

Performance of SD Bioline Tsutsugamushi Assays for the Diagnosis of Scrub Typhus in Thailand

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Objective: To assess the diagnostic capacity of a commercially available test (SD Bioline Tsutsugamushi assay) to aid with the diagnosis of scrub typhus in febrile patients in Thailand.

Material and Method: A commercially available lateral-flow-format immunochromatographic test (ICT) for the detection of *O. tsutsugamushi* IgM, IgG and IgA antibodies was evaluated, using archived serum samples from 102 laboratory confirmed scrub typhus patients and from 63 patients with other causes of fever as the negative control.

Results: The sensitivity, specificity of this rapid immunochromatographic test were 66.7% (95% CI, 57.1 to 75.1%) and 98.4% (95% CI 91.5 to 99.7%) respectively. False positive ICT result occurred in one patient with influenza A infection. Among patients with scrub typhus, 17 out of 38 patients (44.7%, 95% CI 30.2 to 60.3%) with negative IgM antibody test by IFA (titer < 1:50) had positive ICT test. Compared to IFA IgG, 33 out of 54 patients (66.1%, 95% CI 47.8 to 72.9%) with negative IgG antibody test by IFA (titer < 1:50) had positive ICT test.

Conclusion: This rapid ICT test for the diagnosis of scrub typhus was more sensitive than the standard IFA in acute phase specimens.

Keywords: Immunochromatographic assay, Scrub typhus

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Scrub typhus is caused by *Orientia tsutsugamushi*, an obligate intracellular Gram-negative bacterium that has a different cell wall structure and genetic makeup from those of rickettsiae⁽¹⁾. It is transmitted to humans by the bite of the larval stage of trombiculid mites (chiggers). The endemic areas of scrub typhus include rural areas of South-East Asia throughout the Asia Pacific rim and Northern Australia. More than a billion people are at risk of infection and about one million cases occur annually^(1,2). The report incidence of scrub typhus has increased during the previous decade^(1,2). Clinical manifestations of scrub typhus vary widely from a mild and self-limited febrile illness to a more severe course which may be fatal^(3,4). It has been recently recognized as an important cause

of acute undifferentiated fever both in indigenous populations and ill travelers returning from the tropics^(4,5).

Despite the availability of low cost and effective antibiotic treatment, scrub typhus continues to cause significant morbidity and mortality in otherwise healthy adults and children. The greatest challenge to the clinician is diagnosing this infection early in its course, when antibiotic therapy is most effective. Serological diagnosis, using indirect immunofluorescence assay (IFA), or immunoperoxidase test, standard laboratory diagnosis for scrub typhus⁽⁶⁾. These tests are not widely available in rural hospitals. Therefore it is a need for a simple, rapid diagnostic assay for scrub typhus. The objective of the present study described here was to evaluate the diagnostic capacities of a commercial test (SD Bioline Tsutsugamushi assay) for the detection of IgM, IgG and IgA antibodies to aid with the diagnosis of scrub typhus by the use of stored, characterized sera collected from febrile patients in Thailand.

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Material and Method

Indirect immunofluorescence assay (IFA)

IgM and IgG against scrub typhus (*O. tsutsugamushi* pooled Karp, Kato and Gilliam antigens) were detected, using an IFA assay^(4,6). Briefly, patient serum samples were serially diluted two-fold from 1:50 to 1:6,400 in phosphate-buffered saline (PBS) containing 2% (w/v) skim milk powder (Merck), incubated with antigen slide in a humidified atmosphere for 30 minutes at 37°C and washed three times in PBS. Anti-human IgM and IgG fluorescein isothiocyanate conjugate (Jackson Immuno Research Laboratories, West Grove, PA) diluted in PBS-skim milk powder diluent containing 0.00125% (w/v) Evans Blue counter stain was applied to all wells, and wells were incubated in a humidified atmosphere for 30 minutes at 37°C. Slides were examined by fluorescence microscopy (BX50; Olympus, Tokyo, Japan) by two observers at a magnification of 400x. The binding endpoint titer was determined as the highest dilution that showed fluorescence.

SD Bioline *Tsutsugamushi* assay

A commercially available lateral-flow-format immunochromatographic test (Standard Diagnostics Inc, Korea) for the detection of *O. tsutsugamushi* IgM, IgG and IgA antibodies was assessed. Evaluation was performed by the use of specimens collected on admission from acutely ill patients and according to the manufacturer's instructions. Briefly, 10 µl of serum were added to sample well on plastic cassette having immobilized *O. tsutsugamushi* antigen and then 3-4 drops of the assay diluent were added. The results (negative or positive to *O. tsutsugamushi* antibody) were read by one reader (SS), who did not confer, 15-20 minutes after the application of the sample by observing a color line on immunochromatographic test strip, which is visible to naked eye. The results were recorded as positive or negative for the presence of the control and presence or absence of the antibody lines respectively. Samples that gave equivocal results, in which the operator was unsure of the presence of a line, were considered negative and weakly positive lines were considered positive for the purposes of diagnostic evaluation.

Scrub typhus patient samples

Five milliliters of serum samples were collected from 102 patients (64 males and 38 females), aged 15-86 years (mean age 43 years) with acute phase of scrub typhus in 6 hospitals in Thailand between January 2000 and August 2009. Three hospitals are in the

northeastern part of the country (Maharaj Nakhon Ratchasima Hospital, Nakhon Ratchasima Province, Loei Hospital, Loei Province and Banmai Chaiyapod Hospital, Buriram Province), one hospital in the southern part (Chumphon Hospital, Chumphon Province) and two hospitals in the central region (Ratchaburi Hospital, Ratchaburi Province and Siriraj Hospital, Bangkok). Samples were collected as part of studies investigating the causes of fever at these hospitals^(4,7). These clinical studies were approved by the Ethical Review Subcommittee of the Public Health Ministry of Thailand and Siriraj Institutional Review Board, Faculty of Medicine Siriraj Hospital, Mahidol University. Patients provided informed written consent before sample collection. The diagnosis of scrub typhus was made by the detection of either 16S or 56kDa protein encoding gene by nested PCR, or a 4-fold rising titer when paired serum collection was tested by IFA, or an admission-phase sample IgM or IgG IFA assay titer $\geq 1:400$.

Non-scrub typhus patient samples

Serum samples (n = 63) from patients with laboratory confirmed diagnosis of other tropical febrile illness were collected from the same study hospitals as patients with scrub typhus. All non-scrub typhus patient samples were tested by IFA and shown to be negative. The diagnosis of patients in this control group was dengue infection in 17 patients, influenza A or influenza B in 10 patients, murine typhus in 10 patients, leptospirosis in 8 patients, bacteremia from other bacteria such as *Burkholderia pseudomallei*, *Escherchia coli* and *Salmonella* spp in 7 patients, falciparum malaria in 6 patients, suspected spotted fever group rickettsioses in 5 patients. All samples were stored at -70°C until testing.

Data analysis

Diagnostic performance was calculated by comparing the ICT results with the result of the gold standard assay, IFA or PCR for each patient. The reference IFA assay diagnostic criteria for scrub typhus used in the present study was either an admission-phase sample IgM or IgG IFA assay titer $\geq 1:400$ or a four-fold increase between paired admission-phase and convalescent-phase serum samples. Equivocal results were considered negative for the final analysis. A two-by-two table was constructed, in which the gold standard assay result was cross-tabulated with the comparative assay result to define the rates of true-positive, false-positive, false-negative and true-

negative results. The standard diagnostic accuracy indices of sensitivity, specificity, positive predictive value and negative predictive value with 95% confidence intervals (CIs) were calculated, using the SPSS 17.0 software (SPSS Inc, Chicago, IL, USA). Significant differences ($p < 0.05$) between rapid test positivity rates and days of fever and IgM IFA assay titer and assay cross-reactivity using different diagnostic criteria were calculated by using Pearson's Chi-square test.

Results

The diagnosis of scrub typhus was made by the detection of either 16S or 56kDa protein encoding gene from blood in 40 patients (39.2%), a four-four rising in either IgM or IgG antibody titer, using IFA in 46 patients (45.1%) and a high antibody titer on admission in 16 patients (15.7%). The median duration of fever at the time of collection of the admission-phase samples in patients with scrub typhus was 6 days (range 1-47 days) and median interval between obtaining admission phase and convalescence phase samples was 13 days (range 3- 32days).

The sensitivity and specificity of the ICT tests for the detection of IgM, IgG and IgA antibodies against *O. tsutsugamushi* were 66.7% (95% CI, 57.1 to 75.1%), and 98.4% (95% CI 91.5 to 99.7%) respectively. False positive antibody detection was found in only 1 patient with influenza A infection.

Among patients with laboratory confirmed scrub typhus, 17 out of 38 patients (44.7%, 95% CI 30.2 to 60.3%) with negative IgM antibody test by IFA (titer $< 1:50$) had positive ICT test. Compared to IFA IgG, 33 out of 54 patients (66.1%, 95% CI 47.8 to 72.9%) with negative IgG antibody test by IFA (titer $< 1:50$) had positive ICT test. The comparison between IFA IgM and IgG antibody titer, tested on acute serum with the positive rates of the SD Bioline Tsutsugamushi assay are shown in Fig. 1 and 2.

Discussion

Early diagnosis of scrub typhus is important for patient management, to guide appropriate antimicrobial therapy. Empirical therapy with doxycycline is the most cost effective strategy for the management of patients with clinically suspected scrub typhus, because of the absence of rapid, sensitive and affordable diagnostic test in scrub typhus endemic settings⁽⁸⁾. However this could lead to misdiagnosis and patient mismanagement. Standard laboratory diagnosis for scrub typhus is usually based on the

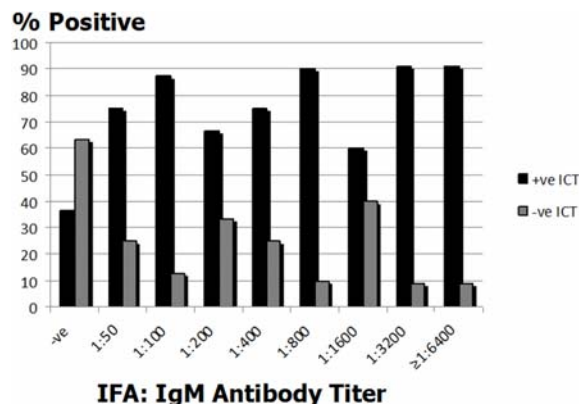


Fig. 1 Percent of +ve ICT and -ve ICT assay vs. IgM antibody titer by IFA

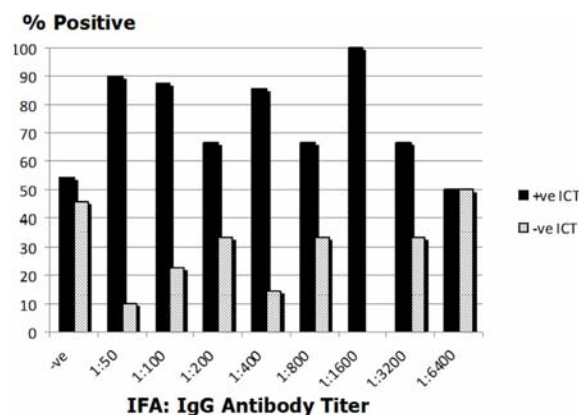


Fig. 2 Percent of +ve ICT and -ve ICT assay vs. IgG antibody titer by IFA

detection of scrub typhus antibodies using IFA. This assay is impractical for routine application in rural settings and has low sensitivity for the diagnosis of scrub typhus in acute phase specimen⁽⁹⁾. There is a clear and urgent need for cheap, accurate and easy assay to use as point-of-care scrub typhus diagnosis.

SD Bioline Tsutsugamushi assay is designed for testing samples collected in the acute phase of infection, which is the "real world" situation for the use of such an assay. In addition, this assay was constructed to detect IgM, IgG and IgA antibodies against *O. tsutsugamushi*. Reinfection with *O. tsutsugamushi* is not uncommon in endemic areas of scrub typhus⁽¹⁰⁾. In the present study, 17 out of 102 patients (16.5%) with scrub typhus developed a four-four rising of IgG or high IgG antibody titer on admission. The pattern of antibody response of reinfection mimics those who had reinfection of dengue infection. Therefore the assay for the detection of both

Table 1. Overall diagnostic accuracy, sensitivity for the rapid ICT for the detection of IgM, IgG, and IgA antibodies compared to the IFA

N(102)	Sensitivity,% (95%CI)	Specificity,% (95%CI)	PPV ^a ,% (95%CI)	NPV ^b ,% (95%CI)
ICT tsutsugamushi	66.7 (57.1 -75.1)	98.4 (91.5-99.7)	98.5 (92.2-99.7)	64.6 (54.6-73.4)

^a Positive Predictive Value

^b Negative Predictive Value

IgM and IgG antibodies at the same time should be more sensitive than those assays which were developed to detect either IgM or IgG antibody against *O. tsutsugamushi*.

However, results of SD Bioline Tsutsugamushi assay was positive in only two-third of the patients with confirmed scrub typhus in the present study. One explanation for this observation is the diversity of antigenically distinct strains of *O. tsutsugamushi* presented in endemic areas. In addition to the three prototype strains; Karp, Kato and Gilliam strains, more than 30 antigenically distinct strains were reported⁽¹⁾. More broadly reactive antigens against various strains of *O. tsutsugamushi* circulating in Thailand should be identified.

In conclusion, the authors have examined SD Bioline Tsutsugamushi, a commercial rapid point-of-care assay for the detection of *O. tsutsugamushi* IgM, IgG and IgA antibodies. Results from this examination of sera from 165 patients with a variety of acute tropical fever diagnoses prevalent in the Southeast Asia suggests that this rapid ICT test for the diagnosis of scrub typhus was more sensitive than the standard IFA in acute phase specimens. Although cross-reactivity between antibodies against *O. tsutsugamushi* with other pathogens was found in only one patient with influenza A virus infection, additional studies are required to determine the true diagnostic utility of the assay as discrepancies between stored samples and prospective studies with the same ICT assays may exist.

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Potential conflicts of interest

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การศึกษาชุดทดสอบเอสดี ไบโอไลน์ ซูซูกามูชิ เพื่อการวินิจฉัยโรคสครับไทฟัสในประเทศไทย

เสาวลักษณ์ ศิลปสาร, ดวงดาว เววา, ศิริวรรณ ชุ่นตระกูล, ชวนพิศ สุทธินนท์, กิตติ โล่สุวรรณรักษ์, ยุพิน ศุภุทธมงคล

วัตถุประสงค์: เพื่อศึกษาความถูกต้องของชุดทดสอบเอสดี ไบโอไลน์ ซูซูกามูชิ ในการวินิจฉัยโรคสครับไทฟัส
วัสดุและวิธีการ: ทำการทดสอบซีรัมผู้ป่วยที่ได้รับการวินิจฉัยว่าเป็นโรคสครับไทฟัส 102 ราย และผู้ป่วยโรคอื่นๆ จำนวน 63 ราย ด้วยชุดทดสอบเอสดี ไบโอไลน์ ซูซูกามูชิ

ผลการศึกษา: ความไวและความจำเพาะของชุดทดสอบเอสดี ไบโอไลน์ ซูซูกามูชิ ในการวินิจฉัยโรคสครับไทฟัส คือ 66.7% (95% CI, 57.1 - 75.1%) และ 98.4% (95% CI 91.5 - 99.7%) ตามลำดับ พบผลบวกลง 1 ราย ในผู้ป่วยที่เป็นไข้หวัดใหญ่ ชนิด เอ ชุดทดสอบนี้ให้ผลบวกในผู้ป่วยสครับไทฟัส 17 รายจาก 38 ราย (44.7%, 95% CI 30.2- 60.3%) ซึ่งตรวจไม่พบอิมมูโนโกลบูลินเอ็มจากการทดสอบด้วยวิธีอิมมูโนฟลูออเรสเซนส์ในการตรวจเลือดครั้งแรก และให้ผลบวกในผู้ป่วย 33 รายจาก 54 ราย (66.1%, 95% CI 47.5- 72.9%) ที่ตรวจไม่พบอิมมูโนโกลบูลินจีจากการทดสอบด้วยวิธีอิมมูโนฟลูออเรสเซนส์ ในการตรวจเลือดครั้งแรก

สรุป: ชุดทดสอบเอสดี ไบโอไลน์ ซูซูกามูชิ มีความไวสูงกว่าการทดสอบด้วยวิธีอิมมูโนฟลูออเรสเซนส์ในการวินิจฉัยโรคสครับไทฟัสเมื่อตรวจเลือดครั้งแรก
