

## COMPARATIVE EVALUATION OF SELECTED DIAGNOSTIC ASSAYS FOR THE DETECTION OF IGG AND IGM ANTIBODY TO *ORIENTIA TSUTSUGAMUSHI* IN THAILAND

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**Abstract.** We compared the performance of 2 commercially available dipstick assays, 2 enzyme-linked immunosorbent assays (ELISAs), and an indirect immunofluorescent antibody (IFA) assay for the diagnosis of scrub typhus, using the indirect immunoperoxidase (IIP) test as the reference standard. The dipstick assays were the Integrated Diagnostics (Baltimore, MD) Dip-S-Ticks Scrub Recombinant (r56) dipstick test (INDX assay) and the PanBio (Brisbane, Australia) Scrub Typhus IgM and IgG Rapid Immunochromatographic test (PanBio assay). One of the ELISAs used pooled cell lysates of Karp, Kato, and Gilliam strain *Orientia tsutsugamushi* as antigen (pooled-antigen ELISA), and the other used a recombinant r56 protein as the antigen (recombinant ELISA). With a panel of 123 positive and 227 negative sera, sensitivity and specificity of the assays were as follows: INDX assay, IgG, 60% and 95%, IgM, 60% and 97%; PanBio assay, IgG, 94% and 96%, IgM, 83% and 93%; IFA (1:400 cutoff), IgG, 91% and 96%, IgM, 85% and 98%; pooled-antigen ELISA, IgG (1:1600 cutoff), 97% and 89%, IgM (1:400 cutoff), 94% and 91%; recombinant ELISA, IgG (1:1600 cutoff), 97% and 92%, IgM (1:400 cutoff), 93% and 94%. Because of its excellent performance and use of a standardized, commercially available antigen, the recombinant ELISA is suitable for use in a diagnostic laboratory, where it may be able to replace the IFA and IIP assays. In contrast, the PanBio dipstick assay was easy to perform and did not require sophisticated equipment, making it suitable for use in rural areas where more sophisticated diagnostic tests such as the ELISA and IFA may not be available.

### INTRODUCTION

Scrub typhus is an acute febrile zoonotic disease resulting from infection with the gram-negative, intracellular bacteria *Orientia* (formerly *Rickettsia*) *tsutsugamushi*.<sup>1</sup> The disease is endemic in southeast Asia, where it accounts for 10–19% of patients admitted to hospitals with acute fever of uncertain origin.<sup>2,3</sup> Approximately 1 million cases occur each year, with >1 billion people at risk.<sup>4,5</sup> Clinical manifestations of scrub typhus range from mild fever with few other symptoms to a fatal syndrome characterized by multiple-organ failure. Treatment with chloramphenicol or other tetracycline antibiotics typically results in rapid and complete recovery.<sup>6</sup>

Serologic testing is the recommended method for the diagnosis of scrub typhus<sup>7</sup>; however, serology is often a confirmatory method because treatment generally is initiated on the basis of a clinical suspicion of the disease.<sup>8</sup> The Weil-Felix test has been widely used in the diagnosis of rickettsial diseases.<sup>9</sup> First described in 1916,<sup>10</sup> the Weil-Felix test is an agglutination test using the OX-19 strain of *Proteus vulgaris* for the diagnosis of *Rickettsia prowazekii* infection. The Weil-Felix test subsequently was modified by incorporation of the Kingsbury (OX-K) strain of *Proteus mirabilis* and has been used for the diagnosis of suspensions in typhus fever patients.<sup>11</sup> The Weil-Felix test is neither sensitive nor specific in the serodiagnosis of rickettsial diseases<sup>12</sup> and has largely been replaced by other assays.

More recently, the detection of antibody to *O. tsutsugamushi* by the indirect immunofluorescent antibody (IFA) test has been considered the standard serologic assay; however, IFA is not widely used because of lack of fluorescence microscopes in the scrub typhus endemic area.<sup>8</sup> The indirect immunoperoxidase (IIP) test is a modification of the IFA that

replaces the fluorochrome with peroxidase, allowing for use of an ordinary light microscope.<sup>13</sup> Although widely used, the IIP test is currently not commercially available, and as a result of variation in antigen preparations used in the assay, sensitivity and specificity vary among test centers.<sup>1</sup> The IIP test has been evaluated in a series of studies<sup>1,11,12,14,15</sup> and seems to perform similarly to the IFA. A variety of studies have evaluated the performance of enzyme-linked immunosorbent assays (ELISAs),<sup>16–19</sup> dot-blot immunoassays,<sup>1,20–23</sup> a rapid flow assay,<sup>24</sup> and a passive hemagglutination assay.<sup>25</sup> Although the performance of some of these assays was as good or better than the IFA or IIP, none of these assays is used routinely on a widespread basis for the diagnosis of scrub typhus. The introduction of recombinant proteins into the development of diagnostic assays for scrub typhus<sup>17–19,24,25</sup> should allow for their increased use, however, because of an enhanced stability and consistency of the antigen preparation and a reduction in cost, transport, and reproducibility problems presently associated with whole-cell antigen preparations. A truncated nonfused recombinant protein of 56 kd antigen from Karp strain (r56) was expressed, purified, and refolded into a structure similar to its native form at the Naval Medical Research Institute.<sup>18</sup> Selection of this protein was based on the fact that sera from 95–99% of acutely ill and convalescent patients with scrub typhus recognize the native 56-kd protein of *O. tsutsugamushi*. Because of its strong immunoreactivity, the r56 recombinant protein has been incorporated into ELISAs<sup>18,19</sup> and dipstick and immunochromatographic<sup>24</sup> assays to diagnose scrub typhus. We evaluate and compare 5 assays (IFA, a pooled-antigen ELISA, a r56 recombinant-antigen ELISA, a Pan-Bio [Brisbane, Australia] dipstick assay, and an Integrated Diagnostics [Baltimore,

MD] dipstick assay) for the diagnosis of scrub typhus, using the IIP test as the reference standard.

## MATERIALS AND METHODS

**Scrub typhus sera samples.** A total of 350 sera samples were used to evaluate the diagnostic assays. All 350 sera samples initially were tested for IgG and IgM to *O. tsutsugamushi* using the IIP assay. After this initial testing, samples were classified as negative ( $n = 227$ ; IgG and IgM titer  $<400$ ), weak positive ( $n = 29$ ; IgG or IgM titer of 400 or 800), or strong positive ( $n = 94$ ; IgG or IgM titer of  $\geq 1,600$ ). Of the sera samples, 164 were from febrile Thai patients reporting to a total of 19 different Bangkok hospitals. Samples were collected between April 9, 1993, and November 27, 1999. There were 56 samples collected from febrile Thai patients reporting to Chiang Rai Provincial Hospitals between January 1991 and November 1992.<sup>16</sup> There were 130 sera samples from Royal Thai Army personnel. Serum samples were frozen at  $-70^{\circ}\text{C}$  before being assayed.

**Sera samples from febrile patients without scrub typhus.** A panel of 40 sera samples that had been confirmed positive for a variety of pathogens and factors that potentially could be mistaken for scrub typhus was established. The panel included sera samples that were positive for the following pathogens and factors: hepatitis B virus ( $n = 5$ ), *Leptospira interrogans* ( $n = 5$ ), *Plasmodium falciparum* ( $n = 2$ ), *Plasmodium vivax* ( $n = 3$ ), *Rickettsia typhi* ( $n = 5$ ), rheumatoid factor ( $n = 5$ ), Japanese encephalitis virus ( $n = 5$ ), dengue virus ( $n = 5$ ), and *Salmonella typhi* ( $n = 5$ ). Ten additional sera samples that were negative for all of the aforementioned pathogens also were included in this evaluation, as were 5 *O. tsutsugamushi*-positive sera samples. The *O. tsutsugamushi*-positive samples were tested at dilutions of 1:50 ( $n = 5$ ), 1:200 ( $n = 5$ ), 1:800 ( $n = 5$ ), 1:3,200 ( $n = 5$ ), 1:12,800 ( $n = 5$ ), and 1:51,200 ( $n = 5$ ). A total of 80 sera samples were included in this evaluation. Each of these samples was tested for IgG and IgM antibody to *O. tsutsugamushi* using each of the diagnostic assays. All samples except for the *O. tsutsugamushi*-positive samples were tested diluted 1:50 in phosphate-buffered saline (PBS). The *O. tsutsugamushi*-positive samples were tested at the above-listed dilutions.

**Indirect immunoperoxidase assay.** Procedures for the IIP assay were modified from those described by Suto<sup>15</sup> and Land et al.<sup>19</sup> In brief, 5  $\mu\text{L}$  each of Karp, Gilliam, and Kato strain *O. tsutsugamushi* (50  $\mu\text{L}$  of purified antigen in 150  $\mu\text{L}$  of PBS) was spotted separately onto a glass slide as antigen. Slides were fixed with acetone, air dried, and stored at  $20^{\circ}\text{C}$  before use. A 1:25 dilution of each test serum sample was prepared by diluting 5  $\mu\text{L}$  of sera in 120  $\mu\text{L}$  of PBS. Further dilutions (1:1) were made in PBS, with a final dilution of 1:12,800. Each serum sample, 10  $\mu\text{L}$ , was added to each of the antigen spots on a slide, incubated for 30 minutes, rinsed 3 times with PBS, and air dried. Horseradish peroxidase (HRP)-conjugated antihuman IgM or IgG specific goat serum (Kirkegaard-Perry Laboratories, Gaithersburg, MD), 10  $\mu\text{L}$ , diluted 1:50 in PBS was added to each spot for 30 minutes at  $37^{\circ}\text{C}$ . Slides were rinsed 3 times with PBS and 1 time with distilled water, then allowed to air dry. Diaminobenzidine (DAB) substrate was added to the slides and incubated for 10 minutes, followed by 3 rinses in PBS and 1 rinse in distilled water. Slides were stained with methylene blue for 5–10 seconds, rinsed with

distilled water, and mounted with Permount (Fisher Chemical, Inc. Atlanta, GA). Slides were observed using bright-field microscopy. The reaction was positive when the rickettsial particles were stained light brown. Titers of antibody were expressed as the reciprocal of the highest dilution with a positive reaction. A test was considered positive if IgG or IgM antibody titers were  $\geq 1:400$ .

**Indirect immunofluorescent antibody assay.** The IFA test was modified from the microimmunofluorescence methods described by Bozeman and Elisberg<sup>26</sup> and Robinson et al.<sup>27</sup> The antigens used were pooled Karp, Kato, and Gilliam strains of yolk sac-propagated *O. tsutsugamushi*, and strains TC586, TA 678, TA 686, TA 716, TA 763, and TH 1817 antigen. The fluorescein conjugates used were rabbit antihuman IgG and IgM. Antigen slides were warmed to room temperature, and antigen microspots were covered with 5  $\mu\text{L}$  of 1:50 dilutions (in PBS) of serum (for screening) or 2-fold dilutions (beginning at 1:50) for titration. Slides were incubated at  $37^{\circ}\text{C}$  for 30 minutes, washed with PBS, and incubated for 30 minutes with fluorescein conjugates. Titers of antibody were expressed as the reciprocal of the highest dilution with a positive reaction. Performance of the IFA was compared with the IIP by assessing sensitivity and specificity of the assay using cutoff thresholds of 1:100, 1:200, 1:400, 1:800, 1:1,600 and 1:3,200.

**PanBio scrub typhus IgM and IgG rapid immunochromatographic assay.** Assays (Catalog No. SCT-25S) were obtained from PanBio (Brisbane, Australia) and used according to the insert (Insert-2-SCT-25S, July 13, 2000). Four drops of buffer were added to a test tube (12  $\times$  75 mm), and a "loop" of undiluted serum was added to the buffer. A test strip was placed in the sample, and results were read after 15 minutes. Validity of the assay was confirmed by the presence of the control band. Test results were recorded as 1 (test line visible) or 0 (test line absent) for IgG and IgM.

**Integrated Diagnostics multitest Dip-S-Ticks scrub recombinant (r56) assay.** Assays (Catalog No. 9999-070; Kit Lot, r56102199-50; Stick Lot, r56-101299) were obtained from Integrated Diagnostics, Inc (Baltimore, MD) and used according to the insert (r56r0.RUO Rev0, October 1999). Validity of the assay was confirmed by the presence of the IgG/IgM positive control. Test results were recorded as the number of dots that were reactive for IgG or IgM.

**Pooled-antigen enzyme-linked immunoassay.** Procedures for the scrub typhus ELISA can be found in the publication by Suwanabun et al.<sup>16</sup> The pooled-antigen used in the assay consisted of 25, 50, and 100 ng of Karp, Gilliam, and Kato strains of *O. tsutsugamushi* in 50  $\mu\text{L}$  of PBS per well. Serum samples assayed for IgM by ELISA were adsorbed with protein G affinity resin (Quick-Sep IgM; Isolab, Inc, Akron, OH). For each sample, 5  $\mu\text{L}$  of undiluted serum was mixed with 40  $\mu\text{L}$  of protein G suspension, shaken, and centrifuged at  $700 \times g$  for 10 minutes. The supernatant was diluted to a final concentration of 1:100 using the ELISA blocking buffer. For the IgG ELISA, the sera initially were diluted in blocking buffer to 1:100 and used directly in the test. The mean optical density (OD) units  $\pm 2$  SD were used as positive cutoff values. Positive sera were titrated against pooled antigen in 2-fold serial dilutions. The greatest dilution giving a net OD value greater than the cutoff OD value was used as the end point titer. Performance of the pooled-antigen ELISA was compared with the IIP by assessing sensitivity and specificity of

TABLE 1

Initial validation of the performance of the IIP test for the detection of IgG and IgM to *Orientia tsutsugamushi* in 350 blinded sera samples

| Assay | Sensitivity (95% CI) | Specificity (95% CI) | Positive predictive value (95% CI) | Negative predictive value (95% CI) | Accuracy | Reliability |
|-------|----------------------|----------------------|------------------------------------|------------------------------------|----------|-------------|
| IgG*  | 98.2% (93.2–99.7)    | 97.9% (94.8–99.2)    | 95.7% (89.8–98.4)                  | 99.1% (96.6–99.9)                  | 98.0%    | 0.96        |
| IgM*  | 99.2% (94.8–100.0)   | 98.7% (95.9–99.7)    | 97.6% (92.5–99.4)                  | 99.6% (97.2–100.0)                 | 98.9%    | 0.98        |

Note. Performance of the IIP was evaluated by comparing results from 2 separate tests. CI, confidence interval.

\* A cutoff titer of 1:400 was used. Titers were expressed as the reciprocal of the highest dilution with a positive reaction. A test was considered positive if IgG or IgM antibody titers were  $\geq$ 1:400.

the assay using cutoff thresholds of 1:100, 1:200, 1:400, 1:800, 1:1,600 and 1:3,200.

#### r56 recombinant-antigen enzyme-linked immunoassay.

Procedures for the r56 recombinant protein ELISA were based on Suwanabun et al.<sup>16</sup> This ELISA was modified by incorporation of a recombinant 56-kd protein<sup>18</sup> as the ELISA antigen. Performance of the r56 recombinant-antigen ELISA was compared with the IIP by assessing sensitivity and specificity of the assay using cutoff thresholds of 1:100, 1:200, 1:400, 1:800, 1:1,600 and 1:3,200.

**Data analysis.** Epi-Info (Center for Disease Control and Prevention, Atlanta, GA) version 6 was used to calculate test

performance and acceptability evaluation indices,<sup>28</sup> with the IIP assay used as the gold standard. Variables measured included the number of true positives (TP), number of true negatives (TN), number of false positives (FP), and number of false negatives (FN). Sensitivity was calculated as TP/(TP + FN), specificity was calculated as TN/(TN + FP), positive predictive value was calculated as TP/(TP + FP), and negative predictive value was calculated as TN/(FN + TN). Test accuracy, the proportion of all tests that gave a correct result, was defined as (TP + TN)/number of all tests. Reliability was expressed as the J index  $([TP \times TN] - [FP \times FN])/([TP + FN][TN + FP])$ .

TABLE 2

Performance of IFA, pooled-antigen ELISA, and r56 recombinant-antigen ELISA assays at selected cutoff titers relative to the IIP test\* for detection of IgG and IgM to *Orientia tsutsugamushi* in 350 blinded sera samples

| Assay                | Cutoff titer  | Sensitivity (95% CI)          | Specificity (95% CI)                                   | Accuracy   |
|----------------------|---|-------------------------------|--|------------|
| IFA                  | 1:3,200<br>1:1,600<br>1:800<br>1:400<br>1:200<br>1:100                            | <i>IgG</i>                    |  |            |
|                      |   | 61.2% (51.8–69.8)             | 98.7% (95.9–99.7)                                      | 85.1%      |
|                      |   | 72.7% (63.7–80.2)             | 98.7% (95.9–99.7)                                      | 89.7%      |
|                      |   | 82.6% (74.5–88.7)             | 98.3% (95.3–99.4)                                      | 93.1%      |
|                      |   | 85.1% (77.2–90.7)             | 98.3% (95.3–99.4)                                      | 94.6%      |
|                      |   | 90.1% (83.1–94.5)             | 96.9% (93.5–98.7)                                      | 93.7%      |
|                      |   | 91.7% (85.0–95.7)             | 95.6% (91.9–97.8)                                      | 94.0%      |
|                      |   | 74.6% (65.4–82.0)             | 96.2% (92.6–98.1)                                      | 89.1%      |
|                      |   | 90.4% (83.0–94.8)             | 94.9% (91.1–97.2)                                      | 93.4%      |
|                      |   | 95.6% (89.6–98.4)             | 91.9% (87.5–95.0)                                      | 93.1%      |
| Pooled-antigen ELISA | 1:400<br>1:200<br>1:100<br>1:3,200<br>1:1,600<br>1:800<br>1:400<br>1:200<br>1:100 | 97.4% (91.9–99.3)             | 89.0% (84.1–92.5)                                      | 91.7%      |
|                      |   | 98.2% (93.2–99.7)             | 86.4% (81.2–90.4)                                      | 90.3%      |
|                      |   | 98.2% (93.2–99.7)             | 85.6% (80.3–89.7)                                      | 89.7%      |
|                      |   | 81.6% (73.0–88.0)             | 97.5% (94.3–99.0)                                      | 92.3%      |
|                      |   | 91.2% (84.1–95.5)             | 96.2% (92.6–98.1)                                      | 94.6%      |
|                      |   | 94.7% (88.4–97.8)             | 93.2% (89.0–95.9)                                      | 93.7%      |
|                      |   | 97.4% (91.9–99.3)             | 91.5% (87.0–94.6)                                      | 93.4%      |
|                      |   | 98.2% (93.2–99.7)             | 86.9% (81.7–90.8)                                      | 90.6%      |
|                      |   | 98.2% (93.2–99.7)             | 81.4% (75.7–86.0)                                      | 86.9%      |
|                      |   | r56 Recombinant-antigen ELISA | 1:3,200<br>1:1,600<br>1:800<br>1:400<br>1:200<br>1:100 | <i>IgM</i> |
| 61.2% (51.8–69.8)    | 98.7% (95.9–99.7)   |                               |  | 85.7%      |
| 72.7% (63.7–80.2)    | 98.7% (95.9–99.7)   |                               |  | 89.7%      |
| 82.6% (74.5–88.7)    | 98.3% (95.3–99.4)   |                               |  | 92.9%      |
| 85.1% (77.2–90.7)    | 98.3% (95.3–99.4)   |                               |  | 93.7%      |
| 90.1% (83.0–94.5)    | 96.9% (93.5–98.7)   |                               |  | 94.6%      |
| 91.7% (85.0–95.7)    | 95.6% (91.9–97.8)   |                               |  | 94.3%      |
| 87.6% (80.1–92.7)    | 98.3% (95.3–99.4)   |                               |  | 78.9%      |
| 91.7% (85.0–95.7)    | 96.9% (93.5–98.7)   |                               |  | 89.4%      |
| 92.6% (86.0–96.3)    | 96.1% (92.4–98.1)   |                               |  | 92.3%      |
| Pooled-antigen ELISA | 1:400<br>1:200<br>1:100<br>1:3,200<br>1:1,600<br>1:800<br>1:400<br>1:200<br>1:100 | 94.2% (88.0–97.4)             | 91.3% (86.6–94.5)                                      | 94.9%      |
|                      |   | 97.5% (92.4–99.4)             | 85.2% (79.7–89.4)                                      | 95.1%      |
|                      |   | 99.2% (94.8–100.0)            | 68.1% (61.6–74.0)                                      | 94.6%      |
|                      |   | 81.0% (72.6–87.3)             | 99.1% (96.5–99.8)                                      | 92.9%      |
|                      |   | 86.0% (78.2–91.4)             | 97.4% (94.1–98.9)                                      | 93.9%      |
|                      |   | 91.7% (85.0–95.7)             | 96.5% (93.0–98.4)                                      | 94.9%      |
|                      |   | 93.4% (87.0–96.9)             | 93.9% (89.7–96.5)                                      | 93.7%      |
|                      |   | 95.9% (90.1–98.5)             | 89.5% (84.6–93.0)                                      | 91.7%      |
|                      |   | 97.5% (92.4–99.4)             | 82.5% (76.9–87.1)                                      | 87.7%      |
|                      |   | r56 Recombinant-antigen ELISA | 1:3,200<br>1:1,600<br>1:800<br>1:400<br>1:200<br>1:100 | <i>IgG</i> |
| 61.2% (51.8–69.8)    | 98.7% (95.9–99.7)   |                               |  | 85.7%      |
| 72.7% (63.7–80.2)    | 98.7% (95.9–99.7)   |                               |  | 89.7%      |
| 82.6% (74.5–88.7)    | 98.3% (95.3–99.4)   |                               |  | 92.9%      |
| 85.1% (77.2–90.7)    | 98.3% (95.3–99.4)   |                               |  | 93.7%      |
| 90.1% (83.0–94.5)    | 96.9% (93.5–98.7)   |                               |  | 94.6%      |
| 91.7% (85.0–95.7)    | 95.6% (91.9–97.8)   |                               |  | 94.3%      |
| 87.6% (80.1–92.7)    | 98.3% (95.3–99.4)   |                               |  | 78.9%      |
| 91.7% (85.0–95.7)    | 96.9% (93.5–98.7)   |                               |  | 89.4%      |
| 92.6% (86.0–96.3)    | 96.1% (92.4–98.1)   |                               |  | 92.3%      |

Abbreviation: CI, confidence interval.

\* A cutoff titer of 1:400 was used for the IIP. Titers were expressed as the reciprocal of the highest dilution with a positive reaction. A test was considered positive if IgG or IgM antibody titers were  $\geq$ 1:400.

## RESULTS

The IIP assay was used as the standard against which each of the other assays was compared. To establish the reproducibility of the IIP, we initially tested each sample ( $n = 350$ ) twice by IIP and assessed test performance by comparing results from the first test with the results from the second test. The IIP assay was 98.2% sensitive and 97.9% specific for IgG and 99.2% sensitive and 98.7% specific for IgM (Table 1). Overall accuracy of the assay was >98% for IgG and IgM, indicating that the IIP was highly reproducible.

To determine optimal cutoff thresholds of the IFA, pooled-antigen ELISA, and r56 recombinant-antigen ELISA, performance characteristics (sensitivity, specificity, and accuracy) of each assay were calculated for IgG and IgM using cutoff thresholds of 1:100, 1:200, 1:400, 1:800, 1:1,600, and 1:3,200, using the IIP as the reference standard (Table 2). Performance of the IFA for IgG and IgM was optimal with a cutoff titer between 1:100 and 1:400. A cutoff titer of 1:400 was selected for all subsequent evaluations of the IFA. Samples with a titer of <1:400 were considered negative, whereas samples with a titer of 1:400 or greater were considered positive. Performance of the pooled-antigen ELISA and the r56 recombinant-antigen ELISA for IgG and IgM were optimal when using cutoff titers of 1:1,600 and 1:400 (Table 2). These values are consistent with those used by Suwanabun et al.<sup>16</sup> and were selected for all subsequent evaluations with these assays.

Complete performance characteristics of each of the assays are presented in Table 3. In general, performance of the PanBio assay, the pooled-antigen ELISA, the r56 recombinant-antigen ELISA, and the IFA were similar to each other, with overall accuracy of each assay 93–95% for IgG and 89–94% for IgM (Table 3). Reading the PanBio assay 24 hours after test completion (rather than 15 minutes as suggested in the insert) increased sensitivity for IgG and IgM; however, accuracy of the assay was not markedly affected (Table 3). Performance of the INDX assay was significantly worse than that of the other assays. The INDX assay was only 59.6% sensitive for IgG and 60.3% sensitive for IgM, with overall accuracy of approximately 84% for IgG and IgM.

The performance of each assay against sera specimens from patients with other febrile diseases is presented in Table 4. The IIP, IFA, and pooled-antigen ELISA assays were negative (IgG and IgM) for all 50 of the non-*O. tsutsugamushi* samples. The PanBio assay was negative for all of the non-*O. tsutsugamushi* samples except for 1 typhoid-positive sample and 1 negative control sample that were IgM positive. The r56 recombinant-antigen ELISA was negative for all 50 non-*O. tsutsugamushi* samples tested by IgM; however, 1 leptospirosis-positive sample yielded a positive IgG titer (1:1,600) against *O. tsutsugamushi* (Table 4). The INDX assay was not tested against this panel of sera samples because of the poor performance of the assay in all previous evaluations.

The efficacy of each assay against increasingly dilute *O. tsutsugamushi*-positive samples is presented in Table 4. The IIP assay, the IFA assay, and both ELISAs consistently detected IgG and IgM antibody against *O. tsutsugamushi* at higher dilutions than did the PanBio assays. When sera samples were diluted 1:800 in PBS, the IIP inexplicably detected fewer samples (IgG and IgM) than at dilutions of 1:3,200 and 1:12,800.

## DISCUSSION

The diagnosis of scrub typhus is hampered by the lack of a reproducible, quantifiable assay. The IFA and the related IIP are generally accepted as the gold standard serologic assays; however, both assays are subjective, semiquantitative tests that require highly trained technicians to prepare the stained slides and to determine end points. Currently the IIP is more widely used than the IFA, owing to the scarcity of fluorescence microscopes in the endemic region. Both assays rely on cultured *O. tsutsugamushi* antigen preparations, and procedures for the production and preparation of antigen can vary greatly among different laboratories, leading to inconsistencies in interpretation of results. In addition, because of risks associated with culturing of live rickettsiae, the U.S. Centers for Disease Control recommends that Biosafety Level 3 (BSL 3) precautions be used when culturing large quantities of *O. tsutsugamushi*.<sup>29</sup>

TABLE 3

Performance characteristics of selected assays relative to the IIP test for detection of IgG and IgM to *Orientia tsutsugamushi* in 350 blinded sera samples

| Assay                         | Cutoff titer* | Sensitivity (95% CI) | Specificity (95% CI) | Positive predictive value (95% CI) | Negative predictive value (95% CI) | Accuracy | Reliability |
|-------------------------------|---------------|----------------------|----------------------|------------------------------------|------------------------------------|----------|-------------|
| <i>IgG</i>                    |               |                      |                      |                                    |                                    |          |             |
| PanBio assay†                 | NA            | 90.4% (83.0–94.8)    | 95.8% (92.1–97.8)    | 91.2% (83.9–95.4)                  | 95.4% (91.6–97.5)                  | 94.0%    | 0.86        |
| PanBio assay‡                 | NA            | 93.9% (87.3–97.3)    | 95.7% (92.1–97.8)    | 91.5% (84.5–95.6)                  | 97.0% (93.6–98.7)                  | 95.1%    | 0.90        |
| INDX assay                    | NA            | 59.6% (50.0–68.6)    | 95.3% (91.6–97.5)    | 86.1% (76.0–92.5)                  | 83.0% (77.9–87.2)                  | 83.7%    | 0.55        |
| IFA                           | 1:400         | 91.2% (84.1–95.5)    | 96.2% (92.6–98.1)    | 92.0% (85.0–96.1)                  | 95.8% (92.1–97.8)                  | 94.6%    | 0.87        |
| Pooled-antigen ELISA          | 1:1,600       | 90.4% (83.0–94.8)    | 94.9% (91.1–97.2)    | 89.6% (82.1–94.3)                  | 95.3% (91.6–97.5)                  | 93.4%    | 0.85        |
| r56 Recombinant-antigen ELISA | 1:1,600       | 91.2% (84.1–95.5)    | 96.2% (92.6–98.1)    | 92.0% (85.0–96.1)                  | 95.8% (92.1–97.8)                  | 94.6%    | 0.87        |
| <i>IgM</i>                    |               |                      |                      |                                    |                                    |          |             |
| PanBio assay†                 | NA            | 78.5% (69.9–85.2)    | 96.5% (93.0–98.4)    | 92.2% (84.8–96.3)                  | 89.5% (84.8–92.9)                  | 90.3%    | 0.75        |
| PanBio assay‡                 | NA            | 82.6% (74.5–88.7)    | 93.4% (89.2–96.2)    | 87.0% (79.1–92.3)                  | 91.1% (86.5–94.3)                  | 89.7%    | 0.76        |
| INDX assay                    | NA            | 60.3% (51.0–69.0)    | 97.4% (94.1–98.9)    | 92.4% (83.6–96.9)                  | 82.3% (77.1–86.5)                  | 84.6%    | 0.58        |
| IFA                           | 1:400         | 85.1% (77.2–90.7)    | 98.3% (95.3–99.4)    | 96.3% (90.1–98.8)                  | 92.6% (88.4–95.4)                  | 93.7%    | 0.83        |
| Pooled-antigen ELISA          | 1:400         | 94.2% (88.0–97.4)    | 91.3% (86.6–94.5)    | 85.1% (77.6–90.4)                  | 96.8% (93.2–98.6)                  | 92.3%    | 0.85        |
| r56 Recombinant-antigen ELISA | 1:400         | 93.4% (87.0–96.9)    | 93.9% (89.7–96.5)    | 89.0% (81.9–93.6)                  | 96.4% (92.8–98.3)                  | 93.7%    | 0.87        |

Abbreviations: CI, confidence interval; NA, not applicable.

\* A cutoff titer of 1:400 was used for the IIP reference assay, with cutoff titers of the other assays as listed. Titers were expressed as the reciprocal of the highest dilution with a positive reaction.

† A test was considered positive if the titer was  $\geq 1:400$ .

‡ Assay read immediately after completion.

§ Assay read 24-hours after completion.

TABLE 4  
Performance of selected assays when assessing sera samples positive for scrub typhus and a variety of other agents

| Sera sample                    | Dilution | No. samples | No. positive by IIP* | No. positive by Pan-Bio | No. positive by IFA* | No. positive by pooled-antigen ELISA* | No. positive by r56 recombinant-antigen ELISA* |
|--------------------------------|----------|-------------|----------------------|-------------------------|----------------------|---------------------------------------|--|
| <i>IgG</i>                     |          |             |                      |                         |                      |                                       |  |
| Scrub typhus positive          | 1:50     | 5           | 9/10 (90%)           | 5/5 (100%)              | 5/5 (100%)           | 5/5 (100%)                            | 5/5 (100%)                                     |
|                                | 1:200    | 5           | 5/10 (50%)           | 2/4 (50%)†              | 5/5 (100%)           | 5/5 (100%)                            | 5/5 (100%)                                     |
|                                | 1:800    | 5           | 2/10 (20%)           | 0/4 (0%)†               | 5/5 (100%)           | 5/5 (100%)                            | 4/5 (80%)                                      |
|                                | 1:3,200  | 5           | 6/10 (60%)           | 0/2 (0%)†               | 0/5 (0%)             | 1/5 (20%)                             | 1/5 (20%)                                      |
|                                | 1:12,800 | 5           | 6/10 (60%)           | ND†                     | 0/5 (0%)             | 0/5 (0%)                              | 0/5 (0%)                                       |
|                                | 1:51,200 | 5           | 0/10 (0%)            | ND†                     | 0/5 (0%)             | 0/5 (0%)                              | 0/5 (0%)                                       |
| Negative control               | 1:50     | 10          | 0/10 (0%)            | 0/10 (0%)               | 0/10 (0%)            | 0/10 (0%)                             | 0/10 (0%)                                      |
| Hepatitis B positive           | 1:50     | 5           | 0/5 (0%)             | 0/5 (0%)                | 0/5 (0%)             | 0/5 (0%)                              | 0/5 (0%)                                       |
| Leptospirosis positive         | 1:50     | 5           | 0/5 (0%)             | 0/5 (0%)                | 0/5 (0%)             | 0/5 (0%)                              | 1/5 (20%)                                      |
| Malaria‡ positive              | 1:50     | 5           | 0/5 (0%)             | 0/5 (0%)                | 0/5 (0%)             | 0/5 (0%)                              | 0/5 (0%)                                       |
| Murine typhus positive         | 1:50     | 5           | 0/5 (0%)             | 0/5 (0%)                | 0/5 (0%)             | 0/5 (0%)                              | 0/5 (0%)                                       |
| Rheumatoid factor positive     | 1:50     | 5           | 0/5 (0%)             | 0/5 (0%)                | 0/5 (0%)             | 0/5 (0%)                              | 0/5 (0%)                                       |
| Japanese encephalitis positive | 1:50     | 5           | 0/5 (0%)             | 0/2 (0%)†               | 0/5 (0%)             | 0/5 (0%)                              | 0/5 (0%)                                       |
| Dengue positive                | 1:50     | 5           | 0/5 (0%)             | 0/5 (0%)                | 0/5 (0%)             | 0/5 (0%)                              | 0/5 (0%)                                       |
| Typhoid positive               | 1:50     | 5           | 0/5 (0%)             | 0/5 (0%)                | 0/5 (0%)             | 0/5 (0%)                              | 0/5 (0%)                                       |
| <i>IgM</i>                     |          |             |                      |                         |                      |                                       |  |
| Scrub typhus positive          | 1:50     | 5           | 10/10 (100%)         | 3/5 (50%)               | 5/5 (100%)           | 5/5 (100%)                            | 5/5 (100%)                                     |
|                                | 1:200    | 5           | 6/10 (60%)           | 4/5 (80%)               | 5/5 (100%)           | 4/5 (80%)                             | 4/5 (80%)                                      |
|                                | 1:800    | 5           | 2/10 (20%)           | 1/5 (20%)               | 5/5 (100%)           | 3/5 (60%)                             | 3/5 (60%)                                      |
|                                | 1:3,200  | 5           | 6/10 (50%)           | 3/5 (60%)               | 0/5 (0%)             | 0/5 (0%)                              | 1/5 (20%)                                      |
|                                | 1:12,800 | 5           | 5/10 (50%)           | 0/5 (0%)                | 0/5 (0%)             | 0/5 (0%)                              | 0/5 (0%)                                       |
|                                | 1:51,200 | 5           | 0/10 (0%)            | 0/5 (0%)                | 0/5 (0%)             | 0/5 (0%)                              | 0/5 (0%)                                       |
| Negative control               | 1:50     | 10          | 0/10 (0%)            | 1/10 (10%)              | 0/10 (0%)            | 0/10 (0%)                             | 0/10 (0%)                                      |
| Hepatitis B positive           | 1:50     | 5           | 0/5 (0%)             | 0/5 (0%)                | 0/5 (0%)             | 0/5 (0%)                              | 0/5 (0%)                                       |
| Leptospirosis positive         | 1:50     | 5           | 0/5 (0%)             | 0/5 (0%)                | 0/5 (0%)             | 0/5 (0%)                              | 0/5 (0%)                                       |
| Malaria‡ positive              | 1:50     | 5           | 0/5 (0%)             | 0/5 (0%)                | 0/5 (0%)             | 0/5 (0%)                              | 0/5 (0%)                                       |
| Murine typhus positive         | 1:50     | 5           | 0/5 (0%)             | 0/5 (0%)                | 0/5 (0%)             | 0/5 (0%)                              | 0/5 (0%)                                       |
| Rheumatoid factor positive     | 1:50     | 5           | 0/5 (0%)             | 0/5 (0%)                | 0/5 (0%)             | 0/5 (0%)                              | 0/5 (0%)                                       |
| Japanese encephalitis positive | 1:50     | 5           | 0/5 (0%)             | 0/5 (0%)                | 0/5 (0%)             | 0/5 (0%)                              | 0/5 (0%)                                       |
| Dengue positive                | 1:50     | 5           | 0/5 (0%)             | 0/5 (0%)                | 0/5 (0%)             | 0/5 (0%)                              | 0/5 (0%)                                       |
| Typhoid positive               | 1:50     | 5           | 0/5 (0%)             | 1/5 (20%)               | 0/5 (0%)             | 0/5 (0%)                              | 0/5 (0%)                                       |

Abbreviation: ND, not done.

\* A cutoff titer of 1:400 was used for the IIP (IgG and IgM), IFA (IgG and IgM), pooled-antigen ELISA (IgM), and r56 recombinant-antigen ELISA (IgM). A cutoff titer of 1:1,600 was used for the pooled-antigen and r56 recombinant-antigen ELISAs (IgG). Titers were expressed as the reciprocal of the highest dilution with a positive reaction. A test was considered positive IgG or IgM antibody titers were equal to or greater than the cutoff titer.

† Positive control lines failed to come up on a certain number of assays in these groups. The number of assays failing was the difference between the total number of samples of each type tested and number of samples listed for each assay.

‡ *Plasmodium falciparum* and *Plasmodium vivax*.

The recombinant 56-kd immunodominant protein from *O. tsutsugamushi* (r56) has been incorporated successfully into a commercially available ELISA, which had a good correlation (IgG and IgM) with an ELISA using native antigen and the IIP assay.<sup>17</sup> The use of the r56 antigen effectively overcomes most of the shortcomings of the native antigen previously used in all scrub typhus assays. Large quantities of the antigen can be prepared safely using *Escherichia coli* and without the need for BSL 3 facilities. The antigen can be stored for long period, and can be provided easily to multiple laboratories. Results from this study confirm that the r56 antigen is sensitive and specific for the detection of IgG and IgM antibody directed against *O. tsutsugamushi*. The r56 recombinant antigen developed by the Naval Medical Research Institute<sup>18</sup> was used in the PanBio dipstick assay and in the recombinant-antigen ELISA. Performance of both assays was acceptable using all criteria evaluated, with overall accuracy of approximately 95% (IgG) and 90% (IgM) when compared with the IIP.

In general, the PanBio assay proved to be better at detecting IgG than IgM (Tables 3 and 4). Of particular concern was the 1 negative-control sample and the 1 *S. typhi* sample that yielded a positive result (Table 4). None of the other assays gave similar results with these 2 samples, suggesting that these

were false-positive results. In contrast to the PanBio assay, the INDX assay provided performance below desired levels. The accuracy and reliability of this assay for IgG were only 84% and 0.55, whereas for IgM the values were 85% and 0.58. These values were significantly lower than the values obtained with each of the other assays. This poor performance was surprising because Pradutkanchana et al.<sup>1</sup> reported that a similar product from the same manufacturer performed as well as the IIP and IFA assays. The INDX assay evaluated by Pradutkanchana et al.<sup>1</sup> used native *O. tsutsugamushi* antigen as described by Weddle et al.,<sup>20</sup> whereas the INDX assay we evaluated used the r56 recombinant antigen.<sup>18</sup> As we have already described, however, the r56 recombinant antigen used in this study has been incorporated into a variety of assays,<sup>18,19,24</sup> in which it has sensitivity and specificity comparable or better than that obtained using native *O. tsutsugamushi* antigen. We can offer no logical explanation for the poor performance of the INDX assay in this study.

Results from this study indicate that the pooled antigen ELISA and r56 recombinant-antigen ELISA are highly accurate for the detection of IgG and IgM antibody induced by *O. tsutsugamushi*. All performance criteria were excellent for both assays (Tables 2 and 3), and both assays seemed quite specific for *O. tsutsugamushi* (Table 4). Although a single

leptospirosis-positive serum sample yielded a positive IgG titer with the r56 recombinant-antigen ELISA, Brown et al.<sup>30</sup> reported that leptospirosis is a major cause of false-positive results in the diagnosis of scrub typhus. Although none of the other assays indicated that this particular sample was positive for *O. tsutsugamushi*, owing to the high sensitivity of the r56 recombinant-antigen ELISA, we cannot rule out the possibility that this individual may have been infected previously with *O. tsutsugamushi*.

In general, the performance of both ELISAs was similar to that of the IIP, suggesting that these assays may serve as gold standard assays for the diagnosis of scrub typhus. The benefits of the r56 recombinant antigen used in the ELISA have been described previously<sup>18,19</sup>; added benefits of the ELISA compared with the IIP and IFA include ability to evaluate large numbers of samples rapidly, standardized methods for evaluation of samples (compared with the subjectivity required by the IIP and IFA), and widespread availability of the required equipment. The r56 recombinant-antigen ELISA should prove to be particularly suitable for testing in moderately equipped laboratories throughout the scrub typhus endemic area. In contrast to the ELISA, the PanBio dipstick assay was easy to perform and had minimal requirements for sophisticated laboratory equipment. Although its performance was not as good as that of the ELISA, the PanBio dipstick assay should prove to be ideal for diagnosis of scrub typhus in many rural areas of the disease endemic area.

The classic serologic diagnosis of rickettsial diseases is based on a  $\geq 4$ -fold rise in the titer between paired acute and convalescent sera determined by a specific test.<sup>7</sup> In contrast, evaluation of the efficacy of diagnostic assays normally is based on establishment of test performance indices using individual sera samples. In this study, we focused on evaluation of test assays compared with a reference assay rather than an actual determination of whether an individual sample was positive or negative. Although it is difficult to extrapolate results from a comparative study to determine how an assay would perform in a clinical setting, all results obtained in this study suggest that the PanBio assay and the r56 recombinant-antigen ELISA should prove to be useful in the clinical diagnosis of scrub typhus.

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