables 2, 3).

In general, IgG antibodies were detected more frequently than IgM during the six-year period. A total of 8.6%, 9.8%, and 9.7% had IgG for OT, RT, and TT respectively, indicat-

ing past exposure. A total of 3.4% of sera had IgM for OT, whereas 3.8% and 6.4% of the sera tested had IgM for RT and TT respectively, indicating recent infection (data not shown).

Table 4 shows the antibody titers of the patients' sera as determined by the IIP tests. Of the 61,501 sera, 90.3%, 89.5%, and 87.1% had no antibodies detected against OT, RT, and

TT respectively. IgG titers of 1:50 to 1:200 and IgM titers of < 1:50 were the commonest IIP titers. A total of 2,986 (4.9%), 1,882 (3.1%), and 1,574 (2.6%) sera had IgG and/or IgM antibody titers of \geq 1:400 for OT, RT, and TT respectively, suggesting active rickettsial infections.

These analyses reflect the seropositivity of the three species of rickettsiae in different regions of Malaysia; the review of the serological results shows the endemicity of rickettsioses in different parts of Malaysia. Despite the large number of sera with IIP titers suggestive of active infections, only 605 cases were reported to the health authority (Annual Report, Vector-Borne Control Section, Ministry of Health, Malaysia) within a 5-year period (1994-1998). The low reported incidence of rickettsial infections reported could be due to the ineffective notification of cases and to the failure of clinicians to submit paired sera for the serological confirmation of a clinical diagnosis. The nonspecific clinical patterns of the diseases also contributed to the underdiagnosis and underreporting of the diseases.

Scrub typhus is the best known and most studied rickettsial disease in Malaysia. The high prevalence of antibody in some rural areas of Malaysia was noted in previous serological surveys, indicating high rates of transmission among the population. A study in Mentakab, a rural district in Malaysia, found that as many as 23% of fevers were due to scrub typhus (Brown *et al*, 1976). A recent study among febrile patients from rural areas of Malaysia demonstrated that scrub typhus remains a public health problem, with a seroprevalence of 24.9% and an estimated annual attack rate of 18.5% (Tay *et al*, 2000). In comparison, a lower seropositivity of OT was observed among patients from various hospitals in this study: this could be due to differences between the group of patients from rural areas and those attending the hospitals. The seroprevalence of antibody for OT reflects the observation of Audy (1961), which was that the epidemiology of scrub typhus is closely related to human occupation and behavior. Most of the earlier

reports of human infections with OT described transmission in agricultural or undeveloped areas (Cadigan *et al*, 1972; Robinson *et al*, 1976). A significantly higher prevalence of rickettsial antibodies was reported among those working in the agricultural sectors than among those not engaged in agricultural activities (Tay *et al*, 2000). Whereas people from rural areas were likely to work in the agricultural sector, most patients attending hospitals were from the town and were, therefore, less likely to be involved in agriculture.

TT118 SFG rickettsiae were used as antigens for the serodiagnosis of tick typhus in this study as well as in others conducted in Southeast Asia (Taylor *et al*, 1986; Strickman *et al*, 1994; Tay *et al*, 1999, 2000). A low prevalence of tick typhus had been reported in Malaysia (Brown *et al*, 1984; Taylor *et al*, 1986) and attempts to isolate SFG rickettsiae have not been successful. Tick typhus was formerly regarded as a mild infection which did not warrant admission to hospital (Brown *et al*, 1984). However, several recent studies showed that antibody to TT118 spotted fever group was more prevalent than OT and RT among febrile patients in Malaysia (Ho *et al*, 1997; Tay *et al*, 1999, 2000). The analysis in this study also demonstrated the higher seropositivity of TT (12.9%) compared with other rickettsiae: this new development could be attributable to changes in ecological factors and human behavior, which may have resulted in the emergence of new disease or the resurgence of existing infectious diseases such as tick typhus. Further studies of the natural hosts, vectors and etiological agents of tick typhus in Malaysia are necessary.

Previous studies suggested that murine typhus was more prevalent in urban than in rural areas (Brown *et al*, 1977, 1984; Taylor *et al*, 1986). However, recent studies showed that murine typhus could be widely distributed in rural areas. A seroprevalence of 28.1% was noted among febrile patients attending health centers in rural areas (Tay *et al*, 2000) and 15.8% of rubber estate workers had antibodies for RT during a dry season (Tay *et al*, 1999).

In this study, a high seropositivity of RT was noted in several parts of Malaysia, indicating the importance of this infection.

The analysis in this study showed a reduction in the seropositivity of murine typhus and tick typhus in Malaysia in recent years. The epidemiology of rickettsial infections is affected by changes in vector-host ecology, environment, and human behavior. Improvement in socioeconomic status, more rigid rodent control, and a more hygienic environment may have contributed to the reduction of the seropositivity to RT, while the clearing of forests in order to allow development may have reduced human exposure to the potential vectors of tick typhus.

Interpretation of the IIP antibody titer requires a certain amount of judgement based on the history of the individual patient and the characteristics of the patient population. One study, of febrile Malaysian patients (Brown *et al*, 1983), showed that a specificity of greater than 95% was achieved when an acute titer of 1:400 (sensitivity of 29% during the first week of illness) or a four-fold rising titer to 1:200 (sensitivity of 54%) was observed. These criteria would serve as good guidance in the endemic area in which they were developed, but would be too conservative for a population that had no history of exposure to scrub typhus (Strickman *et al*, 1994). The IIP test was 93% sensitive in detecting scrub typhus in febrile patients on admission to the hospital, even though more than 50% of the population at large had measurable antibody titers to scrub typhus (Chouriyagune *et al*, 1992). This shows the importance of having baseline data on the prevalence of rickettsial antibodies in each geographical region and a collection of the convalescent sera for confirmation of rickettsial diseases.

The diagnosis of rickettsial disease relies principally on clinical recognition and is prompted by a consideration of relevant local epidemiology, *ie* residence in or recent travel to endemic areas and recent exposure to arthropod vectors. An eschar, rash, and gener-

alized or regional lymphadenopathy are the main features of the illness. However, in many patients, one or more of these findings may not be present (Taylor and Sivarajah, 1986; Tay *et al*, 2000). Symptoms such as headache, fever, and conjunctival infection are not specific enough to allow the differentiation of scrub typhus from other common tropical infections such as leptospirosis, typhoid fever, and dengue. To reduce morbidity and mortality due to rickettsial diseases, epidemiological surveillance and vector surveillance are important. Increasing the awareness of rickettsioses among health personnel will result in the more rapid identification of rickettsial diseases and prompt appropriate antibiotic therapy.

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