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Am. J. Trop. Med. Hyg., 43(3), 1990, pp. 301–307 (89-297)
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MULTI-LABORATORY EVALUATION OF A SCRUB TYPHUS DIAGNOSTIC KIT

DARYL J. KELLY, P. W. WONG, ELSIE GAN, CHAN TEIK CHYE, DAVID COWAN, AND GEORGE E. LEWIS, JR.

U.S. Army Medical Research Unit, Institute for Medical Research, Kuala Lumpur, Malaysia; Walter Reed Army Institute of Research, Washington, DC

Abstract. Scrub typhus is a major cause of febrile illness throughout the Asia-Pacific region. It is commonly undiagnosed, partly because of the lack of a simple, reliable diagnostic test which can be used in clinical laboratories. The indirect immunoperoxidase technique, configured into a test kit, was provided to technicians who were trained in its use. They used the kit during a 2 year field trial in their respective clinical hospital laboratories throughout Malaysia. In an evaluation using 1, 722 consecutive sera tested in those laboratories, the kit was found to have a median sensitivity for IgG detection of 0.85 (range 0.33–0.95), a median specificity of 0.94 (range 0.88–1.00), reproducibility of 0.86, and efficiency of 0.92 when compared to the reference laboratory. In a proficiency survey in which 10 laboratories received 3 coded test samples, all but 2 laboratories had results within 1 dilution of the reference laboratory in quantitating specific IgG, whereas 7 laboratories were within 1 dilution in quantitating IgM. The shelf life of the kit was at least 1 year at 4°C.

Rickettsial diseases are major causes of febrile illness throughout much of the Asia-Pacific region.[1–5] Most febrile patients are admitted to regional hospitals which lack sophisticated laboratory facilities. Thus, they must be managed without the benefit of specific diagnosis. The delay or absence of differential diagnosis has been shown to result in significant mortality, especially in the case of scrub typhus.[6] These problems have resulted in efforts to develop a sensitive, specific, inexpensive, practical, and rapid test for the serodiagnosis of the rickettsial diseases within the hyperendemic region.[7–9] Subsequent to comparative evaluation of the local use of various serodiagnostic techniques, we developed a simplified test kit using indirect immunoperoxidase (IIP) for the detection of IgM and IgG to rickettsial agents.[9] From 1986 to 1989, a series of workshops was conducted by the United States Army Medical Research Unit-Malaysia (USAMRU-M) at the Malaysian Institute for Medical Research (IMR) to instruct medical technologists from Malaysia and neighboring countries in the serodiagnosis of those diseases. Instruction included an introduction to the use of the newly developed IIP test kit with emphasis on scrub typhus. Upon completion of the course, a test kit was provided to each of the participants for use in their own laboratories. Here we present an evaluation of that kit.

MATERIALS AND METHODS

Patient sera

Single acute or convalescent sera were collected from febrile patients presenting at 1 general hospital and 9 district hospitals located in peninsular Malaysia. Sera were submitted to the hospital laboratories for routine testing for reactivity to febrile disease-causing agents, including those causing the rickettsial diseases, especially scrub typhus. The sera were then tested by trained technologists in those laboratories using the Indirect Immunoperoxidase Test Kit for Rickettsial Disease Diagnosis (IIP Test Kit). The stained slide, a copy of reported results, and portions of the tested sera were submitted to the USAMRU-M for corroboration and evaluation. Sequentially submitted sera, no more than 1 serum per patient, were used to ensure actual ratios of reactive to nonreactive sera from the endemic areas were maintained.[10]

Kit stability

The shelf life of the IIP Test Kit was determined by periodically testing the kit reagents, control serum, and antigen slides monthly for the first year, then again at 18 and 24 months. Three clinical specimens of known titer to scrub typhus, typhus (murine), and spotted fever (tick typhus) groups were tested and monitored for

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IgG and IgM titer variability. Monitored kit materials were stored at the USAMRU-M at 4°C and −20°C.

Proficiency survey

The proficiency survey was performed according to the method described by Barnett.[11] Five serum pools were prepared showing serum titers of <50–1, 600 for the scrub typhus, typhus, and spotted fever rickettsial groups. The coded test samples (1 blind sample from each of the 3 pools) plus known positive and negative control serum samples (1 each) were mailed along with instructions to each participating laboratory. All 5 samples were tested simultaneously by the same trained technologist in each laboratory. To control for variability due to prolonged time in the mail, 1 sample set was mailed to a non-participating laboratory and returned for testing at the IMR.

IIP Test Kit assay

The IIP Test Kit immunoassay uses previously evaluated test methods and follows the stepwise procedure.[7-9] All procedures and materials, shown in Table 1, were provided with the kit. The test procedure is outlined in the following sections.

Slide preparation. Remove the antigen slide, which is contained within the micro-slide box, from the −20°C freezer or 4°C refrigerator. Identify each slide in pencil on the frosted surface. Place the slide on the black cardboard plate and immediately place in a 37°C incubator for ~5 min to dry condensation from the slide. A hair dryer, cool air setting, can be used for this or any other step requiring drying.

Serum dilution. Serially dilute serum samples (2-fold, micro [a] or macro [b] method, below).

a. Label side of microtiter plate with dilutions 25, 50, 100, 200, 400, 800. In first well, dilute 5 μl of control or patient's serum with 120 μl phosphate buffered saline (PBS) = 125 μl of 1:25 dilution. Add 50 μl PBS in wells labeled 50 through 800. Using a 50 μl microdiluter or micropipettor, mix serum from the first well (25), transferring serially diluted serum to each subsequent well (50, 100, 200, etc.).

b. Label 6 test tubes 50, 100, 200, 400, 800, 1, 600. In the first tube (50), add 25 μl serum to 1, 225 μl PBS, resulting in a total volume of 1, 250 μl. Add 500 μl PBS to each subsequent tube (100—1, 600). Remove 500 μl of diluted serum from first tube, transfer to second tube, and mix well. Serially transfer 500 μl of mixed diluted serum to each subsequent tube (through 1, 600) resulting in serially diluted sera.

TABLE 1
Reagents and equipment provided in IIP Test Kit

Reagents/material provided in kit
1. Rickettsial antigen slides* prepared from yolk sac propagated rickettsiae; 12 position, 4 antigen dots/position (*Rickettsia tsutsugamushi, R. typhi* [typhus group], TT-118 [spotted fever group], and normal yolk sac control); 25 slides/kit with slide box and dessicant.
2. Sodium acetate (CH_3COONa), 2 vials, 0.5 g/vial.
3. Sodium hydroxide (NaOH), 1 N, 1 vial, 5 ml.
4. pH papers, 1 vial of 20, range 6–8.1.
5. Hydrogen peroxide (H_2O_2), 1 vial, 5 ml, 3%.
6. Phosphate buffered saline (PBS, pH 7.3), 2 vials. NaCl 8.56 g KH_2HPO_4 0.23 g Na_2HPO_4 1.18 g Dissolve in 1,000 ml distilled H_2O. Adjust pH with NaOH and pH papers provided.
7. Conjugate:* Horseradish peroxidase conjugated rabbit anti-human IgG and IgM (Dako, Denmark), 1 vial, 0.2 ml each.
8. Chromagen storage and reaction bottle, dark glass, 100 ml.
9. Chromagen:* 3'3 diaminobenzidine tetrahydrochloride (DAB; Sigma, ST. Louis, MO), 2 vials, 0.15 g.
10. Counterstain: Methylene blue, 1 vial, 2 ml. Dilute in 40 ml distilled H_2O. Store in staining jar up to 8 months.
11. Mounting medium, Permount, 1 vial, 5 ml.
12. Coverslips, glass, 22 mm × 50 mm, 1 box of 25.
13. Microtiter plates, plastic, 96-well, 2 plates.
14. Plastic incubation chamber with wooden applicator sticks, 1.
15. Black plastic plate, 12 cm × 10 cm.
16. Control serum,* human anti-*R. tsutsugamushi* positive and negative, 1 vial each, 0.5 ml.
17. Package insert with complete instructions.

* Reagent transported and stored at 4°C or −20°C.

Add serum to slide. Add ~10 μl of each dilution of serum to different circles on the coated glass antigen slide. Begin from the higher dilution to the lower, using the same micropipette. Place the slide in a moist incubator chamber (Petri dish) to prevent evaporation. Incubate the slide at 37°C for 30 min.

Remove slide from incubator. First rinse with PBS, then wash 3 times by immersing in a staining jar containing PBS (3 changes, 5 min each).

Conjugate. While slide is drying, prepare a 1:100 dilution of the peroxidase conjugated anti-human IgG (HRP-IgG) and anti-human IgM

(HRP-IgM) using PBS as the diluent (5 μl conjugate to 495 μl PBS = 500 μl diluted conjugate). Add ~10 μl of the diluted HRP-IgG or HRP-IgM to each circle containing the serum reacted antigen, using the upper row of antigen spots for IgG and lower row for IgM. Place the slide in the moist Petri dish and again incubate at 37°C for 30 min.

Rinse slide with PBS. Then, using a staining jar, wash the slide twice with PBS (2 changes, 5 min each). Finally, rinse with distilled water for 5 min. Air dry the slide.

Chromagen. Mix 1 vial diaminobenzidine tetrahydrochloride (DAB) and 1 vial sodium acetate in 50 ml distilled water in the chromagen reaction bottle. Add 1 ml sodium hydroxide; mix and adjust pH to 6.2 using pH papers or pH meter. Add 0.35 ml 3% hydrogen peroxide. Filter DAB solution into a staining jar. Incubate the slide in the DAB solution in the dark for 10 min at 37°C. Rinse the slide quickly with distilled water.

Stain slide. Stain slide with diluted methylene-blue solution for ~5 sec. Wash the slide with distilled water and air dry. Mount the coverglass on the slide using 2–3 drops of mounting medium. Examine the slide under an ordinary light microscope (high dry, 40× objective).

Interpretation. The reaction is considered positive when the rickettsiae are visible as light brown particles. The reciprocal of the highest serum dilution with positive reaction is expressed as the IIP antibody titer. Specimens are considered negative if the result obtained using the positive control serum reads within 1 dilution of the given titer and there are no clearly visible organisms seen.

Statistical analysis

Sensitivity was defined as the degree of qualitative agreement for positive reacting sera (≥1:100 by the reference laboratory) and specificity was the degree of qualitative agreement for negative reacting sera (<1:100 by the reference laboratory) using 2-way tables. A cut-off value of 1:100 was selected based upon previously established receiver-operating characteristic curves to optimize sensitivity for the potentially life-threatening rickettsial diseases with the rationale previously described.[9,12] Reproducibility was determined by quantitative IIP titer comparisons between the user laboratories and the reference laboratory on submitted sera. Interlaboratory comparisons of sensitivity, specificity, efficiency, and reproducibility were done using the Wilcoxon Rank-Sum test.[13] Proficiency survey results were examined according to Youden.[11,14]

RESULTS

IIP test kits provided to technologists working at individual hospital laboratories were used to test 1, 722 serum samples collected from febrile patients. In a blind study, all sera were tested at the reference laboratory, which also employed the previously validated IIP technique; multiple samples from the same patient were excluded from the evaluation. The results of the user laboratories were compared with those of the reference laboratory. Table 2 summarizes the sensitivity, specificity, and reproducibility of the results of 10 participating laboratories using 1:100 as the cutoff titer. Among the individual laboratories, there were no significant qualitative differences in specificity, reproducibility, or efficiency when IgG was measured. However, the sensitivity reported in laboratories performing ≤70 tests (laboratories 8–10) during the evaluation period was significantly lower than those performing ≥124 tests (laboratories 1–6) for qualitative IgG detection ($P = 0.02$). In addition, there was an overall association between the number of tests done and sensitivity ($r_s = 0.77$).[13] We could detect no such difference or association when the sensitivity in detecting IgM was examined (data not shown). Findings for specificity, reproducibility, and efficiency were similar when 1, 203 of the sera, tested during the final 6 months of the evaluation, were compared with the entire study period (data not shown).

The quantitative comparison (reproducibility) of titer results of IIP kit users with the reference laboratory using titers for positive reactivity ≥25 is shown in Table 3. Reproducibility did not vary substantially when ≥50 or ≥100 were used as the titers for positive reactivity (data not shown). Reproducibility was high when all sera were considered, due to the large proportion of negative samples, and lower when reactive sera alone were considered.

In order to further evaluate the relative proficiency of the laboratories using the test kit, identical sets of known and unknown serum samples were sent to user laboratories. Figure 1 sum-

TABLE 2

Qualitative comparison of Indirect Immunoperoxidase Test Kit for Rickettsial Disease Diagnosis Rickettsia tsutsugamushi *specific IgG results from evaluating laboratories with actual reference laboratory results: summary of 1,722 sera submitted by selected laboratories performing the evaluation*

Lab no.	n*	Sensitivity†	Specificity	Efficiency	Positive (%)‡
1	238	0.87	0.96	0.93	33.2
2	187	0.85	0.95	0.93	18.2
3	289	0.86	0.93	0.92	21.2
4	124	0.92	0.88	0.90	41.9
5	342	0.95	0.91	0.92	24.6
6	212	0.76	0.94	0.90	19.4
7	159	0.00§	1.00	0.99	1.2
8	69	0.83	0.94	0.91	26
9	47	0.33	1.00	0.91	12.8
10	56	0.50	1.00	0.93	14.3
Median		0.85	0.94	0.92	
Range		0.33–0.95	0.88–1.00	0.90–0.93	1.3–41.9

* Number of sequentially submitted sera; one serum only was scored from each patient.
† Cut-off titer ≥ 1:100 was selected for parameter determinations; TP = true positive, FP = false positive, FN = false negative, TN = true negative; sensitivity = TP/TP + FN; specificity = TN/FP + TN; efficiency = TP + TN/TP + FP + FN + TN. Sensitivity of laboratories 1–6 vs. 8–10 were significantly different ($P < 0.02$ Wilcoxon Rank Sum Test).
‡ Percent of all sera submitted testing positive by the reference lab (≥ 1:100).
§ Only 2 positive sera submitted; laboratory excluded from statistical evaluation.

marizes the results of the proficiency survey. In addition, 1 set mailed to a hospital laboratory 11,000 miles away and returned to the reference laboratory was tested to determine if samples had suffered deleterious shipping effects. Those sera tested within 1 dilution of the original result. The proficiency survey results were examined according to the method of Youden (Fig. 1).[11] The lower titer positive sample (x axis) was plotted against the higher titered positive sample (y axis) for both IgG (Fig. 1A) and IgM (Fig. 1B). As shown in Figure 1A, 8 of 10 participating laboratories fell within 1 doubling dilution for both samples in their IgG results with the 2 remaining laboratories falling in the lower left quadrant. In detecting specific IgM in the samples, 7 of 10 laboratories obtained results within 1 dilution. Two of the 3 results outside the standard were the same as the unsuccessful laboratories shown in Figure 1A. All laboratories correctly reported negative results for the third, unknown sample.

Three control sera of known titers and antigen slides stored at either 4°C or −20°C were periodically tested for 2 years. Specific IgG or IgM serum titers varied by no more than 1 doubling dilution during the first year under any of the storage conditions when tested for specific reac-

TABLE 3

*Reproducibility of anti-*Rickettsia tsutsugamushi *immunoglobulin specific IIP Test Kit results between evaluating laboratories and reference laboratory results*

	All sera				Reactive sera*			
Lab no.	n	IgG	n	IgM	n	IgG	n	IgM
1	238	0.80†	239	0.88	92	0.62	67	0.66
2	187	0.91	187	0.96	53	0.74	7	0.57
3	289	0.85	289	0.94	94	0.63	27	0.67
4	124	0.79	129	0.90	56	0.66	28	0.64
5	342	0.86	342	0.94	107	0.62	34	0.62
6	211	0.82	211	0.87	56	0.52	20	0.60
7	159	0.98	159	0.99	8	0.50	0	
8	69	0.87	69	0.88	23	0.65	12	0.50
9	47	0.87	47	0.89	8	0.25	0	
10	56	0.86	56	0.70	13	0.62	7	0.86
Median		0.86		0.89		0.62		0.63

* All sera reacting at ≥25 were scored as reactive.
† Reproducibility = sera reading within 1 dilution with user laboratories compared to the IIP test performed at the reference laboratory.

tivity to any of the 3 rickettsial groups. Specific anti-*Rickettsia tsutsugamushi* IgM titers and tick typhus (spotted fever group) IgG titers dropped by 2 dilutions when antigen slides and sera were stored in excess of 1 year at 4°C. No other variation in titer beyond 1 dilution was noted during the 2 year period. Negative control sera remained nonreactive throughout the study.

DISCUSSION

The IIP method used in this study and the immunofluorescence antibody test, which is generally accepted as the "standard," have been recommended by the World Health Organization (WHO) as the routine serological diagnostic methods for scrub typhus (WHO meeting of the Task Force on the Serological Diagnosis of Tsutsugamushi Disease [Scrub Typhus], Nishiharacho, Okinawa, Japan, 24 November 1986). The IIP technique has been shown to be effective for the serodiagnosis of scrub typhus within the Asia-Pacific region and most recently in Malaysia.[7-9] In this evaluation, we determined that the early, rapid serodiagnosis of scrub typhus can be made by a trained technologist using an IIP test kit in a district or regional hospital. Over a 2 year period, 10 selected laboratories submitted 1,722 sera collected from consecutive febrile patients and their own results for evaluation and corroboration of their findings.

Technicians using the test kit were trained in the use of the kit prior to performing the test in their respective laboratories. Thus, not surprisingly, there was no clear learning effect evident during the evaluation period. There was little difference in sensitivity, specificity, reproducibility, and efficiency between the overall evaluation and the last 6 months alone. This indicates the initial training in kit use was adequate for accurate performance of the IIP test throughout the study period. In addition, the coefficient of correlation ($r_s = 0.77$) for sensitivity for detection of IgG among the user laboratories indicates there was a strong association between the frequency with which the test was performed and the sensitivity obtained. When inspecting the data received from the user laboratories, there appeared to be a natural break point between the more frequent users (laboratories 1–6) and the infrequent users (laboratories 8–10). The data suggest that labs performing fewer tests did not do them as well as those performing the test more often,

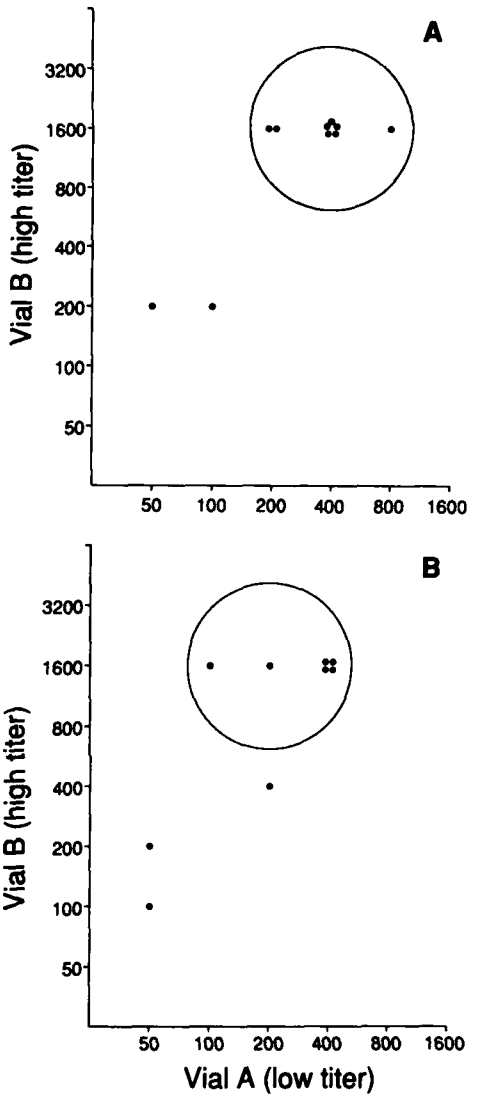

FIGURE 1. Youden plot for scrub typhus proficiency survey. Each point represents the value for 1 laboratory for the 2 positive sample vials, A and B. Circles enclose an area within 1 dilution of the reference laboratory result. Shown are results for IgG (A) and IgM (B).

as demonstrated by the significant differences in sensitivity between laboratories 1–6 and 8–10. From these data, it is not possible to determine what a threshold quantity of tests might be, though we might postulate that a rate of about 100 tests per year would be appropriate. Thus smaller laboratories, which might not annually perform that quantity of tests, might well consider forwarding the samples to their next level of support, which in this case would be a regional

hospital. This finding is consistent with the belief commonly held in clinical laboratories that most assays must be performed with some degree of regularity in order to maintain the proficiency necessary to accurately perform the test. The small percentage of specimens which were reactive for IgM resulted in unstable estimates of sensitivity and specificity, and no patterns could be discerned.

In addition to the evaluation of sera submitted by the user laboratories for corroboration of serological findings, the proficiency survey provided an opportunity to compare the performance of the kit users. The Youden method, widely used by the Australian and American Colleges of Pathology, was used to evaluate the performance of the user laboratories. Results of the majority of laboratories fell scattered within the circle (Fig. 1) representing results within 1 dilution of those of the reference laboratory. Outside the circle, all points are in the lower left quadrant, indicating participants found both values lower than other laboratories and the reference laboratory. This is indicative of a systematic error or consistent bias rather than random error, since titers of both samples were well below the mean. Follow-up of 1 case showed the error was likely to be caused by serum samples sitting several days in extremely hot temperatures. The cause of errors in the other cases was later shown to be inadequate pH verification of the DAB chromogen. Those users were followed-up and, in all cases, the reported known positive control, submitted concurrently with the test samples, was also low, indicative of procedural error or compromised reagents. The 2 laboratories which performed poorly in the evaluation phase (nos. 9 and 10, Table 2), had not participated in the proficiency survey.

The IFA test, long the standard for the serodiagnosis of scrub typhus, is not practical in less than ideal situations. It has not been widely used within the endemic region due to the general lack of fluorescence microscopes. In Malaysia, the IFA test for serodiagnosis of rickettsial diseases is only performed in our own laboratories. The Weil-Felix OXK test is still routinely used in the vast majority of hospital laboratories in Malaysia and throughout the Asia-Pacific region. For this kit, all reagents and supplies used were prepared in pre-measured bottles and vials. No special laboratory equipment, save for a light microscope and laboratory incubator, were required. These kits contained many reagents not commonly supplied with commercial diagnostic kits marketed in the U.S., such as pH papers and microtitration plates. In order to maintain standardization by the users, we recommend periodic monitoring through an annual or semiannual proficiency survey similar to that conducted in this kit evaluation. Such external proficiency testing could further enhance the confidence of the physician in the results produced by the clinical laboratory.

The USAMRU-M, in collaboration with the Malaysian IMR, has introduced an indirect immunoperoxidase test and test kit into clinical laboratories throughout Malaysia and in selected laboratories in other Southeast Asian countries. Although some weaknesses remain, in the hands of trained personnel the Indirect Immunoperoxidase Test Kit for Rickettsial Disease Diagnosis provides a sensitive, specific, reproducible, and practical semiquantitative product which can be used in laboratories where sophisticated equipment is unavailable.

Acknowledgments: The authors wish to thank the following Malaysian laboratory directors and technologists who actively supported the evaluation: Halimah Yahya, Khamdani Alias, Cik Noraini Ariffin, Hospital Besar Tengku Ampuan Rahimah, Kelang, Selangor; Angela Oh, Ng Pey Ping, Loke Siew Kong, Hospital Besar, Pulau Pinang; D. Gregory, Liew Kook Fong, Hospital Besar, Seremban, Negeri Sembilan; Chan Kok Eng, Loo Yon Pong, Hospital Sultanah Aminah, Johore Bahru, Johore; Lau Kong Wah, Cik Robaayah Kamaruddin, Hospital Besar, Melaka; Thomas Chung, Alfred Chee, Jabatan Perkhidmatan Perubatan dan Kesihatan, Kuching, Sarawak; D. Kooshy, M. Puzi Yusof, Hospital Besar, Kuala Terengganu, Terengganu; S. M. Ghani, Tan Chooi Lean, Hospital Besar, Alor Setar, Kedah; Phang Song Kong, Sanat Nasiban, Hospital Daerah, Muar, Johore; M. Jegathesan, N. Ambigadevi, Hospital Besar, Kuala Lumpur; Nasuruddin Abdullah, Cik Noorrela Mokhtar; Universiti Kebangasaan Malaysia, Kuala Lumpur; D. Sukumaran, Puan Zaiton Yusof, Institute for Medical Research, Kuala Lumpur; Bruce Innis, Panor Srisongkram, Armed Forces Research Institute of Medical Science, Bangkok, Thailand. We also thank K. Hechemy for critical review of the manuscript and R. Sherman for statistical assistance. Finally, we are indebted to Lim Teong Wah, past Director, and M. Jegathesan, present Director, Institute for Medical Research, for their enthusiastic support.

Financial support: U.S. Army Medical Research and Development Command grant DAMD 17-85-G-5011.

Authors' addresses: Daryl J. Kelly, Rickettsial Disease Department, Naval Medical Research Institute, Bethesda, MD 20814-5055. P. W. Wong, Elsie Gan, and Chan Teik Chye, U.S. Army Medical Research Unit,

Institute for Medical Research, 50588 Kuala Lumpur, Malaysia. David Cowan, Walter Reed Army Institute of Research, Washington, DC 20307. George E. Lewis, Jr., HQ USAMRDC, Fort Detrick, Frederick, MD 21701.

Reprint requests: Daryl J. Kelly, Rickettsial Disease Department, Naval Medical Research Institute, Bethesda, MD 20814-5055.

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