Dot-ELISA Rapid Test Using Recombinant 56-kDa Protein Antigens for Serodiagnosis of Scrub Typhus

Wuttikon Rodkvamtook, Zhiwen Zhang, Chien-Chung Chao, Erin Huber, Dharadhida Bodhidatta, Jariyanart Gaywee, John Grieco, Narongrid Sirisopana, Manerat Kityapan, Michael Lewis, and Wei-Mei Ching*

Armed Forces Research Institute of Medical Science (AFRIMS), Royal Thai Army, Bangkok, Thailand; Naval Medical Research Center (NMRC), Silver Spring, Maryland; Preventive Medicine and Biometrics Department, Uniformed Services University of the Health Sciences (USUHS), Bethesda, Maryland

Abstract. We developed a rapid dot–enzyme-linked immunosorbent assay (dot-ELISA) using the combination of recombinant 56-kDa protein antigens that exhibited broad reactivity with serum antibodies against the four most prevalent strains (Karp, Kato, Gilliam, and TA763) of *Orientia tsutsugamushi*. The assay is rapid (30 minutes), and can be done at room temperature, and results can be read by the naked eye. Only a simple shaker is required to wash the membrane. Sera from 338 patients suspected of being ill with scrub typhus from rural hospitals around Thailand were tested using this dot-ELISA. Seventy-five (22.2%) patients were found to be positive. The sensitivity and specificity of dot-ELISA were determined using the indirect immunofluorescent assay (IFA) test as the gold standard, with the cutoff titer of immunoglobulin peroxidase conjugate M (IgM)/G (IgG) greater than 1:400/1:400. The dot-ELISA had a sensitivity of 98.5%, a specificity of 96.3%, a positive predictive value of 86.7%, and a negative predictive value of 99.6% for the acute-phase specimens. The results indicate that dot-ELISA rapid test using recombinant 56-kDa protein antigen was comparable with the IFA test and may be very useful for the diagnosis of scrub typhus in rural hospitals, where IFA is not available.

INTRODUCTION

Scrub typhus disease is an acute febrile illness that is caused by the gram-negative intracellular bacteria *Orientia tsutsugamushi*.¹ Infection occurs when humans accidentally enter into the natural lifecycle of *Orientia* spp., which involves arthropod vectors, mite larvae called chiggers, and small mammal reservoirs, such as rodents. An infection is heralded by an eschar at the site of the chigger bite followed by the development of a disseminated fever, malaise, myalgia, anorexia, and papulomaculorash.^{2,3} Clinical manifestations of scrub typhus vary widely from a mild and self-limiting febrile illness to a more severe illness that may be fatal.⁴ Traditionally, the diagnosis of scrub typhus mainly relies on serologic tests. The disease could be diagnosed retrospectively in cases of seroconversion or a more than fourfold rise in antibody titers between acute- and convalescent-phase serum specimens.⁵ Currently, laboratory diagnosis of the disease uses immunological techniques, such as the Weil–Felix (WF) test, passive hemagglutination assay, indirect immunofluorescent assay (IFA) test, indirect immunoperoxidase (IIP) test, and enzyme-linked immunosorbent assay (ELISA).⁶ WF test based on the agglutination of the OXK strain *Proteus mirabilis* is the simplest detection method, which has been widely used for clinical identification at hospitals in tropical countries, especially Thailand.⁷ However, it is neither specific nor sensitive. IFA and IIP tests, although they have high sensitivity and specificity, both will provide satisfactory results in the hands of experienced persons but can be troublesome for inexperienced technicians because of microscopic evaluation.⁸ The rapid dot-ELISA (dot-ELISA) technique using scrub typhus antigens from sonicated cultures of Karp, Gilliam, and Kato⁹ was originally developed by the Armed Forces Research Institute of Medical Sciences (AFRIMS) Royal Thai Army in 2006. This test required the culturing of live *Orientia* in the Biosafety Level 3 (BSL3) Laboratory. The

procedures were labor-intensive and imposed potential risks to staff who worked in the BSL-3 Laboratory.

The purpose of this study is the development and evaluation of a dot-ELISA using the combination of recombinant 56-kDa protein antigens that exhibit broad reactivity with serum antibodies against the four most prevalent strains (Karp, Kato, Gilliam, and TA763; NMRC, Silver Spring, MD). This test is simple to perform and suitable for rural hospitals. We evaluated the clinical use of the dot-ELISA test as compared with the IFA test in the diagnosis of scrub typhus.

MATERIALS AND METHODS

Reagents and solution. Peroxidase conjugated anti-human immunoglobulin M (IgM) and G (IgG) were purchased from KPL (Gaithersburg, MD, catalog number 074-1003; IgG: catalog number 0741002). Skim milk (Difco) and hydrogen peroxide (H₂O₂; 30%) were acquired from Fisher Scientific (Fair Lawn, NJ); 4-cholo-1-naphthol was purchased from Bio-Rad Laboratories (Hercules, CA). Triethanolamine-buffered saline [TBS; pH 7.4; 7.5 g NaCl, 2.8 mL N (CH₂CH₂OH)₃, 17 mL 1 N HCl, 0.1 g MgCl₂ 6H₂O, and 0.02 g CaCl₂ 2H₂O, Sigma-Aldrich, St. Louis, MO] was prepared in volumes up to 1 L in distilled, deionized water.

Sera. In total, 562 sera from 338 patients (114 patients with single serum and 224 patients with paired sera) collected at various locations were used in this study. All sera were obtained from patients presenting at general and district hospitals located in the north, northeast, south, and central areas of Thailand (protocol number S014q/45). Inclusion criteria were patients from the Outpatient Department of hospitals who presented with acute fever, history of fever for no more than 2 weeks, and oral temperature equal to or more than 38°C. At least 3–14 days after the first serum sample was drawn, the second serum sample was collected. All sera were tested for the specific IgM and IgG antibodies to scrub typhus by dot-ELISA and IFA test.

In addition, sera from patients confirmed for other diseases were evaluated for cross-reactivity of the rapid test. There were 15 for leptospirosis (IFA IgM titer against *Leptospira*

*Address correspondence to Wei-Mei Ching, Viral and Rickettsial Disease Program, Infectious Diseases Department, Code 41, Naval Medical Research Center, Silver Spring, MD 20910-7500. E-mail: wei.ching@med.navy.mil

interrogans serovar *bataviae* $\geq 1:400$),¹⁰ 20 for murine typhus (IFA titer against *Rickettsia typhi* $\geq 1:400$),¹¹ 14 for malaria¹² (peripheral smear-positive), and 18 for dengue fever (hemagglutination inhibition antibody titer against dengue virus $\geq 1:10,240$).¹³

56-kDa Protein antigens preparation for dot-ELISA. Cloning, expression, and purification of the 56-kDa protein antigens were carried out as described previously by Chao and others.¹⁴ Briefly, polymerase chain reaction (PCR) amplicons using DNA extracted from indicated strains of *Orientia* were obtained using standard PCR procedures. The amplicons were cloned into pET24a vectors (EMD, Billerica, MA), and the sequence of each clone was confirmed. The plasmid was transformed into BL21(DE3) cells (Invitrogen, Grand Island, NY), induced, and grown for 19 hours. The collected cells were sonicated, and the inclusion body that contained the expressed protein was extracted with buffer A (20 mM Tris HCl, pH 7.5) containing different concentrations of urea (Acros Organics, Pittsburgh, PA). The majority of recombinant 56-kDa protein was in the 6 M urea fraction. The protein was purified using anion exchange chromatography (DEAE) in an high performance liquid chromatography (HPLC) system. The fractions with high purity were collected, and a serial dialysis with descending concentration of urea was performed to remove all residual urea. The final dialysis was carried out against buffer A without any urea.

Antigen disc preparation. A nitrocellulose sheet (P/N66485; BioTrace, Timonium, MD) was cut into 5.5-mm-diameter discs and placed on 96-well flat-bottomed microtiter plates. The mixture of all recombinant 56-kDa protein (Karp, Gilliam, Kato, and TA763) antigens (0.2 μ g in 1 μ L) was dotted on the nitrocellulose discs (the optimum concentrations of scrub typhus antigens were determined by checkerboard titration), air-dried, and stored at room temperature until use.

To analyze a sample, the typical plate design has one well each of IgM control (MC) dotted with human IgM (Sigma; I-8260) and IgG control (GC) dotted with human IgG (Sigma I-2511; Sigma). For test samples, each was evaluated for both IgM (MT) and IgG (GT) on the same plate. An example of a group of four wells of MC, MT, GC, and GT and representative results are shown in Figure 1.

Serum preparation for dot-ELISA. Serum was consistently diluted at 1:625 as originally determined in Thailand by the AFRIMS Royal Thai Army in 2009⁹ as follows. First, add 240 μ L and serum diluent (0.5% skim milk in TBS) to tubes 1 and 2, respectively. Second, add 10 μ L patient serum to tube 1 and mix well. Third, take 10 μ L diluted serum from tube 1, transfer to tube 2, and mix well. This dilution factor may need additional optimization if the assay is performed in other scrub typhus-endemic countries.

Anti-Ig preparation for dot-ELISA. Goat anti-human IgM and IgG were diluted at 1:350 in 0.5% skim milk in TBS.

Procedures for the dot-ELISA. In total, 100 μ L blocking buffer (2.0% skim milk in TBS) was added to each microtiter plate well and distributed over the membrane by tapping the plate against the heel of the hand. The plate was incubated at room temperature for 10 minutes before removal of the buffer.

The diluted serum (50 μ L) from tube 2 (serum preparation as described above) was added to each well, distributed evenly across the membrane, and incubated at room temper-

FIGURE 1. Example of the dot-ELISA test. A typical plate design dot-ELISA test for both IgG and IgM and representative results are shown. For each serum sample to be tested, four wells were used. Among these wells, one well each of IgM control (MC) and IgG control (GC) were dotted with human IgM and IgG, respectively. The other two wells were dotted with the mixture of all recombinant 56-kDa protein (Karp, Gilliam, Kato, and TA763) antigens (0.2 μ g in 1 μ L) for both IgM (MT) and IgG (GT) tests of test serum samples. The preparation and procedure of the dot-ELISA test were performed as described in Materials and Methods. Column A shows positive IgM and IgG. Lane B shows positive IgM and negative IgG. Lane C shows negative IgM and positive IgG. Lane D shows negative IgG and IgM.

ature for 10 minutes. The serum was then removed, and 100 μ L/well washing buffer (0.05% octylphenoxy poly [ethyleneoxy]ethanol [IGEPAL] detergent [CAS 9036-19-5; Sigma] in TBS) was added to each well. After repeating the washing step three times, 50 μ L anti-human IgM or IgG was added to MC/MT and GC/GT wells, respectively. After incubation at room temperature for 10 minutes, the conjugate solution was removed and washed three times as described above. The substrate solution, freshly made by mixing 4-chloro-1-naphthol and substrate buffer (30% H_2O_2 in TBS) at a ratio of 1:4 (e.g., 50 μ L 4-chloro-1-naphthol and 200 μ L substrate buffer), was added (50 μ L/well). After incubation at room temperature for 5–10 minutes, the substrate solution was removed, and TBS buffer (100 μ L/well) was added to stop the reaction and removed after 1 minute.

Reading the results of dot-ELISA (Table 1). Positive: purple-blue dots on the MT and/or GT discs as well as the corresponding control MC and GC discs indicate the presence of scrub typhus-specific antibodies. Negative: no purple-blue dots on the MT and/or GT discs but purple dots on the MC and/or GC discs indicates no presence of scrub typhus-specific antibodies. Invalid assay: no purple-blue dots on the MC and GC discs, regardless the presence or absence of purple-blue dots on the MT and/or GT discs, indicate that the test kit is not working. Test again by using a new test kit. An example of the plate arrangement is shown in Figure 1.

TABLE 1
Interpretation of dot-ELISA results

Purple-blue dots present (+) or absent (-)	Test results
MT (+), GT (+), MC (+), GC (+)	Both IgG- and IgM-positive
MT (+), GT (-), MC (+), GC (+)	IgM-positive only
MT (-), GT (+), MC (+), GC (+)	IgG-positive only
MT (-), GT (-), MC (+), GC (+)	Negative
MC (-) and/or GC (-)*	Invalid

*If either one or both control wells (i.e., MC and GC) do not show purple-blue dots, the test is considered invalid. The sample should be analyzed again to obtain a result.

Indirect IFA. The method was described by Bozeman and Elisberg¹⁵ first and then Robinson and others¹⁶ with some modification. Briefly, antigens used were pooled from *O. tsutsugamushi* Karp, Kato, and Gilliam strains-infected mouse fibroblast cell culture (L929). Fluorescein isothiocyanate (FITC) conjugated to rabbit anti-human IgM and IgG (Santa Cruz Biotechnology, Dallas, TX) was used to detect *O. tsutsugamushi*-specific antibodies in human samples. Serum samples were primarily screened against the pooled antigen at a 1:50 dilution. If a sample showed reactivity, the antibody titer was determined with serial dilutions of 1:100, 1:200, 1:400, 1:800, 1:1,600, 1:3,200, 1:6,400, and 1:12,800. Pooled serum from a *O. tsutsugamushi*-infected patients was used as a positive control, and pooled normal human serum was used as a negative control. A single specimen with an IgM or IgG titer ≥ 400 was interpreted as positive. For paired specimens, if they showed seroconversion or a fourfold or higher rise in titer, they were interpreted as positive.³

Data analysis. The dot-ELISA was assessed for its sensitivity and specificity by 2×2 tables compared with the IFA test.

RESULTS

Sera collected from 338 patients (114 single serum and 224 paired sera samples) suspected of scrub typhus at rural hospitals in Thailand were tested using the dot-ELISA and IFA. Seventy-five (22.2%) patients were found to be positive by dot-ELISA, and 66 (19.5%) patients were found to be positive by IFA tests using the first (i.e., acute-phase) serum of all 338 patients.

The sensitivity and specificity of dot-ELISA using recombinant 56-kDa protein antigen were determined using the IFA test as the gold standard, with the positive cutoff titer of IgM or IgG being greater than 400. The dot-ELISA had a sensitivity of 98.5%, a specificity of 96.3%, a positive predictive value of 86.7%, and a negative predictive value of 99.6% in the first serum samples of all 338 patients, which consisted of the first serum samples from 224 patients who had paired serum samples and 114 serum samples from those who only had single specimens for a total of 338 sera (Table 2).

The performance of dot-ELISA using recombinant 56-kDa protein antigens for the second (i.e., convalescent) serum of 224 paired sera was also determined using the IFA test as the gold standard, with the cutoff titer of greater than 1:400 or a fourfold rise in titer. The results are shown in Table 3. The 98.3% sensitivity, 97.0% specificity, 92.2% positive predictive value, and 99.4% negative predictive value have clearly shown that this dot-ELISA method is as good as the traditional IFA.

To ensure that the recombinant protein antigens used in the dot-ELISA assay do not cross-react with sera from patients with other infectious diseases, 77 additional samples that were

TABLE 2
Comparison of dot-ELISA and IFA to diagnose scrub typhus

Dot-ELISA	IFA	
	Positive	Negative
Positive	65	10
Negative	1	262

Samples tested were first sera from 224 patients from whom paired sera were available and single sera from 114 patients.

TABLE 3
Comparison of dot-ELISA and IFA to diagnose scrub typhus in the second sera of 224 patients

Dot-ELISA	IFA	
	Positive	Negative
Positive	59	5
Negative	1	159

confirmed for infections, including leptospirosis, murine typhus, malaria, and dengue, were tested.

Among 15 sera from leptospirosis patients, only 1 serum sample showed positive results for scrub typhus by both IFA and dot-ELISA (1 of 15). This is possible, because coinfection by *Leptospira* and *O. tsutsugamushi* has been observed in patients with acute febrile illness in Thailand.¹⁷ However, none of the serum samples from patients with murine typhus (0 of 20), malaria (0 of 14), and dengue fever (0 of 18) showed positive results. These results suggested that there was no cross-reactivity between the recombinant proteins used and these disease-specific antibodies.

DISCUSSION

For the diagnosis of scrub typhus, serological testing is the method of choice.¹⁸ The WF test was the first technique developed,¹⁹ but it is neither sensitive nor specific and now, rarely used.²⁰ The IFA test¹⁶ and the IIP²¹ test are now considered the assays of choice. However, use of the IFA is limited, because it requires fluorescence microscopes,²² and the IIP test is not commercially available. Also, because of variations in antigen preparation used in IFA and IIP, the sensitivity and specificity can vary significantly among test centers.²³ Other immunological techniques for the diagnosis of scrub typhus have been developed, including a passive hemagglutination assay,²⁴ ELISAs,²⁵ dot-blot immunoassays,²⁶ and PCR.²⁷ However, none of these methods is routinely used on a widespread basis. Scrub typhus is an endemic infectious disease in rural areas of Asia, and diagnosis is the biggest challenge.⁵ Misdiagnoses and delayed treatment of scrub typhus are frequent in Thailand and often lead to advanced disease, multiorgan failure, and even death.

In this study, we evaluated the performance of the dot-ELISA rapid test using recombinant 56-kDa protein antigens to detect scrub typhus antibody in 562 sera from 338 patients with acute fever illness (5–10 days after the onset of illness) of unknown origin (FUO). The dot-ELISA and the IFA methods performed almost identically. The sensitivity and specificity of dot-ELISA to detect scrub typhus antibody from patients in the acute phase of illness were 98.5% and 96.3%, respectively, using IFA results as the gold standard. In addition, the results in the second serum of the paired sera in 224 patients showed that the sensitivity and specificity are 98.3% and 97.0%, respectively. The high sensitivity and specificity with acute sera by dot-ELISA rapid test using recombinant 56-kDa protein antigen have suggested that the test is suitable for early diagnosis of scrub typhus during the acute phase of illness to avoid delayed treatment and death because of multiorgan failure.

One of the significant advantages of the dot-ELISA rapid test using recombinant 56-kDa protein antigens is that it consists of several recombinant 56-kDa proteins, including

TABLE 4
Comparison of dot-ELISA and IFA tests to diagnose scrub typhus

Condition	Dot-ELISA	IFA test
Antigen preparation	Recombinant 56-kDa protein/BSL-2	L-929 cell culture/BSL-3 facility
Antigen purity	Purified	Crude antigen
Antigen on solid phase	Recombinant proteins	Whole-cell antigens
Antigen kept at	4°C	-20°C
Equipment required	Not required (naked eye)	Fluorescence microscope
Personnel required	Moderate to little experience	Extensive experience
Time performance	30 minutes	2 hours
Result shown as	Purple-blue dot on nitrocellulose	Green fluorescent-labeled <i>Orientia</i> organism

O. tsutsugamushi serotypes Karp, Kato, Gilliam, and TA763, which enhance its sensitivity. In addition, the recombinant protein antigen preparation can be easily standardized in the BSL-2 Laboratory; thus, test result variations among different laboratories can be minimized. This is in contrast to IFA, which requires the propagation of live *Orientia* in the BSL-3 Laboratory, a procedure difficult to standardize among different laboratories. The particular advantages and disadvantages between dot-ELISA and IFA test are shown in Table 4.

Lateral flow tests (LFTs) for scrub typhus diagnosis are commercially available. These tests were constructed using either a single recombinant protein (SD Bioline) or a combination of several recombinant proteins (Access Bios and InBios). Multiple groups have evaluated these LFTs. Although satisfactory clinical performance was observed in general,²⁸⁻³² it is worth noting that the manufacturing of these tests requires a specially equipped facility. On the contrary, the making of dot-ELISA plate needs neither special equipment nor facility, and any laboratory with the supply of recombinant proteins can prepare the dot-ELISA plate. Therefore, the dot-ELISA plate is an alternative to the commercially available LFT, particularly in resource-limited areas.

The traditional gold standard IFA assay is difficult to use in rural areas because of limited sources. A rapid and reliable diagnosis method is urgently needed in Thailand, and we believe that this dot-ELISA rapid test using the recombinant 56-kDa protein antigen is particularly suitable for use in rural settings. The dot-ELISA rapid test kit described in this study is simple to use. It can be used in rural hospitals and is effective for serodiagnosis of scrub typhus. The preparation of scrub typhus recombinant 56-kDa protein antigens is not very complicated. They can be stored at 4°C until use. In addition, after the antigen nitrocellulose discs are prepared, they can be kept at room temperature for more than 1 year without antigenic change.¹⁰ The advantage of this test compared with the IFA test is that it is quick, easy, and relatively inexpensive. In contrast, the IFA test requires a fluorescence microscope, experienced technical staff, growing *Orientia* in large quantities in a BSL-3 facility, and use of sophisticated and expensive equipment available only in referral centers. Our results suggest that the dot-ELISA test is useful in the diagnosis of scrub typhus and suitable for rural hospitals.

In conclusion, the results of this study indicate that the dot-ELISA rapid test using recombinant 56-kDa protein antigens was sensitive and specific compared with the IFA test and may be very useful for the diagnosis of scrub typhus in rural hospitals, where IFA tests are not available. Although the assay has been tested in our laboratory using sera from other parts of Asian Pacific region (data not shown), it is worth

noting that the assay has not been extensively validated in these endemic areas. Therefore, additional studies in other geographic areas, including proper dilution of serum samples, are definitely needed to support the findings of this study.

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Authors' addresses: Wuttikon Rodkvamtook, Dharadhida Bodhidatta, Jariyanart Gaywee, Narongrid Sirisopana, and Manerat Kityapan, Microbiology, Armed Forces Research Institute of Medical Science, Bangkok, Thailand, E-mails: ltwtutti@hotmail.com, dbodhi@hotmail.com, jariyanart@yahoo.com, Narongrid1956@yahoo.com, and kratai_usagi@hotmail.com. Zhiwen Zhang, Chien-Chung Chao, Erin Huber, and Wei-Mei Ching, Viral and Rickettsial Diseases Department, Naval Medical Research Center, Silver Spring, MD, and Preventive Medicine and Biometrics Department, Uniformed Services University of the Health Sciences, Bethesda, MD, E-mails: Zhiwen.zhang@med.navy.mil, chien-chung.chao@med.navy.mil, erin.huber@med.navy.mil, and wei.ching@med.navy.mil. John Grieco and Michael Lewis, Preventive Medicine and Biometrics Department, Uniformed Services University of the Health Sciences, Bethesda, MD, E-mail: johngrieco88@gmail.com and dr.michael.lewis@gmail.com.

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