DEVELOPMENT AND EVALUATION OF A LATEX AGGLUTINATION TEST FOR THE RAPID DIAGNOSIS OF SCRUB TYPHUS

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Abstract. The purpose of this research was to develop a simple and rapid diagnostic test for scrub typhus using a latex agglutination test (LAT) to detect antibodies against *Orientia tsutsugamushi.* Five strains of *O. tsutsugamushi* were propagated in L929 cells. The rickettsiae were purified and concentrated with percoll density gradient centrifugation. A suitable concentration of *O. tsutsugamushi* soluble antigen was used to sensitize latex to prepare the latex antigen. The specificity, sensitivity, and accuracy of the latex antigen were assessed. The LAT, indirect immunofluorescent antibody test (IFA), and Weil-Felix agglutination test (WF) were compared by testing 109 acute febrile illness cases and 100 confirmed non-scrub typhus cases (50 other febrile disease cases and 50 healthy controls). By using the IFA as the standard reference method, the overall sensitivity, specificity, and accuracy of the LAT were 89.1, 98.2, and 93.6%, respectively. By contrast, the sensitivity of the WF, compared with the IFA, was only 47.3%, while the specificity and accuracy were 92.6 and 69.7%, respectively. Thus, the LAT described here is another important alternative test for the diagnosis of scrub typhus.

INTRODUCTION

Scrub typhus is an important rickettsial disease in Southeast Asia and the Asia-Pacific region, including Thailand. It is caused by *Orientia tsutsugamushi*, a small gram-negative intracellular bacterium which is transmitted to humans by the bite of infected larval trombiculid mites. The disease varies in severity from asymptomatic to a febrile syndrome. Severe cases can be fatal from organ failure, as found in up to 30% of patients (Seong *et al*, 2001). Early and accurate diagnosis is needed so that appropriate treatment early in the course of the disease can result in a rapid cure.

Presently, serologic testing is the most frequent method used for diagnosis; the test used is the indirect immunofluorescent antibody test (IFA) (Kelly *et al*, 1990). Although this test generally performs well, expensive equipment and highly skilled technicians are required. Some high-cost diagnostic tests, such as enzyme-linked immunosorbent assay (ELISA) (Jang *et al*, 2003), polymerase chain reaction technique (PCR) (Manosroi *et al*, 2003; Saisongkorh *et al*, 2004) or dipsticks (Chinprasatsak *et al*, 2001), may not easily be applied in developing countries. In Thailand, the current diagnostic method is the Weil-Felix (WF) test. Although it is simple, it results in a large number of false-positive and false-negative reactions (Brown *et al*, 1983; Chouriyagune *et al*, 1992). There is still a need for a simple, rapid and sensitive diagnostic test that can be performed locally in small laboratories, because the infection is usually acquired in rural areas.

In our study, a latex agglutination test (LAT) was developed and compared with IFA and WF for the diagnosis of scrub typhus.

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MATERIALS AND METHODS

Serum specimens

One hundred and nine serum samples from

patients with suspected or confirmed scrub typhus, admitted to Maharat Nakhon Ratchasima Hospital, Nakhon Ratchasima, Northeast Thailand, were included in this study. Fifty healthy blood donor sera were obtained from blood donors at the blood bank of the same hospital. A further 50 sera from patients with other febrile diseases (5 influenza, 5 dengue fever, 10 leptospirosis, 10 typhoid fever, 10 malaria, and 10 OX-19 positive sera), were obtained from the Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, to be used as controls for checking cross-reactions with scrub typhus. All the serum samples were kept at -20ºC until used.

Scrub typhus antigen

Orientia tsutsugamushi (Karp, Kato, Gilliam, KR1 and KR2) were grown in L929 mouse fibroblast cells at 35ºC in MEM medium containing 5% fetal bovine serum (Gibco, USA) as described by Kelly *et al* (1988). When an 80-90% cytopathic effect occurred, the infected cells were harvested by rocking with glass beads. The rickettsiae and infected cells were washed 3 times with PBS, pH 7.2, by centrifugation at 200*g* (Beckman, USA), 4ºC for 15 minutes. Then, the pellet was divided into 2 parts: one part was diluted with PBS pH 7.2 to contain $1x10^6$ infected cells/ml and was used as a scrub typhus whole cell antigen for the IFA test; the other part was diluted to an equal volume and homogenized by a glass grinder in 0.033 M Tris-sucrose buffer, pH 7.4, and the homogenates obtained were centrifuged at 200*g* for 10 minutes. The supernatant fluid was mixed with Percoll (Pharmacia Fine Chemical, Sweden) following the purification method described by Tamura *et al* (1982). The *O. tsutsugamushi* obtained after Percoll gradient centrifugation were disrupted with an ultrasonic disintegrator (MSE, USA) followed by centrifugation at 10,000*g* (Beckman, USA) for 10 minutes at 4ºC. The supernatant fluid was collected and used as soluble antigen (SoAg) for the latex agglutination test. The SoAg was stored in small aliquots and kept at -70ºC until used. The protein content of the SoAg was determined by the method of Lowry *et al* (1951). The SoAg from each strain of *O. tsutsugamushi* (Karp, Kato, Gilliam, KR1 and KR2) were pooled at the same protein concentration and used as pooled SoAg for the sensitization of the latex particles.

Detection of antibodies by IFA test

Five microliters of whole cell antigen (Karp:Kato:Gilliam:KR1:KR2= 2:1:1:1:1) were dropped onto each well of a teflon multiwell slide (BDH, England) and thoroughly air dried; the slide was fixed in cold acetone for 20 minutes, thoroughly air-dried and stored in the dark at -20ºC until used.

The IFA test was performed as described by Robinson *et al* (1976). Ten microliters each of seven two-fold-diluted sera, starting with a dilution of 1:200, were incubated with the antigen in each well, at 37ºC for 30 minutes. Positive and negative controls were included in the test. After washing in PBS 3 times for 5 minutes, the slides were air-dried and then incubated with 1:40 fluorescein-isothiocyanate (FITC)-labeled rabbit anti-human immunoglobulin (DAKO Patts, Denmark) and 1:10,000 Evan blue as a counterstain at 37ºC for 30 minutes, then washed as previously described. Each slide was mounted in two or three drops of mounting buffer and then observed under a fluorescence microscope (Olympus BH-2, Japan). The results were taken as the last dilution to show the green fluorescence of the microorganisms. A single cut-off titer of ≥1:400 was regarded as a positive result (Brown *et al*, 1983).

Weil-Felix test (OX-K)

The Weil-Felix test (OX-K) was performed according to the manufacturer's instructions (Biotech, Thailand). Tested serum was 2-fold diluted from 1:10 to 1:320. One drop of OX-K antigen was added into 1 ml of each serum dilution, including the last tube containing 1 ml of 0.85% NaCl as a buffer control. All tubes were mixed by agitation and incubated for 4 hours at 50ºC. The results were reported as titers of final dilution that gave 50% agglutination. Titers of ≥1:160 were considered positive (Brown *et al*, 1983).

Latex agglutination test

A latex agglutination technique described by Hechemy *et al* (1980) was used, with slight modification. The latex suspension (0.81μm; Difco Laboratories, USA) was adjusted with 0.1

M glycine-buffered saline (pH 8.1) to an optical density of 0.3 at 650 nm (Beckman DU-30 Spectrophotometer, USA). Then, 0.5 ml of the latex suspension was mixed with 0.3 ml of pooled SoAg at various protein concentrations and 0.2 ml of 0.1% fatty-acid-free bovine albumin (Sigma Chemical, USA) with sodium azide added, for a final concentration of 1%. The mixture was incubated at 37ºC for 30 minutes and kept overnight at 4ºC. The optimum concentration of pooled SoAg was determined by checkerboard titration with known reactive human anti-*O. tsutsugamushi* serum. In this study, the optimum pooled SoAg concentration for the sensitization of the latex particles was found to be 300 μg/ml. To perform the test, specimens were diluted in two-fold serial dilutions, from 1:4 to 1:8 and then 1:16 in glycine-buffered saline fatty acid-free bovine albumin. Twenty microliters of diluted serum were mixed with 20 μl of the sensitized latex on each well of a six-well latex agglutination card. The reaction components were mixed and spread to cover a circular area of about 10 mm in diameter using a wooden applicator stick. The card was then placed in a humid box on a rotator (AH Thomas, USA) and rotated gently for 5 minutes. The test was read and reported as positive if there was evidence of agglutination of the latex.

Statistical analysis

Statistical analysis for the diagnostic sensitivities, specificities, and accuracies of the LAT and WF test for the diagnosis of scrub typhus was conducted by the method of Griner *et al* (1981), when compared with the results of the IFA test, as a standard.

RESULTS

The purpose of this study was to develop a simple one-step serodiagnosis for scrub typhus infection. Thus, the cut-off titer was determined first by a panel of negative controls, which included 50 confirmed non-scrub typhus febrile disease cases and 50 healthy blood donors. Table 1 shows the results of both the IFA and

Table 1
Sera tested for possible non-specific cross-reactivity with the latex agglutination test for the detection of scrub typhus antibody.

Other febrile diseases and healthy controls (Number of tested sera)	IFA			LAT		
	<1:200	1:200	1:400	≤1:4	1:8	1:16
Influenza (n=5)	3	2	-	5	-	-
Dengue fever (n=5)	5	-	-	5	-	
Leptospirosis (n=10)	9	1	-	7	3	-
Typhoid fever (n=10)	8	2	-	8	2	-
Malaria (n=10)	6	4	-	8	2	-
OX-19 positive sera (n=10)	7	3	-	9	1	-
Healthy blood donors (n=50)	50	-	-	45	5	-
Total (n=100)	88	12	-	87	13	-

Table 2
Comparison of LAT or WF to IFA for the detection of scrub typhus antibodies in 109 acute febrile patients.

IFA	LAT		Total	WF		Total
	Positive	Negative		Positive	Negative	
Positive	49	6	55	26	29	55
Negative	1	53	54	4	50	54
Total	50	59	109	30	79	109

Table 3
Comparison of the sensitivity, specificity, and accuracy of scrub typhus antibody detection by IFA, LAT, and WF tests in 109 patients with suspected or confirmed scrub typhus.

Test	Sensitivity (%)	Specificity (%)	Accuracy (%)
IFA	100.0	100.0	100.0
LA	89.1	98.2	93.6
WF	47.3	92.6	69.7

LAT for the negative controls in which all had titers of less than 1:400 and 1:16 in the IFA and LAT, respectively. Accordingly, a minimum titer of 1:16 was considered seropositive for scrub typhus infection by the LAT in this study. Further evaluation of this test was done with 109 sera from patients with suspected or confirmed scrub typhus and compared to the results of the IFA and WF test. As the results show in Table 2, forty-nine patient sera were positive by both IFA and LAT. Six patients had positive titers by IFA but were negative by LAT. One negative IFA patient serum showed a positive LAT. The WF test results were relatively insensitive, with more than half (29/55) of the IFA positive cases not being detected. Furthermore, 4 cases were positive by WF but negative by IFA. The overall sensitivities, specificities, and accuracies of LAT and WF test compared to the IFA standard method are shown in Table 3. The sensitivity, specificity, and accuracy of the LAT were 89.1, 98.2, and 93.6%, while those of the WF test were 47.3, 92.6 and 69.7%, respectively.

DISCUSSION

The latex agglutination test (LAT) appears attractive, since this test is simple and rapid. The LAT has previously been developed to detect antibodies to several rickettsial infections, including *Rickettsia rickettsii* (Hechemy *et al*, 1980, Kaplan and Schonberger, 1986), *R. typhi* and *R. prowazekii* (Hechemy *et al*, 1981) and *R. conorii* (Hechemy *et al*, 1986). Those results indicated that the LAT is sensitive and specific when compared with IFA, and can serve as a first-line screening test for rickettsial diseases. To our knowledge, LAT for the detection of antibodies against *O. tsutsugamushi* has never been reported elsewhere.

In our study, we used pooled SoAg collected from 3 commonly used prototypes (Karp, Kato, Gilliam) and 2 local Thai strains of *O. tsutsugamushi* for coating the latex particles. The addition of the 2 new Thai isolates collected locally gave a better agglutination result than the combination of the 3 prototypes alone (data not shown). The reason for this is not clear. It may be due to the different antigenic components from the different strains (Ohashi *et al*, 1988, 1996). In routine laboratory work, the prototypes, Gilliam, Karp and Kato, are commonly used as antigens. Since the local rickettsiae have some antigenic cross-reactivity with one or more of the prototypes, the sera of patients who had a local type should show some antibody titers to the prototype antigens. However, the same sera should have clearer antibody elevation if the local antigen is used. Initially, the sensitized latex reagent was tested with a panel of positive and negative controls. The results corresponded with the positive and negative controls and no cross-positive reactions were detected at a cut-off titer of 1:16 (Table 1). We further evaluated this test with 109 sera from patients with suspected or confirmed scrub typhus using a cut-off titer of ≥1:16. The test was compared with the IFA test, and the overall sensitivity, specificity, and accuracy were 89.1, 98.2, and 93.6%, respectively. We confirmed the WF test lacks sensitivity (47.3%), similar to several studies in Thailand and other Asian countries (Brown *et al*, 1983; Silpapojakul *et al*, 1987; Sirisantana and Poneprasert, 1989; Kelly *et al*, 1990; Chouriyagune *et al*, 1992; Pradutkanchana *et al*, 1997). In conclusion, the LAT has comparable sensitivity and specificity to the IFA test, and is superior to the WF test. Moreover, the speed of the LAT procedure, its simplicity, and its adaptability to any laboratory make it an excellent replacement for the WF screening test. Studies are underway to evaluate this test on a larger scale before the application of routine laboratory testing.

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