USE OF THE RECOMBINANT K39 DIPSTICK TEST AND THE DIRECT AGGLUTINATION TEST IN A SETTING ENDEMIC FOR VISCERAL LEISHMANIASIS IN NEPAL

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Abstract. We evaluated the field use of two serologic tests for visceral leishmaniasis (VL), the direct agglutination test (DAT) and rK39 dipstick test, in the context of a case-control study. Most VL cases in Nepal are currently diagnosed on clinical grounds and with relatively non-specific tests such as the formal-gel test. Among 14 newly diagnosed VL patients with bone-marrow slides confirmed positive in two independent laboratories, the sensitivity of both tests was 100%. Among 113 controls with no personal or household history of VL, the specificity of the rK39 was 100% while that of the DAT was 93%. The rK39 was less expensive than DAT, and has the advantages of ease of use and obtaining results within minutes. The wider use of the rK39 dipstick test could improve the specificity of VL diagnosis in Nepal.

INTRODUCTION

The diagnosis of visceral leishmaniasis (VL) is complex under the most favorable circumstances. Definitive diagnosis requires demonstration of parasites by smear or culture in tissue, usually spleen, bone marrow, or lymph node, and thus entails at least one invasive procedure. These procedures are less than 100% sensitive, and there is no accepted “gold standard” for VL diagnosis. For example, the sensitivity of lymph node aspirate was estimated at 50% in the Sudan, where lymphadenopathy is a common sign of VL. Furthermore, the sensitivity of bone marrow-aspirate smear is estimated at 70% or lower. If the first procedure does not identify parasites and the clinical index of suspicion is high, repeat sampling from the same or another site is often recommended. Smears from splenic aspirates have sensitivity greater than 90%, but the procedure carries a risk of potentially fatal hemorrhage.

In many VL-endemic locations, the situation is further complicated by limited infrastructure and health care funds, and by the scarcity of trained personnel. Under these conditions, performing multiple invasive procedures is even more problematic. In Nepal, for example, splenic aspiration is rarely performed because of the risk of hemorrhage and the absence of experienced medical personnel. Bone-marrow aspirates are carried out in only a few Nepali hospitals and private facilities, and only patients with sufficient resources can travel across the border to India to undergo a splenic aspirate. Most cases of VL in Nepal are diagnosed and treated based on clinical findings of prolonged fever and splenomegaly, and nonspecific tests such as the total white blood cell count and the formal-gel or aldehyde test, which is a qualitative measure of hypergammaglobulinemia.

Efforts to improve VL diagnosis in Nepal have recently focused on the direct agglutination test (DAT), a field-applicable serologic test which, in contrast to standard ELISA tests, requires minimal equipment and can be read visually. The DAT, in common with many other serologic tests for VL, employs an antigen preparation based on whole *Leishmania* promastigotes. The DAT is highly sensitive for VL, but populations in endemic areas have high background DAT seropositivity. In a large survey in endemic districts of Bangladesh, the ratio of seropositive persons without VL to those with VL was 6 to 1. A positive serologic test in the absence of clinical illness may represent past infection, asymptomatic infection, pre-clinical VL, or cross-reactivity with another infectious organism.

A recently-developed nitrocellulose dipstick test that detects antibody to the recombinant amastigote antigen K39 (rK39) is highly sensitive and specific for the diagnosis of acute VL in clinical settings. The rK39 dipstick test is not yet widely used in Nepal, in part because of concerns about cost. As part of a case-control study of VL in Nepal, we assessed the field use of the DAT and rK39 dipstick tests. Specifically, we determined the sensitivity and specificity in patients with bone-marrow confirmed VL, and in villagers from the same endemic communities who had no personal or household history of VL.

METHODS

We conducted the fieldwork in the VL-endemic districts of Dhanusha, Mahottari, and Siraha. We identified VL patients through the registries of the 4 area government hospitals, and from patient logs of 2 health-care practitioners who performed diagnostic bone-marrow aspirates. In addition, we recruited individuals with clinical findings consistent with VL from the villages we visited, and those with documented parasitologic diagnosis or a convincing record of clinical diagnosis of VL and response to therapy. We referred sick patients to the nearest government hospital for diagnosis and treatment. Standard drug treatment for VL was provided free by the Ministry of Health. For a subset of the newly diagnosed patients, bone-marrow slides were read both by local health care personnel, and by the staff of the Biology and Diagnostics Branch of the Division of Parasitic Diseases, Centers for Disease Control and Prevention (CDC), Atlanta, Georgia. Centers for Disease Control and Prevention staff were not informed of the results of readings by Nepali workers. The slides were prepared and stained in Nepal.

All VL patients had a history of 2 or more weeks of fever plus at least one of the following: weight loss, abdominal
swelling, abdominal pain, and/or darkening of the skin. In addition, all previously-diagnosed patients had a history of treatment with sodium stibogluconate or amphotericin B, and resolution of symptoms after treatment.

Control households were chosen at random from the voting registry of the Village Development Committees (VDC) where we found the VL cases. Village Development Committees are local political units that comprise 3 to 8 villages with a combined population of 3,000–12,000. Controls were frequency-matched by age and sex with the cases from their respective VDCs. To minimize the likelihood of including VL-infected persons among our controls, we excluded potential control households if there was a history of prolonged febrile illness consistent with VL, or if there had ever been a diagnosis of VL in any household member.13,14

A capillary-puncture blood specimen was collected for rK39 dipstick testing in the field (InSure Rapid Test for Detection of Visceral Leishmaniasis, InBios International Ltd., Seattle, WA). One to two drops of blood followed by one to two drops of buffer were placed on the pad of the dipstick, which was then observed for approximately 5 minutes. Following the manufacturer’s instructions, a test was positive when two bands, a control band and a positive test band, appeared within 5 minutes. The test was negative if only the control band appeared. The test is qualitative, and the manufacturer indicates that a faint test band should be considered positive. If no control band appeared, the dipstick was considered invalid and a new dipstick was used.

The DAT was performed either on blood eluted from filter paper, or in the case of newly-diagnosed cases, serum specimens. The direct agglutination test processing was carried out at the Vector-Borne Disease Research and Training Center, His Majesty’s Government of Nepal Ministry of Health, Hetauda, Nepal, following standard methods and using antigen purchased from the Prince Leopold Institute of Tropical Medicine in Antwerp, Belgium.69,13 Each microtiter plate included positive and negative controls. In whole blood, the test was considered negative at a titer of < 1:2,000, borderline at 1:2,000, and positive at ≥ 1:4,000. In serum, the test was considered negative at a titer of < 1:3,200, borderline at 1:3,200, and positive at ≥ 1:6,400. All titers refer to dilution of serum or whole blood after addition of antigen.

† Time between bone-marrow aspirate and blood drawing for serologic testing; usually done simultaneously and within 5 days in all cases.
‡ Controls were negative at a titer of 1:6,400. All titers refer to dilution of serum or whole blood after addition of antigen.
§ Four controls had positive DAT titers (range 1:4,000–1:32,000) and four had borderline DAT titers (1:2,000).
¶ Four case-patients had positive DAT, but negative rK39. Two were negative for both tests.
# Four controls had positive DAT titers (range 1:4,000–1:32,000) and four had borderline DAT titers (1:2,000).

We recruited a total of 92 VL patients and 113 controls between November 15 and December 15, 1999. Sixty-two patients (67%) were diagnosed with VL between January and November 1999, prior to contact with the study team. Of these, 48 had finished a full course of treatment and 14 were under treatment at the time of interview. During the month of fieldwork, we found 30 additional persons with clinical illness consistent with VL, and referred them to the nearest government hospital for definitive diagnosis. Of the 30 new patients, 29 underwent diagnostic bone-marrow aspiration. Of these, 23 were reported positive and 6 negative for Leishmania amastigotes. All 30 patients were considered to have VL by the attending physicians and all were treated with anti-leishmanial therapy.

Bone marrow-aspirate slides from 23 of 29 new patients were also examined by CDC laboratory staff. Of these, 14 were positive and six were negative according to both the Nepali practitioner and the CDC laboratory staff. Three slides were read as positive in Nepal and negative in Atlanta. According to the Nepali readings, all of the discordant specimens had few amastigotes, whereas the slides that were read as positive in both laboratories showed a range of parasite quantities (4 few, 4 moderate, 4 many, and 2 very many parasites). For each case, only one, or in a few cases, two, of the multiple slides prepared and read in Nepal was available for reading in Atlanta. The rK39 dipstick was performed on specimens from all cases and controls; DAT results were missing for one case and one control.

We considered the 14 newly-diagnosed cases with bone-marrow confirmation both in Nepal and Atlanta as “true positives,” and the 113 controls with no household or personal history of VL as “true negatives.” All true positive cases had positive rK39 results, and all had DAT titers greater than 1:204,800 (Table 1). Among cases of VL diagnosed clinically or parasitologically in Nepal or India, more than 90% were positive by both rK39 and DAT. Five of 6 VL cases with negative rK39 results, and 1 of 2 cases with negative DAT results, had completed treatment at least 2 months

### Table 1

<table>
<thead>
<tr>
<th>Case classification</th>
<th>rK39 dipstick positive no/total tested (%)</th>
<th>DAT positive or borderline* no/total tested (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow confirmed positive in two laboratories† (&quot;true positives&quot;)</td>
<td>14/14 (100)</td>
<td>13/13 (100)</td>
</tr>
<tr>
<td>Bone marrow or splenic aspirate reported positive in Nepal or India‡</td>
<td>46/51 (90)</td>
<td>49/51 (96)</td>
</tr>
<tr>
<td>Clinical diagnosis of VL in Nepal§</td>
<td>26/27 (96)</td>
<td>27/27 (100)</td>
</tr>
<tr>
<td>All VL cases</td>
<td>86/92 (93)†</td>
<td>89/91 (98)†</td>
</tr>
<tr>
<td>Controls (&quot;true negatives&quot;)</td>
<td>0/113 (0)</td>
<td>8/112 (7)†#</td>
</tr>
</tbody>
</table>

* In whole blood, the direct agglutination test (DAT) was considered to be negative at a titer of <1:2,000, borderline at 1:2,000, and positive at ≥1:4,000. In serum, the test was considered to be negative at a titer of <1:3,200, borderline at 1:3,200, and positive at ≥1:6,400. All titers refer to dilution of serum or whole blood after addition of antigen.
† Time between bone-marrow aspirate and blood drawing for serologic testing; usually done simultaneously and within 5 days in all cases.
‡ Forty-six bone-marrow aspirates done in Nepal, 5 splenic aspirates done in India. Mean time between aspirate and serologic testing was 72 days, range 9 days (when done simultaneously) to 353 days. Includes 3 patients whose bone-marrow aspirate was read as positive in Nepal but negative at the Centers for Disease Control and Prevention (CDC), all of whom had positive rK39 and DAT.
§ Includes 6 patients whose bone-marrow aspirates were read as negative both in Nepal and at CDC. Mean time between diagnosis/treatment initiation and serologic testing was 37 days, range 0 days (when done simultaneously) to 120 days.
† Four case-patients had positive DAT, but negative rK39. Two were negative for both tests.
# Four controls had positive DAT titers (range 1:4,000–1:32,000) and four had borderline DAT titers (1:2,000).
Among VL cases who finished treatment more than 1 month before testing, of the 113 potential controls, none was rK39 dipstick positive. Of 112 controls tested, 4 (3.5%) had a positive DAT, ranging in titer from 1:4,000 to 1:32,000, and 4 (3.5%) had a borderline DAT titer of 1:2,000. Based on these limited data, both the rK39 dipstick test and the DAT were 100% sensitive for the diagnosis of acute VL. The specificity of the rK39 dipstick test in this study population was 100%. Specificity for the DAT was 93% if borderline titers were considered positive, or 96% if borderline titers were counted as negative.

Results of the rK39 dipstick test and the DAT were consistent (Table 2). Among the 91 VL cases for whom results were available, discordant test results were found in 3 patients with a positive DAT, ranging in titer from 1:32,000 to >1:512,000, but negative rK39 dipstick results. All had completed treatment for VL at least 2 months before the study. Two of 3 had positive bone marrow aspirates before treatment.

The rate of positive rK39 results tended to be lower in cases who completed treatment more than 1 month prior to the study compared with newly diagnosed untreated cases (Table 3). There was no difference in positive DAT results for these 2 groups, and DAT titers among treated patients were somewhat higher than for newly-diagnosed individuals (median titer 1:512,000 versus 1:204,800). Among patients who had finished treatment more than 1 month before testing, those with negative rK39 results tended to have a shorter median duration of fever than those who remained rK39 dipstick-positive.

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>VL CASES AND CONTROLS*</th>
<th>CONTROLS ONLY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rK39 positive</td>
<td>rK39 negative</td>
</tr>
<tr>
<td>DAT positive or borderline</td>
<td>85</td>
<td>11</td>
</tr>
<tr>
<td>DAT negative</td>
<td>0</td>
<td>139</td>
</tr>
<tr>
<td>DAT positive or borderline</td>
<td>85</td>
<td>3</td>
</tr>
<tr>
<td>DAT negative</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

*Among all cases and controls, the kappa coefficient of agreement between rK39 and DAT results was 0.89. However, like positive and negative predictive values, the kappa coefficient for a test varies with the prevalence of disease in the population tested.
†Four controls had positive DAT titers, and four had borderline DAT titers.

### Table 3

<table>
<thead>
<tr>
<th>Interval between VL treatment and testing</th>
<th>rK39 positive (n = 54)</th>
<th>DAT positive* (n = 54)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Finished treatment ≥ 1 month before testing</td>
<td>38/43 (88)†</td>
<td>41/43 (95)†</td>
</tr>
<tr>
<td>Newly diagnosed, untreated at time of testing</td>
<td>30/30 (100)‡</td>
<td>29/29 (100)‡</td>
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Among VL cases who finished treatment ≥1 month before testing

<table>
<thead>
<tr>
<th>rK39 dipstick test result</th>
<th>Positive (n = 34)</th>
<th>Negative (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median duration of fever before treatment (range)</td>
<td>3.0 mo. (0.25–8)§</td>
<td>2.0 mo. (0.5–4.75)§</td>
</tr>
</tbody>
</table>

*In whole blood, the DAT was considered to be negative at a titer of <1:2,000; borderline at 1:2,000, and positive at ≥1:4,000. In serum, the test was considered to be negative at a titer of <1:3200, borderline at 1:3200, and positive at ≥1:6400. All titers refer to dilution of serum or whole blood after addition of antigen.
†P = 0.07 by 2-tailed Fisher exact test for those treated ≥ 1 month before testing compared with newly-diagnosed, untreated VL cases. Patients whose treatment ended less than one month before or who were receiving treatment at the time of testing were excluded from analysis.
‡P = 0.5 by 2-tailed Fisher exact test for those treated ≥ 1 month before testing compared with newly-diagnosed, untreated cases.
§P = 0.22 by Wilcoxon 2-sample test for those with positive rK39 compared with those with negative rK39 dipstick test result.

### Discussion

Our study focused on the field use of two serologic tests in a VL-endemic area with limited diagnostic options. We found that both the DAT and the rK39 dipstick test were highly sensitive for detecting newly-diagnosed cases of VL. Individuals can remain positive for both tests for months after treatment and clinical resolution of VL, although rK39 positivity may be slightly less persistent than DAT positivity. Greater duration of rK39 positivity in patients with a longer symptomatic period may be related to persistence of parasites after clinical resolution of symptoms, a phenomenon reported for cutaneous leishmaniasis and post kala-azar dermal leishmaniasis. Indeed, data from animal models suggest that sterile cure of leishmaniasis may be the exception rather than the rule. An alternative explanation is that the titer of anti-K39 antibody achieved during active disease is higher and therefore more persistent among patients with prolonged illness.

The performance of the DAT and the rK39 test has been evaluated previously in patients with parasitologically confirmed VL in India and other parts of the world. The predictive values of either test depend on the prevalence of VL in the population. However, for DAT, the threshold titer chosen to define a positive test is also crucial. Several studies have reported high sensitivity for DAT; but each used a different definition of a positive test, with threshold titers ranging from 1:800 to 1:6,400. In addition, one multicenter study suggests that variations in handling of the antigen can affect reproducibility of the DAT.

Our results suggest that in VL-endemic districts of Nepal, the rK39 dipstick would be a useful confirmatory test for VL in patients with clinically-consistent disease. The rK39 dipstick has several major advantages compared with DAT in the field setting. Ease of use and rapidity of the rK39 dipstick are especially important in a setting such as rural Nepal, where bone-marrow aspirates can be performed by only a few practitioners, few laboratories can perform DAT, and travel to a referral center is difficult. In addition, our data suggest that the rK39 may be more specific than the
DAT for acute disease in VL-endemic settings. None of our potential controls had a positive rK39 dipstick test, compared with a 7% rate of positive or borderline DAT in the same group. Although the specificity of DAT was high, costs could be improved by increasing the threshold titre; it was not as simple to perform or interpret as the rK39 dipstick test. The specificity of rK39 needs to be assessed in a wider survey of people living in VL-endemic communities, and in household contacts of VL patients, a group we deliberately excluded. Compared with other members of the same VL-endemic communities, household contacts and close neighbors of VL patients have a higher frequency of positive DAT and leishmanin skin tests.14 A study from the Sudan suggests that antibodies to rK39 can be detected in the absence of clinical VL.15 Community-based studies would therefore be useful to assess the extent to which subclinical VL and asymptomatic infections occur in South Asia.

In our study, the cost of the rK39 dipstick was approximately US$1.20 per test, compared with approximately US$8 per DAT using imported antigen (source of cost data, Environmental Health Project, Washington, DC, 1999). Local production of antigen could lower the cost of the DAT, but would introduce important issues of quality control. A recent analysis identifies the use of DAT for confirmation of clinically-suspected VL cases as the most cost-effective diagnostic strategy in VL-endemic areas.4 According to this analysis, treatment of all clinically suspected cases would prevent 88% of VL-related deaths, but would require treating many persons without VL. Treating only patients with parasitologic diagnosis would avert only 53% of deaths, whereas treatment based on clinical suspicion plus serologic confirmation would prevent 85% of possible deaths. The cost estimate for the latter two strategies was nearly equal, while treatment of all suspect cases cost more than twice as much per death averted. These results apply equally to the use of DAT for serodiagnosis followed by antigenic dipstick or interpret as DAT, as the most cost-effective diagnostic strategy in VL-endemic areas.4

According to this treatment, all clinically suspected cases would prevent 88% of VL-related deaths, but would require treating many persons without VL. Treating only patients with parasitologic diagnosis would avert only 53% of deaths, whereas treatment based on clinical suspicion plus serologic confirmation would prevent 85% of possible deaths. The cost estimate for the latter two strategies was nearly equal, while treatment of all suspect cases cost more than twice as much per death averted. These results apply equally to the use of DAT for serodiagnosis followed by antigenic dipstick or interpret as DAT, as the most cost-effective diagnostic strategy in VL-endemic areas.4

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