Evaluation of Enzyme-Linked Immunosorbent Assay Using Recombinant 56-kDa Type-Specific Antigens Derived from Multiple *Orientia tsutsugamushi* Strains for Detection of Scrub Typhus Infection

Su-Lin Yang,¹ Kun-Hsien Tsai,² Hsiang-Fei Chen,¹ Jun-Yu Luo,¹ and Pei-Yun Shu^{1*}

¹Center for Diagnostics and Vaccine Development, Centers for Disease Control, Ministry of Health and Welfare, Taipei, Republic of China;

²Department of Public Health and Institute of Environmental Health, College of Public Health, National Taiwan University, Taipei, Republic of China

Abstract. Scrub typhus is caused by the intracellular bacterium *Orientia tsutsugamushi*. The 56-kDa type-specific antigen (TSA) displays a significant antigenic variation across different *O. tsutsugamushi* strains. To minimize the influence of the antigenic diversity of TSA on assay sensitivity, we developed a mixed-TSA enzyme-linked immunosorbent assay (mixed-TSA ELISA) using a mixture of recombinant TSAs of prototype (Karp, Gilliam, and Kato) and local (TW-1, TW-10, TW-19, and TW-22) *O. tsutsugamushi* strains as antigens to detect immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies against *O. tsutsugamushi*. These four local strains covered a major part of the total genetic diversity of TSA gene of *O. tsutsugamushi* in Taiwan. A total of 109 acute-phase serum samples from *O. tsutsugamushi* polymerase chain reaction–positive, scrub typhus patients, and 82 negative control serum samples from non-scrub typhus cases were used for evaluation of the recombinant TSA-based ELISA. We compared the performance of the mixed-TSA ELISA with immunofluorescence assay (IFA), which is considered the gold standard method for the serological diagnosis of scrub typhus. The results indicated that the sensitivity of IgM mixed-TSA ELISA (80.7%) was significantly higher than that of IgM IFA (68.8%). We demonstrated that the mixed-TSA ELISA had a high sensitivity and specificity and can be used for screening of scrub typhus patient in the early phase of the disease.

INTRODUCTION

Scrub typhus is caused by the intracellular bacterium *Orientia tsutsugamushi* following the bite of an infected larval-stage trombiculid mite (chigger)¹ and is endemic in most countries across the Asian–Pacific region and Northern Australia.^{2,3} An estimated one million infections occur every year, and the case fatality rate can be up to 30–70%, if not treated appropriately.^{4–6} Scrub typhus is the most important rickettsial disease in Taiwan and has been designated a notifiable infectious disease since 1955. The number of laboratory-confirmed cases of scrub typhus between 2011 and 2016 were 322, 460, 538, 414, 494, and 488, respectively (<http://www.cdc.gov.tw>). The current case number of scrub typhus has exceeded that of dengue and constitutes a major vector-borne infectious disease in Taiwan.

Symptoms of scrub typhus include fever, headache, eschar, rash, cough, body aches, and muscle pain. Severe manifestations may include pneumonia, meningitis, encephalitis, disseminated intravascular coagulation, and multi-organ failure.^{7–9} Scrub typhus is often undiagnosed or misdiagnosed because of its clinical symptoms, which are similar to many other febrile infectious diseases, such as murine typhus, leptospirosis, and dengue virus infections. Early diagnosis and treatment of patients with scrub typhus are crucial for improving the outcome of the disease. The diagnosis of scrub typhus has relied on the detection of antibodies against *O. tsutsugamushi* in paired acute and convalescent serum samples. At present, the indirect immunofluorescence assay (IFA) is considered the gold standard method for the serological diagnosis of scrub typhus.^{10,11} However, the IFA procedure is time consuming and requires specialized equipment and well-trained personnel; therefore, it is necessary to develop the more time-effective, accurate, and

labor-saving diagnostics to process large numbers of samples in highly endemic areas, including Taiwan.

The 56-kDa type-specific antigen (TSA) is a major outer membrane protein of *O. tsutsugamushi* and is the immunodominant target of human antibody response.^{12–14} We previously demonstrated that there were many TSA sequence types of *O. tsutsugamushi* strains cocirculating in Taiwan, and some of these strains were unique to Taiwan.¹⁵ Recent studies also indicated that more divergent genotypes of *O. tsutsugamushi* prevail in Taiwan compared with other endemic countries in the Asian–Pacific region.^{16,17} Therefore, it is necessary to consider the impact of broad genetic diversity of TSA gene of *O. tsutsugamushi* strains on diagnosis of scrub typhus in Taiwan.

Recombinant TSA proteins derived from prototype (such as Karp, Gilliam, and Kato strains) or local strains have been used to develop several diagnostic assays and have shown promising diagnostic accuracy.^{18–24} However, it is worth noting that the wide antigenic diversity of TSA and the different patterns of the geographical distribution of *O. tsutsugamushi* genotypes may restrict the performance of these assays in different countries.

To develop a rapid and sensitive method for the diagnosis of scrub typhus, three prototype strains (Karp, Gilliam, and Kato) and four Taiwanese endemic strains (TW-1, TW-10, TW-19, and TW-22) of *O. tsutsugamushi* were cloned and expressed in an *Escherichia coli* expression system. The recombinant TSA proteins from these seven strains were pooled together and served as antigens for the development of the mixed-TSA IgM and IgG ELISA for the detection of *O. tsutsugamushi* infection. Because early diagnosis is essential to provide proper medical treatment for scrub typhus patients, we evaluate the mixed-TSA ELISA using acute-phase serum samples from *O. tsutsugamushi* polymerase chain reaction (PCR)–positive, scrub typhus patients and compared the results with those of IFA.

MATERIALS AND METHODS

Human samples. Scrub typhus is a notifiable infectious disease in Taiwan and suspected cases must be reported to

* Address correspondence to Pei-Yun Shu, Center for Diagnostics and Vaccine Development, Centers for Disease Control, Ministry of Health and Welfare, No. 161, Kunyang St., Taipei 11561, Republic of China. E-mail: pyshu@cdc.gov.tw

Taiwan Centers for Disease Control (Taiwan CDC). A suspected scrub typhus case is a patient presenting signs or symptoms such as fever, headache, body aches, chills, night sweats, swollen lymph nodes, rash, and eschar. If a patient with abovementioned signs or symptoms has recently experienced field activity, the doctor might suspect that it is scrub typhus and reported to the Taiwan CDC. Both whole-blood and serum samples from patients with suspected scrub typhus were collected and sent to the Taiwan CDC for laboratory diagnosis. Peripheral blood mononuclear cells (PBMCs) isolated from blood samples were used for real-time quantitative PCR (qPCR) and paired serum samples were used for serological diagnosis.¹⁵ The samples were considered positive for scrub typhus based on positive results by qPCR, or a seroconversion or at least a 4-fold increase in titer of the IgM or IgG in paired acute and convalescent serum samples and a titer equal to or greater than 1:80 in acute or convalescent serum sample for IgG tested by IFA. A total of 109 serum samples collected from PCR-positive, scrub typhus patients were used to evaluate the performance of the recombinant TSA-based ELISA. These samples were collected from Taiwan (the Main Island) and offshore islands representing different geographical regions of the country. Supplemental Figure 1A showed geographic distribution of 109 PCR-positive, scrub typhus patients. The median fever day of these specimens was 6 days after onset of disease (interquartile range [IQR] 3–8 days). The median age of the patients was 57 years (IQR, 45–65 years), and 41 patients (37.6%) were male. A total of 82 serum samples with negative PCR and IFA results of scrub typhus were used as negative control group, including samples collected from patients with fever of unknown origin ($n = 48$), murine typhus ($n = 10$), dengue fever ($n = 10$), Japanese encephalitis ($n = 12$), Chikungunya ($n = 1$), and Zika virus infection ($n = 1$). Supplemental Figure 1B showed geographic distribution of 82 non-scrub typhus cases. All human samples used in this study were collected during 2016–2017.

Ethics statement. The study protocol was reviewed and approved by the Taiwan CDC Institutional Review Board (104128). The informed consent requirement was waived by the board.

Real-time PCR. Peripheral blood mononuclear cells isolated from acute-phase blood samples of suspected scrub typhus patients were used for the detection of *O. tsutsugamushi* infection by real-time PCR. Peripheral blood mononuclear cells were purified from 4 mL of blood sample by Ficoll-Paque™ Plus (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and then washed and resuspended in 400 μ L of phosphate buffered saline (PBS) containing 2% fetal calf serum. A 200- μ L volume of PBMC suspension was subjected to DNA extraction using the QIAamp DNA blood Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. Primer sets targeting the TSA gene (RST-14F: 5'-CCATTTGGTGG TACATTAGCTGCAGGT-3'; RST-6R: 5'-TCACGATCAGC TATACTTATAGGCA-3') and the 16S ribosomal RNA gene (OTF7: 5'-CCAGYGGGTRATGCCGGGAACAT-3'; OTR6: 5'GGCAGTGTGTACAAGGCCCGAGAA-3') of *O. tsutsugamushi* were used in the SYBR Green I-based real-time PCR reaction.²⁵ Real-time PCR amplification was performed using the Fast Start Essential DNA Green Master kit (Roche Diagnostics, Basel, Switzerland) with the following parameters: 94°C for 15 minutes, 40 cycles of 94°C for 15 seconds (denaturation), 55°C for 30 seconds (annealing), 72°C for 20

seconds (extension), and 77°C for 30 seconds (data collection). Following amplification, a melting curve analysis was performed to verify the correct product by its specific melting temperature (T_m). Melting curve analysis consisted of a denaturation step at 95°C for 1 minute, lowered to 65°C for 30 seconds, and followed by a gradual increase in temperature (1°C/30 seconds) to 90°C with continuous reading of fluorescence. The results were analyzed with the melting curve analysis software of the LightCycler 96 Real-Time PCR System (Roche Diagnostics, Mannheim Germany). Polymerase chain reaction–positive samples are defined as positive results using both primer sets targeting the TSA gene (RST-14F and RST-6R) and the 16S rRNA gene (OTF7 and OTR6). Polymerase chain reaction products obtained using RST-14F and RST-6R primer set were subjected to direct sequencing for confirmation of *O. tsutsugamushi* infection.

Indirect IFA. *Orientia tsutsugamushi* whole-cell antigens, Karp, Gilliam, and Kato strains at an equal weight ratio were dotted on Teflon-coated spot glass slides, followed by fixation and permeabilization with ice-cold acetone/methanol (1:1) for 10 minutes. The slides were air-dried and blocked with PBS containing 1% goat serum. Serum samples for the detection of IgM antibodies were pretreated with IgM pretreatment diluent (Focus Diagnostics, Cypress, CA) according to the manufacturer's protocol. Serum samples were serially diluted (from 1:10 to 1:5,120) with PBS containing 2% (w/v) skim milk powder and incubated with the antigen-coated spot on a slide in a humidified atmosphere for 30 minutes at 37°C. The samples were then washed three times with PBS containing 0.05% Tween-20 (PBST) and air-dried. Subsequently, fluorescein isothiocyanate-conjugated anti-human IgM and IgG (Sigma, St. Louis, MO) were diluted with PBS containing Evans blue counterstain (Sigma Chemical Company), applied to an antigen-coated spot in a humidified atmosphere for 30 minutes at 37°C and washed three times with PBST. The slides were examined by epifluorescence microscopy (Zeiss) by two observers at a magnification of $\times 400$. The binding

FIGURE 1. Analysis of *Escherichia coli* expressing recombinant type-specific antigen (TSA) proteins. **(A)** Polymerase chain reaction–amplified products of the 56-kDa TSA from genomic DNA of *Orientia tsutsugamushi* strains, including TW-1, TW-10, TW-19, TW-22, Kato, Gilliam, and Karp (lane 1–7). Lane M, 1-Kb DNA ladder marker. **(B)** Purified TSA proteins were resolved by 12% Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and stained with Coomassie Blue. Lane 1–7, TSA of TW-1, TW-10, TW-19, TW-22, Kato, Gilliam, and Karp. Lane M, protein molecular weight marker. **(C)** Western blot analysis of recombinant TSA proteins from different strains. An anti-His tag antibody and anti-TSA monoclonal antibody were used to detect recombinant TSA proteins on membrane. This figure appears in color at www.ajtmh.org.

endpoint titer was determined as the highest dilution with a positive fluorescence reaction.

Type-specific antigen gene cloning. Nucleotide sequences of TSA gene fragments of TW-1, TW-10, TW-19, TW-22, Karp, Gilliam, and Kato (GenBank accession numbers GQ332742, GQ332751, GQ332760, GQ332763, AY956315, DQ485289, and AY836148, respectively) strains of *O. tsutsugamushi* were amplified by PCR using a forward primer: 5'-CA GGATCC GAG TGC GAT AGA ATT GGG -3' (underline shows the *Bam*HI restriction enzyme site) and a reverse primer: 5'-GC CTCGAG GCC AAC CAT ACT CAG -3' (underline shows the *Xho*I restriction enzyme site) to yield fragments encoding the truncated TSA protein of each strain. The truncated TSA proteins consist of 411–423 amino acid residues with the first 20 amino acids of the N-terminus deleted. The amplified fragments were digested with *Bam*HI and *Xho*I and then cloned into the *Bam*HI and *Xho*I site of the pET47b vector (Novagen, Madison, WI). The partial TSA gene fragment inserts of each *O. tsutsugamushi* strain were confirmed by DNA sequencing (ABI Prism 3730XL DNA sequencer; Applied Biosystems, Foster City, CA).

Expression and purification of recombinant TSA protein. Nucleotide sequences encoding the truncated TSA protein of each *O. tsutsugamushi* strain were cloned into the expression vector and expressed in *E. coli* BL21. The resulting recombinant TSA protein contained a 6× histidine (His) tag at its 5' end. Isolation of recombinant TSA protein from *E. coli* was performed using the BugBuster Extraction Reagent (Novagen) according to the manufacturer's instructions. The inclusion body that contained the recombinant protein was resolved with 8 M urea at room temperature for 2 hours and then purified by His-Bind Kits (Novagen) according to the manufacturer's instructions. The recombinant TSA protein was dissolved in the elution buffer and was further dissolved using sequential dialysis into 4 M urea and 2 M urea in 20 mM Tris buffer (pH 8.0) and finally with urea-free Tris buffer.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis. The purified TSA protein was analyzed by SDS-PAGE. The polyacrylamide gels were either stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Hercules, CA) and scanned with an ImageQuant LAS 4000 biomolecular imager

FIGURE 2. The distribution of immunofluorescence assay (IFA) IgM and IgG titers. (A) One hundred and nine serum samples from qPCR-positive, scrub typhus patients. (B) Eighty-two serum samples from non-scrub typhus cases. The numbers shown on the top of each bar indicate the number of samples with their respective IFA titer.

(GE Healthcare Bio-Sciences AB) for quantitation and analysis of purity of recombinant TSA protein or transferred onto an iBlot® 2 nitrocellulose regular stacks by iBlot® 2 gel transfer device (Thermo Fisher Scientific, Carlsbad, CA) for immunoblotting. Antibodies used in the immunoblotting including anti-TSA monoclonal antibody (1:100 dilution; YH560001, Yao-Hong biotechnology Inc., New Taipei City, R.O.C.), anti-poly-His tag monoclonal antibody (Sigma) and serum samples (1:100 dilution) from scrub typhus patients. Proteins of interest were detected using the appropriate IgG-horseradish peroxidase secondary antibody and enhanced chemiluminescent reagent. Blotted proteins were scanned with an Image-Quant LAS 4000 mini biomolecular imager (Fuji Film, Tokyo, Japan).

Recombinant TSA-based ELISA. Recombinant TSA proteins of TW-1, TW-10, TW-19, TW-22, Karp, Gilliam, and Kato strains at a fine-tuning weight ratio of 4:2:2:2:4:1:1 (the mixed-TSA ELISA), or Karp, Gilliam, and Kato strains at an equal weight ratio (the KGK ELISA) were pooled together and served as antigens for the detection of IgM and IgG against *O. tsutsugamushi*. Each well of 96-well microtiter plates (Corning, Corning, NY) was coated with pooled recombinant TSA proteins at 2.5 µg/mL (100 µL/well) in 0.05 M Na₂CO₃/NaHCO₃, pH 9.5, and incubated overnight at 4°C. After washing with PBST, the wells were blocked with 200 µL of PBS-1% bovine serum albumin for 1 hour at 37°C. After washing, serum samples were added at a 1:100 dilution in PBST-4% normal goat serum-2% normal rabbit serum and incubated for 1 hour at 37°C. Goat anti-human IgM or IgG conjugated to alkaline phosphatase (Jackson Immuno Research Laboratories, West Grove, PA) was then added and incubated for 1 hour at 37°C. The enzyme activity was developed with the addition of the substrate *p*-nitrophenylphosphate (Chemicon, Temecula, CA), and the optical density (OD) was determined 30 minutes later at the dual wavelengths of 405 and 630 nm with an ELISA reader (Spectra max 250, Molecular Devices, Sunnyvale, CA).

Determination of cutoff value of the recombinant TSA-based ELISA. Cutoff values for IgM and IgG ELISA were determined by two different calculations. The first cutoff value was defined as the mean OD of negative control sera (82 samples) plus three standard deviations (SDs).²⁶ The second cutoff values were determined by generating a receiver operating characteristic (ROC) curve using GraphPad Prism v

5.03 (GraphPad Software Inc., San Diego, CA). The cutoff values of the IgM and IgG ELISAs were selected as the values that maximized the sum of sensitivity and specificity using Youden's index.²⁷

Statistical and genetic analyses. To reduce the risk of bias in assessment, the interpretation of PCR, IFA, and ELISA test results was carried out in a blinded manner. Diagnostic accuracy was calculated by comparing the results of ELISA and IFA. A two-by-two table was constructed, variables measured included the numbers of true positives (TPs), true negatives (TNs), false positives (FPs), and false negatives (FNs). Statistical methods used to compare ELISA and IFA included: sensitivity = TP/(TP + FN), specificity = TN/(TN + FP), positive predictive value = TP/(TP + FP), and negative predictive value = TN/(FN + TN). To test accuracy, the proportion of all tests that represent a correct result was defined as (TP + TN)/number of all tests. Data analysis was performed using the Microsoft Excel and GraphPad Prism v 5.03. Statistical analysis was performed using Chi-square test. The *P*-value < 0.05 was considered statistically significant. The phylogenetic analysis was performed using MEGA version 7 (<http://www.megasoftware.net/>).²⁸ To construct the phylogenetic trees, the neighbor-joining method using the maximum composite likelihood as a substitution model were used. The reliability of the analysis was evaluated by a bootstrap test with 1,000 replications.

RESULTS

Expression of recombinant TSA proteins from various *O. tsutsugamushi* strains. Recombinant TSA protein genes from various *O. tsutsugamushi* strains were cloned and expressed in a prokaryotic expression system. The TSA genes of different strains of *O. tsutsugamushi* were amplified by PCR. Figure 1A shows the PCR products of TW-1, TW-10, TW-19, TW-22, Karp, Gilliam, and Kato strains that were electrophoresed and visualized on agarose gel (approximately 1.3–1.4 kb). The PCR products of TSA genes were cloned and expressed in pET expression system. Figure 1B shows the predicted 55–57-kDa molecular mass of purified recombinant TSA proteins by SDS-PAGE; the high purity (92–95%) of

TABLE 1

Performance of the IFA, the KGK ELISA, and the mixed-TSA ELISA using cutoff values based on non-scrub typhus negative control specimens at the mean plus three standard deviations

ELISA	IFA		KGK ELISA		Mixed-TSA ELISA	
	IgM	IgG	IgM	IgG	IgM	IgG
Cutoff value	40	80	0.34	0.35	0.28	0.32
TP (n)	75	96	83	103	88	106
TN (n)	81	82	80	80	80	80
FP (n)	1	0	2	2	2	2
FN (n)	34	13	26	6	21	3
Sensitivity (%)	68.8	88.1	76.1	94.5	80.7	97.2
Specificity (%)	98.8	100.0	97.6	97.6	97.6	97.6
PPV (%)	98.7	100.0	97.6	98.1	97.8	98.1
NPV (%)	70.4	86.3	75.5	93.0	79.2	96.4
Accuracy (%)	81.7	93.2	85.3	95.8	88.0	97.4

ELISA = enzyme-linked immunosorbent assay; IFA = immunofluorescence assay; KGK ELISA = Karp, Gilliam, and Kato strains at an equal weight ratio; TSA = type-specific antigen. Table shows number of samples (n) from scrub typhus patients and non-scrub typhus cases for true positive (TP), true negative (TN), false positive (FP), and false negative (FN). Sensitivity, specificity, positive predict value (PPV), and negative predict value (NPV) are shown as percentage.

TABLE 2

Performance of the IFA, the KGK ELISA, and the mixed-TSA ELISA among 109 specimens from polymerase chain reaction (+) scrub typhus patients using calculated cutoff values derived from receiver operating characteristic analyses

ELISA	IFA		KGK ELISA		Mixed-TSA ELISA	
	IgM	IgG	IgM	IgG	IgM	IgG
Cutoff value	40	80	0.30	0.37	0.27	0.36
TP (n)	75	96	83	103	89	105
TN (n)	81	82	80	81	80	81
FP (n)	1	0	2	1	2	1
FN (n)	34	13	26	6	20	4
Sensitivity (%)	68.8	88.1	76.1	94.5	81.7	96.3
Specificity (%)	98.8	100.0	97.6	98.8	97.6	98.8
PPV (%)	98.7	100.0	97.6	99.0	97.8	99.1
NPV (%)	70.4	86.3	75.5	93.1	80.0	95.3
Accuracy (%)	81.7	93.2	85.3	96.3	88.5	97.4

ELISA = enzyme-linked immunosorbent assay; IFA = immunofluorescence assay; KGK ELISA = Karp, Gilliam, and Kato strains at an equal weight ratio; TSA = type-specific antigen. Table shows number of samples (n) from scrub typhus patients and non-scrub typhus cases for true positive (TP), true negative (TN), false positive (FP), and false negative (FN). Sensitivity, specificity, positive predict value (PPV), and negative predict value (NPV) are shown as percentage.

FIGURE 3. The distribution of optical density (OD) values obtained by the KGTK enzyme-linked immunosorbent assay (ELISA) on non-scrub typhus and scrub typhus specimens. (A) For IgM ELISA, the cutoff values derived from mean plus three SDs (dotted line) was 0.34 and the cutoff value derived from receiver operating characteristic (ROC) analysis (solid line) was 0.30. (B) For IgG ELISA, the cutoff values derived from mean plus three SDs (dotted line) was 0.35 and the cutoff value derived from ROC analysis (solid line) was 0.37. This figure appears in color at www.ajtmh.org.

recombinant proteins was observed. Figure 1C demonstrates that the anti-His and anti-TSA monoclonal antibodies recognized the purified recombinant TSA proteins derived from various strains of *O. tsutsugamushi* by Western blot analysis.

Distribution of IFA titer of acute-phase serum samples from PCR-positive, scrub typhus patients and non-scrub typhus cases. Figure 2 summarizes the distribution of IgM and IgG IFA titers of acute-phase serum samples obtained from 109 *O. tsutsugamushi* PCR-positive, scrub typhus patients and 82 non-scrub typhus cases. Among 109 acute-phase scrub typhus specimens, 68.8% (75/109) had IgM titers equal or greater than 1:40 and 88.1% (96/109) had IgG titers equal or greater than 1:80. Among 82 non-scrub typhus specimens, one had an IgM titer of 1:40, three had 1:20, 11 had 1:10, and the remaining 67 specimens were negative at 1:10. For IgG antibodies, one of the non-scrub typhus specimens had a titer of 1:20, seven had 1:10 and the remaining 74 specimens were negative at 1:10. For IgM IFA, a sensitivity of 68.8% (95% confidence interval [CI] = 59.1–77.1%) and a specificity of 98.8% (95% CI = 92.5–99.9%) was found when the cutoff was set at 1:40 and a sensitivity of 54.1% (95% CI = 44.3–63.6%) and a specificity of 100.0% (95% CI = 94.4–100.0%) when the cutoff was set at 1:80. For IgG IFA, a sensitivity of 93.6% (95% CI = 86.8–97.2%) and a specificity of 100% (95% CI = 94.4–100.0%) was found when the cutoff was set at 1:40 and a sensitivity of 88.1% (95% CI = 80.1–93.2%) and specificity of 100% (95% CI = 94.4–100.0%) when the cutoff was set at 1:80. In this study, a titer equal to or

greater than 1:40 for IgM and a titer equal to or greater than 1:80 for IgG are considered a positive IFA result (Tables 1 and 2). Supplemental Tables 1 and 2 showed IFA and ELISA results of serum samples collected from 109 PCR-positive, scrub typhus patients and 82 non-scrub typhus cases, respectively.

Performance evaluation of ELISAs using cutoff values based on non-scrub typhus negative control specimens at the mean plus three SDs. Figures 3 and 4 illustrate the distributions of OD values obtained by the KGTK ELISA and the mixed-TSA ELISA, respectively, of non-scrub typhus and scrub typhus specimens. The performance of the KGTK ELISA and mixed-TSA ELISA using cutoff values of the mean plus three SDs is summarized in Table 1. The resulting IgM and IgG cutoff values were 0.34 and 0.35, respectively, for the KGTK ELISA, and 0.28 and 0.32, respectively, for the mixed-TSA ELISA (at 99% CI). Using these cutoff values, the positive rates for the KGTK ELISA were 76.1% (83/109) for IgM and 94.5% (103/109) for IgG; and the positive rates for the mixed-TSA ELISA were 80.7% (88/109) for IgM and 97.2% (106/109) for IgG. The results indicated that the sensitivities for the IgM and IgG mixed-TSA ELISA were higher than those of the KGTK ELISA and IFA (Table 1). The sensitivity of IgM mixed-TSA ELISA (80.7%) was significantly higher than that of IgM IFA (68.8%) (Chi-square test, $P < 0.05$).

Performance evaluation of ELISAs using cutoff values determined using ROC analysis. The performance of the KGTK ELISA and mixed-TSA ELISA using cutoff values derived from ROC analysis is summarized in Table 2. The resulting IgM

FIGURE 4. The distribution of optical density (OD) values obtained by the mixed-TSA enzyme-linked immunosorbent assay (ELISA) on non-scrub typhus and scrub typhus specimens. (A) For IgM ELISA, the cutoff values derived from mean plus three SDs (dotted line) was 0.28 and the cutoff value derived from receiver operating characteristic (ROC) analysis (solid line) was 0.27. (B) For IgG ELISA, the cutoff values derived from mean plus three SDs (dotted line) was 0.32 and the cutoff value derived from ROC analysis (solid line) was 0.36. This figure appears in color at www.ajtmh.org.

FIGURE 5. Correlation of the mixed-TSA enzyme-linked immunosorbent assay (ELISA) optical density (OD) values with immunofluorescence assay (IFA) titers. (A) The correlation of IgM ELISA OD values and IgM IFA titers. The Pearson correlation coefficient was 0.59 and the Spearman correlation coefficient was 0.61 ($P < 0.0001$). (B) The correlation of IgG ELISA OD values and IgG IFA titers. The Pearson correlation coefficient was 0.47 and the Spearman correlation coefficient was 0.48 ($P < 0.0001$). This figure appears in color at www.ajtmh.org.

and IgG cutoff values were 0.30 and 0.37, respectively, for the KGK ELISA, and the cutoff values were 0.27 and 0.36, respectively, for the mixed-TSA ELISA (at 99% CI) (Figures 3 and 4). The areas under the ROC analysis of the KGK ELISA and mixed-TSA ELISA are shown in Supplemental Figures 2 and 3. Using these cutoff values, the positive rates for the KGK ELISA were 76.1% (83/109) for IgM and 94.5% (103/109) for IgG; the positive rates for the mixed-TSA ELISA were 81.7% (89/109) for IgM and 96.3% (105/109) for IgG. The results indicated that the sensitivities for the IgM and IgG mixed-TSA ELISA were higher than those of the KGK ELISA and IFA. The sensitivity of the IgM mixed-TSA ELISA (81.7%) was significantly higher than that of IgM IFA (68.8%) (Chi-square test, $P < 0.005$).

Correlation between IFA titers and the mixed-TSA ELISA OD values. Figure 5 shows the correlation between OD values of the mixed-TSA ELISA and IFA titers. A strong correlation between ELISA OD values and IFA titers for IgM

was observed: the Pearson correlation coefficient was 0.59 ($P < 0.0001$) and the Spearman correlation coefficient was 0.61 ($P < 0.0001$). A medium correlation between mixed-TSA ELISA OD values and IFA titers for IgG was observed: the Pearson correlation coefficient was 0.47 ($P < 0.0001$) and the Spearman correlation coefficient was 0.48 ($P < 0.0001$).

DISCUSSION

We previously demonstrated that several distinct sequence types of TSA gene of *O. tsutsugamushi* strains were identified in Taiwan, and analysis of these TSA gene sequences showed a high level of genetic diversity.¹⁵ To improve the sensitivity of serological diagnosis for scrub typhus, recombinant TSA proteins derived from four dominant Taiwanese local strains (TW-1, TW-10, TW-19, and TW-22) and three prototype strains (Karp, Gilliam, and Kato) were selected and served as

FIGURE 6. A phylogenetic tree of *Orientia tsutsugamushi*. The phylogenetic tree is based on the complete 56-kDa type-specific antigen gene (TSA) sequences of *O. tsutsugamushi* strains. The tree was constructed by the neighbor-joining method and the maximum composite likelihood model. Bootstrap support values greater than 75 are shown. The Taiwanese TSA sequence types are designated in solid circles (●) and prototype TSA sequences are designated in solid triangles (▲). Type-specific antigen sequences were identified by using the nomenclature of OT (*Orientia tsutsugamushi*)/country/strain/year of isolation/GenBank accession number/sequence type. The scale bar on the left indicates substitutions per site.

antigens for the development of the mixed-TSA ELISA. TW-1, TW-10, and TW-19 strains belonged to the Karp-, TA763-, and Kawasaki-related genotypes, respectively, whereas TW-22 is a genotype of *O. tsutsugamushi* unique to Taiwan (Figure 6). These four local strains covered a major part of the total genetic diversity of TSA gene of *O. tsutsugamushi* in Taiwan.¹⁵ Because the mixed-TSA ELISA contains a combination of recombinant TSA proteins with wide sequence diversity, this ELISA may be not only suitable for use in Taiwan but also in other endemic areas.

The performance of the mixed-TSA ELISA was compared with the KGK ELISA, which contains recombinant TSA proteins of Karp, Gilliam, and Kato prototype strains, the results showed that the mixed-TSA ELISA was more sensitive than KGK ELISA for both IgM and IgG tests (Tables 1 and 2) using cutoff values determined by both mean plus three SDs and ROC analysis. The results indicated that using a combination of recombinant TSA proteins containing the local and prototype strains can increase the assay sensitivity for the detection of *O. tsutsugamushi* infection. The performance of recombinant TSA-based ELISA was also compared with IFA, which is considered the gold standard method for the diagnosis of scrub typhus. As shown in Tables 1 and 2, the TSA-based ELISAs are more sensitive than IFA for both IgM and IgG tests. In addition, the performance of the ELISA among 22 PCR-negative, paired IFA-positive (by 4-fold rise) scrub typhus patients (Supplemental Table 3) was evaluated using cutoff values of the mean plus three SDs (Supplemental Table 4) and cutoff values derived from ROC analysis (Supplemental Table 5). The results also indicated that the sensitivities for the IgM and IgG mixed-TSA ELISA were higher than those of the KGK ELISA and IFA.

Early diagnosis and prompt treatment are essential to the successful management of scrub typhus. Because both acute- and convalescent-phase serum samples are required for serological tests, a delay of diagnosis is inevitable. In practice, clinicians should not wait for the final results of serological diagnosis before starting treatment. Therefore, it is proper to evaluate the usefulness of the mixed TSA ELISA on acute-phase specimens. In addition, recent study demonstrated that because of the low specificities of the scrub typhus infection criteria, this method may not be suitable for the evaluation of new diagnostic tests.²⁹ In our study, we used acute-phase serum samples from PCR-positive, scrub typhus patients and IFA to evaluate the performance of the mixed-TSA ELISA. The results showed that the mixed-TSA ELISA is a sensitive method for diagnosing scrub typhus patients during the acute-phase of infection.

Confirmed cases of scrub typhus have been identified throughout the country, although most cases (60.6%) were from eastern Taiwan and offshore islands during 2011–2016 (<https://www.cdc.gov.tw/>). To reduce the influence of sample selection bias on evaluation results, serum samples from scrub typhus and non-scrub typhus cases used in this study were collected from Taiwan Main Island and offshore islands representing different geographical regions of the country. Our results showed that most of the serum samples from non-scrub typhus cases had low IgM and IgG IFA titers; among 82 specimens, only one had an IgM titer of 1:40 that was a confirmed case with Japanese encephalitis, and the remaining specimens were all < 1:40 for both IgM and IgG IFA titers. Whether the low IFA titers in non-scrub typhus cases reflects

the low seroprevalence in Taiwan requires further study. Interestingly, the low cutoff titer used for a positive IFA diagnosis has also been reported in Japan and Korea.³⁰

In our routine laboratory diagnosis for scrub typhus, approximately 60–70% of scrub typhus cases were confirmed by IFA, and the remaining cases were identified by real-time PCR and/or bacterial isolation. An accurate, cost-effective and high-throughput method is needed for screening of a large number of specimens from suspected scrub typhus cases. In this study, we demonstrated that the mixed-TSA ELISA had a high sensitivity and specificity and can be used for screening of scrub typhus patient in early phase of the disease.

Received May 8, 2018. Accepted for publication November 8, 2018.

Published online December 10, 2018.

Note: Supplemental figures and tables appear at www.ajtmh.org.

Acknowledgments: We thank Yeou-Lin Hsueh, Hsiu-Ying Lu, and Ming-Yen Lin for their expert technical assistance.

Financial support: This work was supported in part by grants MOHW104-CDC-C-315-000115 and MOHW105-CDC-C-315-123504 from Centers for Disease Control, Ministry of Health and Welfare, Taiwan, Republic of China.

Conflicts of Interest: PYS and SLY have a patent detection kit for diagnosis of scrub typhus and detection method thereof pending.

Authors' addresses: Su-Lin Yang, Hsiang-Fei Chen, Jun-Yu Luo, and Pei-Yun Shu, Center for Diagnostics and Vaccine Development, Centers for Disease Control, Ministry of Health and Welfare, Taipei, Republic of China, E-mails: cerline@cdc.gov.tw, xiangfei@cdc.gov.tw, lions8022@cdc.gov.tw, and pyshu@cdc.gov.tw. Kun-Hsien Tsai, Department of Public Health and Institute of Environmental Health, College of Public Health, National Taiwan University, Taipei, Republic of China, E-mail: kunhtsai@ntu.edu.tw.

REFERENCES

1. Tamura A, Ohashi N, Urakami H, Miyamura S, 1995. Classification of *Rickettsia tsutsugamushi* in a new genus, *Orientia* gen. nov., as *Orientia tsutsugamushi* comb. nov. *Int J Syst Bacteriol* 45: 589–591.
2. Walker DH, 2003. Rickettsial diseases in travelers. *Trav Med Infect Dis* 1: 35–40.
3. Kelly DJ, Fuerst PA, Ching WM, Richards AL, 2009. Scrub typhus: the geographic distribution of phenotypic and genotypic variants of *Orientia tsutsugamushi*. *Clin Infect Dis* 48: S203–S230.
4. Watt G, Strickman D, 1994. Life-threatening scrub typhus in a traveler returning from Thailand. *Clin Infect Dis* 18: 624–626.
5. Paris DH, Shelite TR, Day NP, Walker DH, 2013. Unresolved problems related to scrub typhus: a seriously neglected life-threatening disease. *Am J Trop Med Hyg* 89: 301–307.
6. Xu G, Walker DH, Jupiter D, Melby PC, Arcari CM, 2017. A review of the global epidemiology of scrub typhus. *PLoS Negl Trop Dis* 11: e0006062.
7. Seong SY, Choi MS, Kim IS, 2001. *Orientia tsutsugamushi* infection: overview and immune responses. *Microbes Infect* 3: 11–21.
8. Brown GW, Saunders JP, Singh S, Huxsoll DL, Shirai A, 1978. Single dose doxycycline therapy for scrub typhus. *Trans R Soc Trop Med Hyg* 72: 412–416.
9. Chen CC, Juan CJ, Juan CW, Zeng XC, Huang M, 2007. Multi-organ dysfunction caused by scrub typhus initially misinterpreted as acute tonsillitis. *J Emerg Crit Care Med* 18: 161–166.
10. Robinson DM, Brown G, Gan E, Huxsoll DL, 1976. Adaptation of a microimmunofluorescence test to the study of human *Rickettsia tsutsugamushi* antibody. *Am J Trop Med Hyg* 25: 900–905.
11. Koh GCKW, Maude RJ, Paris DH, Newton PN, Blacksell SD, 2010. Diagnosis of scrub typhus. *Am J Trop Med Hyg* 82: 368–370.

12. Hanson B, 1985. Identification and partial characterization of *Rickettsia tsutsugamushi* major protein immunogens. *Infect Immun* 50: 603–609.
13. Tamura A, Ohashi N, Urakami H, Takahashi K, Oyanagi M, 1985. Analysis of polypeptide composition and antigenic components of *Rickettsia tsutsugamushi* by polyacrylamide gel electrophoresis and immunoblotting. *Infect Immun* 48: 671–675.
14. Ohashi N, Tamura A, Ohta M, Hayashi K, 1989. Purification and partial characterization of a type-specific antigen of *Rickettsia tsutsugamushi*. *Infect Immun* 57: 1427–1431.
15. Lu HY, Tsai KH, Yu SK, Cheng CH, Yang JS, Su CL, Hu HC, Wang HC, Huang JH, Shu PY, 2010. Phylogenetic analysis of 56-kDa type-specific antigen gene of *Orientia tsutsugamushi* isolates in Taiwan. *Am J Trop Med Hyg* 83: 658–663.
16. Yang HH, Huang IT, Lin CH, Chen TY, Chen LK, 2012. New genotypes of *Orientia tsutsugamushi* isolated from humans in eastern Taiwan. *PLoS One* 7: e46997.
17. Kim G et al., 2017. Diversification of *Orientia tsutsugamushi* genotypes by intragenic recombination and their potential expansion in endemic areas. *PLOS Negl Trop Dis* 11: e0005408.
18. Ching WM, Wang H, Eamsila C, Kelly D, Dasch G, 1998. Expression and refolding of truncated recombinant major outer membrane protein antigen (r56) of *Orientia tsutsugamushi* and its use in enzyme-linked immunosorbent assays. *Clin Diagn Lab Immunol* 5: 519–526.
19. Blacksell SD, Jenjaroen K, Phetsouvanh R, Tanganuchitcharnchai A, Phouminh P, Phongmany S, Day NP, Newton PN, 2010. Accuracy of rapid IgM-based immunochromatographic and immunoblot assays for diagnosis of acute scrub typhus and murine typhus infections in Laos. *Am J Trop Med Hyg* 83: 365–369.
20. Ching WM, Rowland D, Zhang Z, Bourgeois A, Kelly D, Dasch G, Devine P, 2001. Early diagnosis of scrub typhus with a rapid flow assay using recombinant major outer membrane protein antigen (r56) of *Orientia tsutsugamushi*. *Clin Diagn Lab Immunol* 8: 409–414.
21. Jiang J, Marienau KJ, May LA, Beecham HJ, Wilkinson R, Ching W-M, Richards AL, 2003. Laboratory diagnosis of two scrub typhus outbreaks at Camp Fuji, Japan in 2000 and 2001 by enzyme-linked immunosorbent assay, rapid flow assay, and Western blot assay using outer membrane 56-kD recombinant proteins. *Am J Trop Med Hyg* 69: 60–66.
22. Jang WJ, Huh MS, Park KH, Choi MS, Kim IS, 2003. Evaluation of an immunoglobulin M capture enzyme-linked immunosorbent assay for diagnosis of *Orientia tsutsugamushi* infection. *Clin Diagn Lab Immunol* 10: 394–398.
23. Blacksell SD, Jenjaroen K, Phetsouvanh R, Wuthiekanun V, Day NP, Newton PN, Ching WM, 2010. Accuracy of AccessBio immunoglobulin M and total antibody rapid immunochromatographic assays for the diagnosis of acute scrub typhus infection. *Clin Vaccine Immunol* 17: 263–266.
24. Chao CC, Huber ES, Porter TB, Zhang Z, Ching WM, 2011. Analysis of the cross-reactivity of various 56 kD recombinant protein antigens with serum samples collected after *Orientia tsutsugamushi* infection by ELISA. *Am J Trop Med Hyg* 84: 967–972.
25. Tsai KH, Lu HY, Tsai JJ, Yu SK, Huang JH, Shu PY, 2008. Human case of *Rickettsia felis* infection, Taiwan. *Emerg Infect Dis* 14: 1970–1972.
26. Frey A, Di Canzio J, Zurakowski D, 1998. A statistically defined endpoint titer determination method for immunoassays. *J Immunol Methods* 221: 35–41.
27. Kumar R, Indrayan A, 2011. Receiver operating characteristic (ROC) curve for medical researchers. *Indian Pediatr* 48: 277–287.
28. Thompson JD, Higgins DG, Gibson TJ, 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22: 4673–4680.
29. Lim C, Paris DH, Blacksell SD, Laongnualpanich A, Kantipong P, Chierakul W, Wuthiekanun V, Day NP, Cooper BS, Limmathurotsakul D, 2015. How to determine the accuracy of an alternative diagnostic test when it is actually better than the reference tests: a re-evaluation of diagnostic tests for scrub typhus using Bayesian LCMs. *PLoS One* 10: e0114930.
30. Kim DM, Lee YM, Back JH, Yang TY, Lee JH, Song HJ, Shim SK, Hwang KJ, Park MY, 2010. A serosurvey of *Orientia tsutsugamushi* from patients with scrub typhus. *Clin Microbiol Infect* 16: 447–451.