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Comparative Evaluation of a Commercial Enzyme Immunoassay for the Detection of Human Antibody to *Rickettsia typhi*

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A commercial enzyme immunoassay kit called the Dip-S-Ticks (DS) for the detection of total immunoglobulin (Ig) G and IgM human antibodies to *Rickettsia typhi* was evaluated. In tests with 340 serum samples from patients with diagnosed cases of rickettsial diseases, patients suffering from other febrile illnesses, and normal subjects, the DS compared favorably with the standard indirect fluorescent-antibody (IFA) test. At IFA cutoff titers of ≥1:64 and ≥1:128, the DS showed sensitivities of 88.2 and 91.4% and specificities of 91.8 and 87.7%, respectively. The DS test correlated significantly with both the IFA IgG ($r = 0.84$, $P < 0.0005$) and IgM ($r = 0.63$, $P < 0.0005$) titers. Only 80% of IgG and 82% of IgM IFA readings determined by two technicians were within one dilution, while the DS was more reliable, with 100% within one dot. The rapidity, reliability, and simplicity of the DS suggest that it is a suitable test for use in clinical laboratories unable to perform the IFA test.

Rickettsia typhi, the etiologic agent of murine and endemic typhus, is distributed worldwide. *Rattus norvegicus* and *Rattus rattus* are the principal reservoirs, and humans are incidental hosts. The oriental rat flea, *Xenopsylla cheopis*, is the primary vector (1). The infection is usually acquired by self-inoculation of infected flea feces into the skin by scratching of the flea bite site, but there is also recent evidence to suggest that the agent can also be transmitted by flea bite (1). Although the agent can be isolated from the patient's blood during the acute stages of illness, the techniques are difficult, time consuming, and hazardous, requiring biosafety level 3 practices (5, 15, 16). Acute disease chemical and hematological laboratory tests are not diagnostic; hence, serological confirmation of the clinical diagnosis is necessary. Unfortunately, the inadequate sensitivity of antibody assays can cause false-negative results due to such factors as the timing of the specimen collection and antibody responses which may be either delayed or abrogated in patients treated with antibiotics (7). Epidemiologic factors, clinical findings, routine serological laboratory results, and prior exposure in regions where the disease is hyperendemic must be considered when a diagnosis of murine typhus is contemplated.

The indirect fluorescent-antibody (IFA) test has become the standard procedure for serological confirmation of the illness (15, 16). However, this test is not routinely available and requires equipment often not available, especially in third world locations. More recently, the latex agglutination test has proven to be useful in serodiagnosis, especially for acute- and early convalescent-phase samples, since it is primarily an immunoglobulin (Ig) M assay detecting group lipopolysaccharide-reactive antibodies (8, 9, 19). However, latex reagents are expensive, and the product is no longer commercially available. A commercial enzyme immunoassay for the *R. typhi*-specific antibody called the Dip-S-Ticks (DS) for in vitro diagnostic use for the detection of total IgG and IgM antibodies was recently introduced as an aid in the presumptive diagnosis of typhus (11, 13). We show here that the DS compares favorably with the IFA test. Its promise of rapidity and simplicity makes the DS a potentially realistic replacement for the insensitive, nonspecific Weil-Felix test which is still commonly used for the serodiagnosis of murine typhus (10, 15, 16, 19).

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MATERIALS AND METHODS

Sera. Most of the sera used in this study were provided by the Department of Rickettsial Diseases, Walter Reed Army Institute of Research, Washington, D.C., and the Rickettsial Disease Program, Naval Medical Research Institute, Bethesda, Md., and were from clinically documented cases of rickettsial diseases as previously published (7, 9, 19). Selected serum panels had been collected sequentially from personnel with laboratory-acquired murine or epidemic typhus infection (7). Sera were also collected from febrile patients and healthy people with no evidence of murine or epidemic typhus or as defined by the presence of an alternate diagnosis. Sera from people in various locales who had the following conditions were used (the providers of the sera are noted): Peru, bartonellosis ($n = 6$), cholera ($n = 8$), typhoid ($n = 3$), leptospirosis ($n = 6$), and malaria ($n = 6$) (D. Watts, Naval Medical Research Institute Detachment, Lima, Peru); Indonesia, typhoid ($n = 13$) (J. Olson, Naval Medical Research Unit 2, Jakarta, Indonesia); United States, tularemia ($n = 10$) (M. Clements, Johns Hopkins University, Baltimore, Md.); Somalia, malaria ($n = 11$), dengue ($n = 6$), shigellosis ($n = 3$), and hepatitis E ($n = 1$) (J. Burans and T. Sharp, Naval Medical Research Institute); and United States, anti-nuclear antibody ($n = 10$) and rheumatoid factor ($n = 10$) (R. Haberberger, Naval Medical Research Institute). There were 60 healthy controls (12).

IFA test. The IFA test was a modification of the one developed by Robinson et al. for the detection of rickettsial antibodies in human serum (18). *R. typhi* Wilmington VR-144, *Rickettsia tsutsugamushi* Karp VR-150, and *Rickettsia conorii* Moroccan VR-141 were propagated in cell cultures with murine fibroblast L cells which were gamma irradiated with 3 kilorads prior to infection (23). The harvested cell pellet was irradiated with 200 kilorads to render the rickettsiae noninfectious and diluted to yield approximately 500 organisms per 400× microscopic field. Approximately 0.2 μl of each of the three rickettsial and single L-cell control antigen spots was applied with a P2 micropipetor (Pipetman; Ranin Instrument, Woburn, Mass.) to yield nine clusters of the four antigens per slide. The slides were dried at room temperature for 30 min in a laminar flow hood, acetone fixed for 10 min at 4°C, and then stored with desiccant in slide boxes at −20°C. Prior to use, the slides were rapidly brought to room temper-

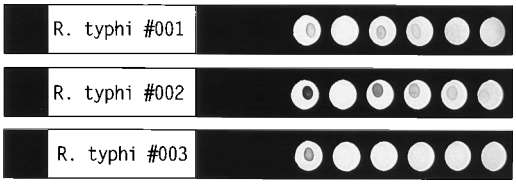

FIG. 1. DS strips containing positive reagent control (window 1, closest to handle), negative control (window 2), and fourfold serial dilutions of *R. typhi* antigen (windows 3 to 6). Negative serum control is shown at the bottom, 4+ murine typhus patient serum is shown in the middle, and 2+ murine typhus patient serum is shown at the top. The 2+ serum is reactive in windows 3 and 4, and the 4+ serum is reactive in windows 4 through 6.

ature with an air blower to remove condensation formed on the surface. Each of the nine clusters was covered with 20 μl of either a 1:64 screening serum dilution or, if positive in the screen, a series of twofold serum dilutions from 1:64 to 1:16,384. Sera were diluted with phosphate-buffered saline (PBS), pH 7.2. The slides were incubated for 30 min in a humidified chamber at 37°C, washed twice with PBS for 10 min in a staining jar with a magnetic stirrer at room temperature, and air dried at room temperature with an air blower. Twenty microliters of the diluted conjugate was then added to each cluster, and the slides were incubated again in the same manner as that for the serum incubation. The fluorescein-conjugated goat IgG fraction to the human IgG Fab fragment or IgM (5Fc μ specific) (Cappel, Organon Teknika Corp., Durham, N.C.) was diluted with Eriochrome black (Integrated Diagnostics, Baltimore, Md.) diluted 1:1,000 with PBS, pH 7.2, as a counterstain. Conjugates were optimized by conventional checkerboard titration with control serum. Following incubation, washing, and drying, coverslips (22 by 50 mm) were mounted onto slides with 90% glycerin with 10% 0.1 M PBS, pH 8.0 to 8.6. The slides were read with a Zeiss Universal fluorescence microscope (Carl Zeiss, Oberkochen, Germany) at a 40× fluorescence objective (high dry). The endpoint was defined as the highest serum dilution with discernible fluorescing rickettsiae.

Enzyme immunoassay. The DS murine typhus test kit (Integrated Diagnostics), an assay based on an enzyme-linked immunoassay dot technique for the detection of IgG and IgM antibodies, was evaluated in this study. Kits were provided by Integrated Diagnostics. The kits, which required refrigeration, were used according to the manufacturer's instructions as follows. For each sample assay, the kit contained an antigen strip, four liquid reagents, and reaction cuvettes. For the immunoassay, the *R. typhi* antigen was prepared from fresh pools of infected yolk sacs of embryonated eggs by the method of Halle and Dasch (7). The manufactured stick contained a series of six windows (Fig. 1). The first window, or dot, of the assay strip, that closest to the handle, was the positive reagent control consisting of human IgG. The second window was the negative control consisting of PBS. Windows three to six contained fourfold serial dilutions of formalin-treated whole-cell *R. typhi* antigen (7). To perform the test, 2 ml of each of four liquids contained in the kit was added to a series of four reaction cuvettes in sequence (the following was adapted from the package insert): liquid 1 was added to cuvette 1 containing sample diluent consisting of diluent buffer salts, sodium azide as a preservative, and inert liquid material, pH 6.2 to 7.6; liquid 2 was added to cuvette 2 containing enhancer consisting of sodium chloride, sodium azide, and inert liquid materials; liquid 3 was added to cuvette 3 containing conjugate consisting of alkaline phosphatase-conjugated goat anti-human IgG and IgM antibodies in diluent buffer salts, sodium azide, and inert liquid materials (pH 6.2 to 7.6); and liquid 4 was added to cuvette 4 containing developer consisting of 5-bromo-4-chloro-3-indolylphosphate and *p*-nitroblue tetrazolium chloride in buffered diluent salts, sodium azide, and inert liquid materials, pH 9.0 to 11.0. Temperature was maintained at 50°C with a variable-temperature heating block (Lab-Line Instruments, Inc., Melrose Park, Ill.) or an eight-well fixed-temperature heating block (50 ± 0.1°C) (Integrated Diagnostics). Ten microliters of the serum was added to the first cuvette, rendering a dilution of 1:200. The antigen strips were premoistened in distilled water for at least 4 min and inserted into the first cuvette, allowing patient antibodies reactive with the test antigen to bind to antigen on the strip's solid support membrane during a 5-min incubation. Following a rapid rinse in distilled water, the strip was placed in the second cuvette for 5 min, during which nonspecifically bound materials were removed. After a rinse, the strips were placed for 15 min in the third cuvette containing a mixture of alkaline phosphatase-conjugated anti-human IgG and IgM, which reacted with the bound patient antibodies. The strips were again rinsed and then transferred for 5 min into the fourth cuvette containing the substrate reagent, 5-bromo-4-chloro-3-indolylphosphate, and *p*-nitroblue tetrazolium chloride, which reacted with the bound alkaline phosphatase to produce a colored spot. Following a final rinse, the strips were allowed to air dry. A positive reaction produced a blue dot with an easily seen, distinct border, with the outer perimeter of the window colored white to pale gray.

Data analysis. All IFA and DS results were determined by one technician, blinded to the identity of the serum and the previous test outcome. A second technician with equivalent training was given random samples of serum, which were also evaluated in a blinded fashion.

DS and IFA results as determined by the initial technician were used in the data analysis. The maximum IgG or IgM endpoint titer for a serum was used as the IFA result. The association of DS values and IFA titer results was examined by Spearman rank correlation. The sensitivity and specificity of the DS were examined, with IFA cutoff titers of 1:64 and 1:128 (6, 16) being used as the "gold standard." Proportions are presented together with 95% confidence intervals. DS sensitivities among disease groups were compared by Fisher's exact test (two tailed). DS reliability and IFA test reliability for the same serum were compared by McNemar's test for matched proportions.

TABLE 1. Reactivities of the Integrated Diagnostics *R. typhi* DS test and IFA test for sera from healthy and diseased people

Condition	Total no.	No. reactive for *R. typhi*			No. reactive for *R. conorii* by IFA[a]		No. reactive for *R. tsutsugamushi* by IFA[a]	
		DS[b]	IFA[a]					
			IgG	IgM	IgG	IgM	IgG	IgM
Uninfected healthy controls	60	0	0	0	0	2	0	0
Dengue	6	0	0	0	1	0	0	0
Hepatitis E	1	0	0	0	0	0	0	0
Malaria	17	0	1	0	2	1	0	0
Bartonellosis	6	0	0	0	1	0	0	0
Cholera	8	0	1	1	4	2	0	0
Leptospirosis	6	0	0	0	1	1	0	0
Typhoid	16	5	4[c]	0	0	0	0	0
Tularemia	10	0	0	0	0	0	0	0
Shigellosis	3	1	0	0	0	0	0	0
Anti-nuclear antibody	10	1	0	0	3	2	0	0
Rheumatoid factor	10	2	4	0	3	2	1	1
Scrub typhus	9	2	3	1	0	0	8	3
Rocky Mountain spotted fever	8	4	2	1	8	4	0	0
Murine typhus	87[d]	73	72	46	31[e]	24[e]	0	0
Epidemic typhus	64[d]	48	51	29	30	11	0	0
Brill-Zinsser	3	3	3	0	3	0	0	0
Prerickettsial disease[f]	16	4	2	1	0	1	0	0

[a] Positive, ≥1:64.
[b] DS positive, ≥1 dot.
[c] All four sera were reactive by the first DS dot.
[d] Includes sera from acute, nonseroreactive cases.
[e] Only 79 of 87 serum samples were tested.
[f] Sera were from epidemic and murine typhus patients prior to the acquisition of the illness by the patients.

RESULTS

Correlation of *R. typhi* DS reactivity and IFA titer. A total of 340 serum samples were tested by *R. typhi* DS titers and IFA titers for IgG and IgM. Table 1 shows the reactivities by the DS and the IFA test of sera collected from healthy people and from diseased people having rickettsial and nonrickettsial illnesses. Of the 60 serum samples from healthy controls, 2 were reactive with *R. conorii* antigen by IgM, whereas none reacted with the DS.

Fewer rheumatoid factor-positive sera were reactive with the DS than with the *R. typhi* or *R. conorii* IgG IFA. Also, fewer anti-nuclear antibody-positive sera were reactive with the DS than with the *R. conorii* antigen in the IFA test. As expected, sera from Rocky Mountain spotted fever patients were cross-reactive with the *R. typhi* antigen and sera from murine typhus patients cross-reacted with *R. conorii* antigen, as measured by the IFA test. The *R. typhi* DS also detected these cross-reactive antibodies in the Rocky Mountain spotted fever patient sera.

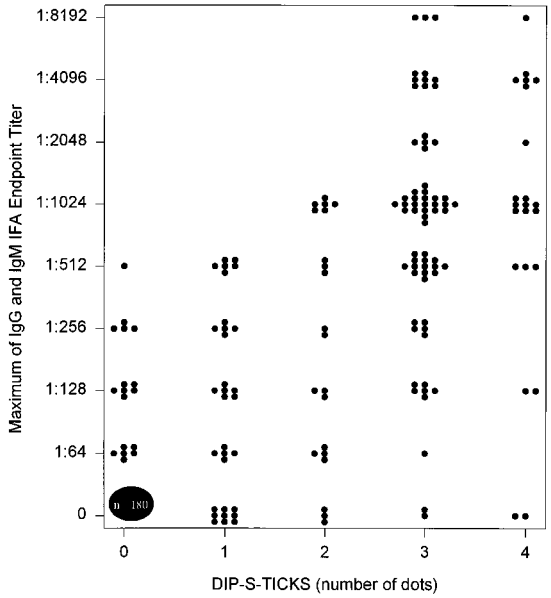

FIG. 2. Association between DS results and maximum endpoints of IgG and IgM titers for each serum ($r = 0.85, P < 0.0005$). Each solid circle represents one DS.

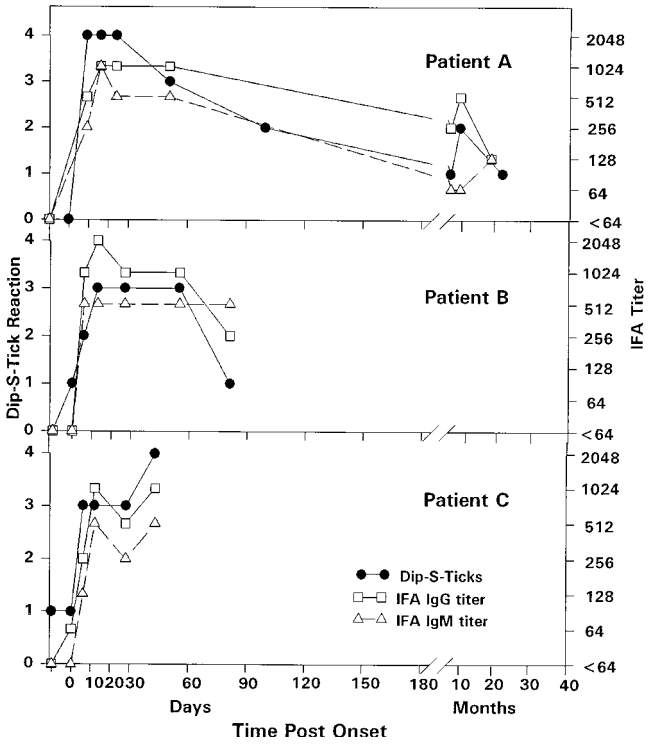

FIG. 3. Patterns of antibody response to *R. typhi* infections. Patients A and B received prompt antibiotic therapy, whereas patient C received delayed antibiotic treatment. Time 0 represents the initial onset of symptoms. Preonset sera were collected from patients A, B, and C at 10 months, 5 months, and 9 years previous to onset, respectively.

Four of eight serum samples from cholera patients were reactive against *R. conorii* by the IFA test, but they were not DS positive. These sera were collected in Peru, where spotted fever group illnesses have been reported. Only typhoid and scrub typhus patient sera exhibited unusually frequent reactivity to *R. typhi* by both the IFA test and DS but not to *R. conorii* antigen by the IFA test. The prerickettsial disease group includes sera from laboratory workers who may have seroconverted without showing clinical illness.

There was a significant correlation between the DS and IFA IgG results ($r = 0.84, P < 0.0005$) and between the DS and IFA IgM results ($r = 0.63, P < 0.0005$) (Fig. 2).

With the maximum IFA results for IgG and IgM being used as the criterion for the presence of disease, the highest sensitivity of the DS, 91.4% (95% confidence interval, 84.8 to 95.4%), occurred when IFA titers of ≥1:128 were considered positive for disease, and the highest specificity, 91.8% (95% confidence interval, 86.8 to 95.1%), occurred with cutoff titers of ≥1:64 (Table 2). At the IFA cutoff value of ≥1:64, there was no significant difference between sensitivities for murine typhus (90.3%, $n = 72$) and epidemic typhus–Brill-Zinsser disease (94.4%, $n = 54$) when sera from proven cases alone were compared ($P = 0.51$, Fisher's exact test).

Reproducibility. Between-run precision for the *R. typhi* DS test was explored by assaying a single reactive serum (positive control serum) 31 times on 8 days over a 9-month period with the same reagent lots. Twenty-nine (94%) of the tests produced three positive dots on the DS, and two (6%) tests produced two dots. The latter two runs were repeated, subsequently giving three positive dot control results. For the same control serum as that used in the IFA test, 23 runs on 23 separate days gave 1 titer of 1:4,096, 4 titers of 1:2,048, 16 titers of 1:1,024, and 2 titers of 1:512.

TABLE 2. Sensitivity and specificity of the DS *R. typhi* test as judged by IFA *R. typhi* results

IFA cutoff[a]	% (no. of sera in agreement/total no. of sera)	
	Sensitivity[b]	Specificity
1:64	88.2 (127/144)	91.8 (180/196)
1:128	91.4 (117/128)	87.7 (186/212)

[a] Using the maximum IgG or IgM titer.
[b] Positive DS test results were defined as one or more positive dots.

Reliability. To determine the reliability of the readings of the DS and IFA tests, a second technician independently read the results for a random sample of the sera.

For the IgG IFA test, 98 serum samples were evaluated. Thirty-five samples (35.7%) had identical readings, and 78 (79.6%) were within 1 dilution. Of the 21 serum samples read as negative by the first technician, 4 (19.0%) were read as positive by the second technician.

For the IgM IFA test, 76 serum samples were randomly selected for evaluation. Thirty-nine samples (51.3%) had identical readings, and 62 (81.6%) were within 1 dilution. Of the 34 serum samples read as negative by the first technician, 2 (8.8%) were read as positive by the second technician.

A total of 323 serum samples were evaluated for DS reliability. While 298 readings were identical for the two readers (92.3%), all 323 samples were within one dot. Of the 186 samples read as negative by the first technician, 5 (2.7%) were read as positive by the second technician.

To compare DS reliability and IgG IFA reliability, results were evaluated for the same 98 serum samples. The DS results were identical for the two technicians for 83 serum samples (84.7%), which compares with a 35.7% perfect-agreement rate for the IgG IFA test ($P < 0.00001$, McNemar's test).

Representative patterns of antibody responses in isolate-

positive murine typhus cases are shown in Fig. 3. Panels A and B illustrate two cases which were recognized early and treated promptly with tetracycline. Antibody responses measured by both the DS and the IFA (both IgG and IgM) test rose rapidly following the onset of symptoms and subsequently declined approximately 90 days later. The antibody response remained measurable in sera collected from patient A for up to 2 years postinfection. Panel C illustrates the response of a patient for whom therapy was delayed for 5 days after the onset of symptoms. Both IgG and IgM antibodies rose rapidly to high levels. In addition to these three patients, paired acute- and convalescent-phase sera from four other patients were used to determine if seroconversion could be detected with the DS. Six pairs showed fourfold rises in the levels of IgM and four did so for IgG by the IFA test, and four showed at least a one-dot rise by DS.

DISCUSSION

The first commercially available test for the specific detection of human antibody to typhus group rickettsiae was the Weil-Felix test (22). This test, which became popular in the 1920s, remains simple, cheap, and widely used. However, during the past several years, it has been shown to be of dubious sensitivity and specificity, particularly for spotted fever and scrub typhus infections (10, 12, 16, 19, 20). Consequently, some investigators have recommended its use be discontinued (16). The IFA test has become the "gold standard" against which other tests for serodiagnosis of rickettsial diseases are compared (16, 20). The subjectivity of the IFA test, the relative expense of antigen reagents, and the expensive equipment have generally discouraged the routine use of this test by most clinical laboratories. It requires relatively sophisticated and sensitive instrumentation and a highly trained technician to prepare the stained slides and read the endpoints. The DS test is a commercially available semiquantitative enzyme immunoassay for the detection of total IgG and IgM antibodies to *R. typhi*, which aids in the presumptive serodiagnosis of typhus group infections. The relative simplicity of this test, which requires only the kit and either an electric heating block or a simple water bath, gives it broad utility, even in field laboratory environments and especially in places which may not have the resources to perform the IFA test (3, 21). It does not require sophisticated or delicate instrumentation, such as the fluorescence microscope needed for the IFA test, or a highly trained technician (12, 21). In the present study, all repeat DS readings agreed within one positive dot, which was markedly better than the performance of the IFA test, in which endpoints of only about 80% of the sera agreed within 1 dilution. While our results for the DS for a single serum between separate runs were within 1 dilution, additional testing of the reproducibility of the IFA test and DS is required.

The manufacturer suggests that DS reactivity can be used quantitatively to determine specific IFA-equivalent antibody titers. We found that the number of dots in the DS test significantly correlated with both the IFA IgG ($r = 0.84$) and IgM ($r = 0.63$) results. If DS dots correlate to IFA reactivity results spanning the clinically significant range, it should be possible to detect rises in specific titer. In fact, the manufacturer recommends testing with a sample collected 2 to 3 weeks later to confirm recent specific antibody. Single serum titers of $\geq$1:64 have been suggested to be diagnostic for typhus infections (16). Therefore, for the evaluation of sensitivity and specificity of the DS, we selected IFA IgG and IgM cutoff values of $\geq$1:64 and $\geq$1:128. Using the IFA test as the standard, we found the peak DS sensitivity to be 91%. Nevertheless, the relative contributions of IgG and IgM to DS reactivity are not known, and we believe that future formulations for the DS might be more informative if individual conjugates were available.

The cross-reactivity of a test can result in significantly reduced specificity. Because of the presence of common antigens within the same rickettsial groups and among different rickettsial groups, the serological tests with native *R. typhi* antigen may react with those of other rickettsial species. There are no commercially available tests which can be used to distinguish between antibodies to *R. typhi* and *Rickettsia prowazekii*, for example (16, 17). We found sera from epidemic typhus and Brill-Zinsser (recrudescent typhus) patients to be highly reactive with the *R. typhi* DS (Table 1), which suggests it should prove useful in detecting antibody to either disease (17). While it is of important epidemiologic utility, the capacity to distinguish between the two diseases is arguably of little clinical value. The primary need of the clinician is to distinguish the treatable typhus diseases from viral, parasitic, and other bacterial diseases which mimic their clinical presentations. Hence, it could be considered useful that the DS can detect both *R. typhi*- and *R. prowazekii*-induced antibodies. In regions where conditions for epidemic typhus exist, the DS might prove useful in detecting outbreaks. Similarly, the DS may prove useful for state health departments and commercial laboratories for testing in nontraditional regions of epidemic typhus which now report typhus caused by the enzootic flying squirrel agent (2, 15). Koay and Cheong (13) report that sera from murine typhus patients are reactive to spotted fever group rickettsial antigens in the DS format, as does Broadhurst et al. (3). The reactivity of some sera from Rocky Mountain spotted fever patients with typhus group antigens, as previously reported (17), was also detected here with the DS (Table 1). Reactivity of sera from patients with nonrickettsial illnesses with rickettsial antigens was minimal, with the exception of sera from proven typhoid cases. There are no reports of either *R. tsutsugamushi* or *Salmonella typhi* sharing antigenic components with *R. typhi*. Since all reactive typhoid patient sera were collected from people living in areas within Indonesia where murine typhus is endemic, they may contain specific anti-*R. typhi* antibody from active or previous exposures. Similarly, this might explain the reactivity of *R. typhi* antigen with sera from three scrub typhus patients also from Indonesia (14).

There are inherent weaknesses in the DS. In addition to the problems with specificity, the liquid reagents require refrigeration, somewhat limiting the field utility because of transport limitations. As with most serological tests for infectious disease, including the IFA test, the manufacturer suggests that a confirmatory diagnosis requires paired sera to show specific rises or falls in titers. Thus, reliable serodiagnosis remains retrospective, so that treatment will continue to be initiated on the basis of the clinical impressions of the attending physician. Until accurate agent detection systems such as the PCR for specific nucleic acid detection (4, 15), antigen capture assays, and direct staining of rickettsiae in tissue samples are adequately developed, evaluated, and more widely available, serodiagnosis remains the sole widespread method for confirmation of diagnosis of rickettsial disease.

In addition to offering clinical utility, the simple DS product could be used to identify foci of endemicity within suspect geographical regions. For example, the DS could be used in seroprevalence studies to determine which areas might require more intensive evaluation. Since the kits and the testing capability are portable, on-the-spot testing could be performed in the town or village where febrile illnesses have been reported. We found that the reactive DS faded only after several months of storage; thus, it could be used in areas where the disease is

endemic and quantitative densitometry of the results could take place later at a central laboratory.

This study clearly demonstrates that the DS test is similar in reactivity to the IFA test. Thus, the many practical benefits of the DS suggest that it should be the test of choice for use in clinical laboratories lacking the ability to routinely perform the IFA test and in laboratories which do not currently perform tests for rickettsial diseases or in which only the Weil-Felix test is presently performed.

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