LABORATORY EVALUATION OF A DOT-BLOT ENZYME IMMUNOASSAY FOR SEROLOGIC CONFIRMATION OF ILLNESS DUE TO RICKETTSIA CONORII

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Abstract. Of the 169 United States Army soldiers who deployed on a field training exercise to a remote area of Botswana for two weeks in January 1992, more than 30% developed a febrile illness within five days of their return. A diagnosis of South African tick typhus was suggested by soldiers’ exposure to ticks, as well as the presence of eschars and vesicles at the site of tick bites, and tender regional lymphadenopathies. This high attack rate, experienced during such a short exposure period, emphasizes the hazard of illness due to Rickettsia conorii to persons visiting endemic areas. A rapid, diagnostic, semiquantitative enzyme immunoassay (DS) for detection of IgG and IgM antibodies to R. conorii was performed on 209 acute and convalescent sera from soldiers in the outbreak and on 75 control sera. For the acute sera from soldiers meeting the probable case definition of having both regional lymphadenopathy and tick bite eschar, as judged by an IgG indirect fluorescent antibody (IFA) test, the resulting sensitivity and specificity of the DS test were 100% and 48%, respectively. In the analysis of the acute sera, the DS test identified as reactive more of the probable cases (62%) than either the IgG (16%) or IgM (55%) IFAs. This simple and rapid diagnostic test could be useful in establishing a preliminary diagnosis of R. conorii rickettiosis in remote settings when immediate confirmation by IFA is impossible.

Rickettsia conorii causes significant morbidity worldwide. Historically, there are various names for the disease it causes in different geographic areas, such as Mediterranean spotted fever, South African tick typhus, Boutonneuse fever, and Indian tick typhus. However, the disease diagnosis is often unsupported by laboratory results due to the limited capabilities of the laboratories in many of the areas where R. conorii is present. As a result, confirmation of suspected clinical disease and correct and timely treatment are often lacking. A sensitive, specific, simple, and rapid diagnostic kit has not previously been commercially available. The indirect fluorescent antibody (IFA) test and other sophisticated procedures are available only in large population centers using skilled personnel. This creates a diagnostic dilemma for clinicians treating not only indigenous patients but visitors to endemic areas who are coming into contact with the vectors of R. conorii for the first time.

During a U.S. military training exercise in Botswana in January 1992, soldiers had their first presumed contact with the vectors of R. conorii. The exercise involved 169 soldiers of the 3rd Battalion of the U.S. Army’s 325th Infantry Regiment from Vicenza, Italy, who deployed for two weeks to an isolated area of southeastern Botswana. Upon their return to Vicenza, nearly 30% of the soldiers sought medical attention. The preliminary clinical diagnosis for the soldiers with the signs and symptoms of tick bite, regional lymphadenitis, and febrile myalgias was South African tick typhus. A new diagnostic test kit (DS) using a dot-blot enzyme immunoassay technology aided in making the diagnosis in 23 of these soldiers who were on location in a military hospital with very basic laboratory capabilities. Because of this positive performance, further testing of a larger group of the sera from these soldiers was performed, and the results are presented here.

MATERIALS AND METHODS

Sera. Evaluation of the DS test, including testing of sera and comparison with the gold standard IFA was performed at the Naval Medical Research Institute (Bethesda, MD). The study used 209 sera, (69 pairs, seven acute, and 64 convalescent only) drawn from 140 soldiers in the outbreak. The acute and convalescent sera were collected in Vicenza 2–10 and 56–60 days after onset of signs and symptoms. In addition, six sera from individuals with various confirmed nonrickettsial illnesses (herpes simplex virus, hepatitis A, cytomegalovirus, varicella zoster, mumps, and assorted pooled febrile sera) (J. Buruns, Naval Medical Research Institute) and sera from 54 uninfected healthy people were also tested using the DS test. Control sera from 15 patients diagnosed as having rickettsial diseases caused by R. conorii (Mediterranean spotted fever) (two), R. rickettsia (Rocky Mountain spotted fever) (eight), R. typhi (murine typhus) (two), R. prowazekii (louse-borne typhus) (one), and Orientia tsutsugamushi (scrub typhus) (two) were tested by the DS test.

Indirect fluorescent antibody test. The IFA test was used as a modification of that developed by Philip and others for the quantification of rickettsial antibodies in human serum. The R. conorii Moroccan strain (ATCC VR-141) was propagated in cell culture using murine fibroblast L-cells that were gamma irradiated with 3 Krads prior to infection. The remainder of the IFA test was carried out as previously described.

Dipsticks. The R. conorii DS test used an enzyme-linked immunoassay dot technology to quantify IgG and IgM antibodies. Rickettsia conorii DS testing was performed following kit instructions and as previously described using kits obtained from Integrated Diagnostics (Baltimore, MD). The kit contained an antigen strip, four liquid reagents, reaction cuvettes and detailed instructions. The R. conorii (Moroccan strain) antigen used on the strips was prepared by the manufacturer from fresh pools of infected yolk sacs of embryonated eggs using the method of Halle and Dasch. Kits were received and maintained at refrigeration temperature.

Data analysis. The DS tests were performed by a clinician previously untrained in their use and blinded to the
identity of the sera or previous IFA test outcomes. The IFA tests were performed in a similarly blinded fashion by a technician highly trained to perform this test. For sera analyzed by the IFA test using single specimens, a reciprocal IgG titer greater than or equal to 64 was considered positive.

To identify all cases, we considered any level of reactivity (1–4 dots) as positive. The manufacturer’s criteria were used to define positive and negative results for tests of sera by the DS test. Positive results were dots with easily seen borders visible in the center of the window. If no dot was seen or a dot was difficult to see, it was read as negative.

Clinical cases were defined as soldiers having the classic skin lesion of South African tick typhus, tache noire (black spot) or tender regional lymphadenopathy by physical examination performed by one of two physicians.³ Cases were further subdivided as probable cases (lymphadenopathy and tache noire) or possible cases (lymphadenopathy or tache noire).

The sensitivity of the DS test is defined as the proportion of IFA-positive sera (reciprocal IgG titer greater than or equal to 64) that were also positive by the DS test, while specificity of the DS test is defined as the proportion of IFA-negative sera (reciprocal IgG titer less than 64) that were also negative by the DS test. Reproducibility was determined by reading two tests done in the same run and the kappa statistic was calculated using the method of Fleiss.¹¹

### RESULTS

The DS assays of the negative serum panel indicated good specificity (57 of 60 = 95%). None of the nonrickettsial disease panel were reactive; two sera from the healthy patients were reactive at the first dot and one was reactive at the second dot. In the rickettsial diseases panel, as expected, all sera except one from a scrub typhus patient were reactive at one or more dots.

In the analysis of the acute sera (Table 1), the DS identified as reactive more of the probable cases (62%) than either the IgG IFA (16%) or the IgM IFA (55%) assays. The DS test also identified fewer asymptomatic soldiers as positive than the IgM IFA assay. When only convalescent sera were tested (Table 2), the DS test identified as reactive nearly all (93%) the probable cases, and identified fewer (51%) asymptomatic soldiers as reactive than either the IgM IFA (95%) or the IgG IFA (54%).

The results presented in Table 3 are for acute and convalescent sera from soldiers defined as probable cases only. The sensitivity of the DS test for the sera as judged by IgG IFA results was 100% for both acute and convalescent sera. The sensitivity as judged by IgM IFA results was also high (92%) for convalescent sera. The DS test specificity, however, as judged by the IgG IFA and the IgM IFA assays was low for both acute and convalescent specimens.

Although only four of 10 negative DS test results were negative by repeat testing, 29 of 30 positive results were positive by repeat testing. This resulted in a kappa statistic of 0.44, which is considered an indication of good reproducibility.

### DISCUSSION

The strength of the DS test is that it appears to be a sensitive test, as discussed above, with regard to the 100% sensitivity with IgG IFA results for probable cases. The negative predictive values, although not presented here, were also extremely high; therefore, if a DS test result was negative, the clinician could be reasonably certain that the individual had not been exposed to R. conorii and could concentrate on other possible diagnoses. The kit was especially important in the initial evaluation of this outbreak because it provided

### Table 1

Reactivity of the *Rickettsia conorii* dot-blot enzyme immunoassay (DS) test and indirect fluorescent antibody (IFA) test for acute sera from Botswana outbreak

<table>
<thead>
<tr>
<th>Condition</th>
<th>% reactive for <em>R. conorii</em> by DS test*</th>
<th>% reactive for <em>R. conorii</em> by IFA test†</th>
<th>Speciﬁcity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asymptomatic soldiers</td>
<td>49% (18/37) 22% (8/36) 86% (31/36)</td>
<td>57% (17/30) 1:64</td>
<td>95% (35/37)</td>
</tr>
<tr>
<td>Possible cases‡</td>
<td>25% (2/8) 0% (0/9) 66% (6/9)</td>
<td>51% (49/97) 1:64</td>
<td>88% (7/8)</td>
</tr>
<tr>
<td>Probable cases§</td>
<td>62% (16/26) 16% (5/31) 55% (17/31)</td>
<td>95% (26/27)</td>
<td>96% (26/27)</td>
</tr>
</tbody>
</table>

* DS reactive ≥ one dot.
† IFA reactive ≥ 1:64.
‡ Lymphadenopathy or tache noire.
§ Lymphadenopathy and tache noire.

### Table 2

Reactivity of the *Rickettsia conorii* dot-blot enzyme immunoassay (DS) test and indirect fluorescent antibody (IFA) test for convalescent sera from Botswana outbreak

<table>
<thead>
<tr>
<th>Condition</th>
<th>% reactive for <em>R. conorii</em> by DS test*</th>
<th>% reactive for <em>R. conorii</em> by IFA†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asymptomatic soldiers</td>
<td>51% (49/97)</td>
<td>54% (50/93)</td>
</tr>
<tr>
<td>Possible cases‡</td>
<td>66% (6/9)</td>
<td>50% (4/8)</td>
</tr>
<tr>
<td>Probable cases§</td>
<td>93% (25/27)</td>
<td>78% (21/27)</td>
</tr>
</tbody>
</table>

* DS reactive ≥ one dot.
† IFA reactive ≥ 1:64.
‡ Lymphadenopathy or tache noire.
§ Lymphadenopathy and tache noire.
for early/accurate diagnosis in a very basic laboratory facility. The greatest advantage in the field is that the test is quick, easy, and relatively inexpensive. In contrast, despite the fact that the IFA test is the gold standard, its subjectivity, expense, and requirement for sophisticated and sensitive equipment and highly trained technicians discourage its use by most clinical laboratories. The IFA test was not an option in our early effort to evaluate the outbreak.

The DS test specificity using the negative serum panel was high (95%), which is an additional strength of the test. However, the specificity of the DS test was relatively low using the outbreak sera, and because of the high prevalence of false-positive results, confirmation by a more specific assay when available might be desirable. These results are in accordance with the manufacturer’s statement that in a screened population that includes noncases, their serologic test results are not specific. As a result, many false-positive results and the resulting low positive predictive value are expected. This is not only a disadvantage but a danger in some situations if it discourages a clinician to vigorously pursue the true diagnosis that could be a more deadly illness. For example, *Plasmodium falciparum* malaria is prevalent in many of the same areas as *R. conorii* and has similar signs and symptoms. Empiric treatment should be given to a febrile patient with tache noire and/or regional lymphadenopathy with a history of tick exposure in a geographic region known to be endemic for spotted fever group rickettsiosis whether an enzyme immunoassay test result is positive or not. Other disadvantages of the test include requirements for strict temperature limits during test performance and refrigeration during shipment and storage. It is often difficult to meet these requirements in a field setting.

Although there are problems with using the IFA as the gold standard to evaluate the DS test because there is an inherent sensitivity and specificity of the IFA test itself, the DS test evaluations for other rickettsial diseases validate such comparisons. The cut-off value of 1:64 for positive IgM IFA and IgG IFA test results is within the range 1:32–1:128 found elsewhere in the literature. By changing the cut-off value, there would have been different criteria for positive and negative results, and, therefore, different summary statistics.

The Moroccan strain of *R. conorii* used in testing is different from the strain that probably caused the illness. There has been some evidence to suggest that the rickettsia in Botswana is either another strain of *R. conorii* or a new rickettsia (*R. africae*). Finally, the background exposures of the soldiers to rickettsia that might have caused serologic cross-reaction with both the IFA and DS tests are unknown. As expected, sera from known spotted fever and typhus group rickettsial disease cases were uniformly positive with the DS test. This demonstrated the potential use of this test to detect other rickettsial diseases, but these results could be misleading if antibody persists from a previous rickettsial illness. The positive DS test result for one of the sera from the scrub typhus cases was unexpected since there is no known serologic cross-reactivity between scrub typhus and other rickettsial groups. This finding could be due to the patient’s coinfection with another rickettsial agent.

In the outbreak situation, the DS test was actually used upon the return of the soldiers to their Army base in Italy to identify the outbreak and detected several cases as early as two days after the onset of symptoms. The outbreak was later confirmed by the IFA test in the United States to be caused by *R. conorii*. Until that time, the DS test was the only laboratory evidence available in those first few days that the soldiers were receiving proper treatment for the most likely diagnosis. This rapid diagnostic aid proved to be invaluable in the diagnosis of *R. conorii*-induced illnesses, which are often prevalent in areas that lack sophisticated laboratories.

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