Evaluation of a rapid immunochromatographic test for diagnosis of kala-azar & post kala-azar dermal leishmaniasis at a tertiary care centre of north India

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Background & objectives: Definitive diagnosis of kala-azar requires demonstration of parasites by diagnostic protocols based on invasive organ aspirations. We evaluated in the present study the diagnostic utility of an immunochromatographic test (ICT) for detection of anti-rK-39 antibodies for the non-invasive diagnosis of kala-azar and post kala-azar dermal leishmaniasis (PKDL) at a tertiary care centre of north India.

Methods: The study was conducted in the Department of Microbiology, All India Institute of Medical Sciences, New Delhi, from July 2003 to October 2004. Of the 120 samples tested, 57 were found to be positive by ICT; of which, 51 were diagnosed as kala-azar and 6 as PKDL. The controls included individuals from endemic (50) and non endemic (19) areas with malignancies, haemolytic disorders, chronic liver diseases, hypersplenism, portal hypertension, metabolic disorders and sarcoidosis. In addition, 47 sera from confirmed cases of tuberculosis, malaria, typhoid, filariasis, leptospirosis, histoplasmosis, toxoplasmosis, invasive aspergillosis, amoebic liver abscess, AIDS, leprosy, cryptococcosis, strongyloidiasis, cyclosporiasis, patients having collagen vascular diseases and hypergammaglobulinaemia were also tested to check the specificity of the test.

Results: Of the 51 cases with kala-azar 43 were males, children accounted for 25 per cent of these cases. All had fever of duration ranging from <1 month to 1.5 yr (median 4.5 months). All PKDL patients (n=6, 4 males) gave a history of having suffered from kala-azar in the past, and their slit skin test smears were microscopically positive for Leishman-Donovan (LD) bodies. The strip test was positive in all the cases of kala-azar and PKDL (estimated sensitivity 100%), all control sera were negative by the ICT (specificity 100%).

Interpretation & conclusion: The rK-39 ICT is a highly sensitive and specific test, and may be suitable for a rapid, cost-effective and reliable field diagnosis of kala-azar and PKDL.

Key words Immunochromatographic test - kala-azar - low prevalence region - PKDL - rK-39 - visceral leishmaniasis
Visceral leishmaniasis (VL, kala-azar), a systemic infection of the reticulo-endothelial system caused by protozoa of the genus *Leishmania* has an estimated prevalence of 0.5 million cases worldwide. India alone accounts for 40-50 per cent of the world’s burden of the disease. The clinical features of VL resemble those of several other infectious and non-infectious diseases, which may have an overlapping geographical distribution with VL. Since kala-azar is fatal in 90 per cent of the untreated cases and the treatment consists of prolonged courses of highly toxic drugs, an early and accurate diagnosis is essential.

The definitive diagnosis of kala-azar requires demonstration or isolation of parasites from samples collected by invasive organ aspiration. These techniques often have low sensitivity, require extensive facilities and expertise and therefore, generally not feasible where it is most needed. Serological tests like enzyme linked immunosorbent assay (ELISA), direct agglutination test (DAT) and immunofluorescent assay, used for diagnosis of kala-azar have a variable sensitivity and low specificity and reproducibility. In addition, equipped laboratories are required for their performance.

Due to these limitations, an accurate, rapid, field applicable and non-invasive diagnostic method would be of immense utility. Following the discovery of a kinesin related gene in *Leishmania chagasi*, which encodes for a 39 amino acid residue (k-39), and is conserved in amastigote forms of VL causing Leishmania strains (*L. donovani*, *L. infantum* and *L. chagasi*), detection of antibodies to the recombinant product of the K-39 (rK-39) has been used in the format of ELISA and immunochromatographic test. The ELISA was found to be highly sensitive but lacked portability, and the dipstick formats have shown a variable sensitivity and specificity. Moreover, most of the studies have been done in high prevalence populations. The present study was undertaken to evaluate the performance of a new generation of rK-39 strip test for the diagnosis of kala-azar and post kala-azar dermal leishmaniasis (PKDL) at a tertiary care hospital situated in a low prevalence area of northern India.

**Material & Methods**

The study was conducted over a period of 15 months (July 2003 to October 2004) at the Department of Microbiology of the All India Institute of Medical Sciences (AIIMS), New Delhi. A total of 120 consecutive samples of bone marrow/splenic aspirates and serum from cases of pyrexia of unknown origin (PUO), with or without splenomegaly were sent to our laboratory to rule out kala-azar. In addition, split skin smears and serum from 6 suspected patients of PKDL were also received. A detailed history including clinical features, physical and laboratory findings [haemogram, serum albumin/globulin (A/G) levels, liver and kidney function tests] were recorded for each patient. The diagnosis of kala-azar and PKDL was established in clinically suspected patients by Giemsa stained bone marrow/splenic or skin aspirates respectively, positive for Leishman-Donovan (LD) bodies. In a few patients who were microscopically negative for LD bodies, the diagnosis was based on suggestive clinical, epidemiological and laboratory findings, exclusion of other possible diagnoses and a response to specific antileishmanial treatment. Response to treatment was defined as resolution of fever, regression of spleen size and improvement of haemoglobin, leucocyte and platelet counts.

In addition, 34 serum samples from confirmed cases of tuberculosis (5), malaria, typhoid, filariasis (4 each), leptospirosis (3), histoplasmosis, toxoplasmosis, invasive aspergillosis, amoebic liver abscess, AIDS (2 each), leprosy, cryptococcosis, strongyloidiasis and cyclosporosis (1 each) were tested to check the specificity of the rK-39 strip test. Apart from these, 13 serum from patients having collagen vascular diseases (10) [rheumatoid factor positive (6), antinuclear antibody positive (2), systemic lupus erythematosus (SLE) (2)] and hypergammaglobulinaemia (3) were also tested for cross reactions.

The rK-39 immunochromatographic test (rK-39 ICT, Kala-azar Detect, lot no. DM 1083, Bios International, Seattle, USA), was performed and interpreted according to the manufacturer’s instructions. The test was considered positive when
two bands (a control and a test band) appeared within 10 min and negative when only the control band appeared (Fig.). All the strips were read independently by two medical personnel and one technical staff.

ELISA for antileishmanial antibodies was done for a few patients of VL, PKDL and controls using a commercial kit (Ridascreen® Leishmania Ab, R-Biopharm, Dolivostr, Germany), according to manufacturer’s instructions. The aldehyde test was done for all cases of VL, PKDL and controls according to standard protocols. The test was positive when gellification and egg white opacity occurred within 20 min; if this occurred within 3 min, the test was interpreted as strongly positive.

Of the 51 patients of kala-azar, only 10 could be followed up at the end of three months (3 patients), six months (3 patients) and one-year (4 patients) post-treatment. The spleen size (if any), haemoglobin percentage, total leucocyte count, platelet count and serum total proteins/albumin/globulin levels were recorded and an aldehyde test and rK-39 ICT was repeated at the time of follow up for each of these patients.

Approvals for various laboratory investigations were obtained at the time of admission through the Institution Review Boards. Informed consent was obtained from all patients and parents or legal guardians of minors.

The Chi-square test was used for statistical analysis.

Results & Discussion

Of the 126 bone marrow/spleen aspirate/skin smears and serum samples tested, 57 were positive for rK 39 antibodies. Of these, 51 were diagnosed as cases of VL, and six as PKDL. Final diagnoses of the remaining 69 cases were malignancies (18), haemolytic disorders (15), chronic liver diseases (15), hypersplenism (7), portal hypertension (6), metabolic disorders (4), sarcoidosis (1) and fever of unknown etiology (3).

Of the 51 cases of VL, 43 (84%) had a bone marrow/splenic aspirate positive for LD bodies and eight patients had a microscopically negative aspirate. However, these eight patients were from the endemic areas who had presented with prolonged fever, weight loss and hepatosplenomegaly. They were also found to have pancytopenia and hypergammaglobulinaemia along with a positive aldehyde test and ELISA positive for Leishmania antibodies (Table). On this basis and after exclusion of other possible diagnoses, they were treated with antileishmanials and all of them responded to the treatment. Of the 51 cases, 41 (80%)
were from Bihar, six (12%) from Uttaranchal, two (4%) from Nepal and one each from Assam and Delhi. Forty three of the 51 (84%) kala-azar cases were males; with age ranging from six months to 65 yr (median 32 yr). Children (n=13) accounted for 25 per cent of all VL patients. All had fever, the duration of which ranged from less than one month to 1.5 yr (median duration 4.5 months). All the six cases of PKDL (4 males; 2 females, aged 15-28 yr, median age 24 yr) were from Bihar and their slit skin smears were microscopically positive for LD bodies. All gave a history of having suffered from VL in the past. The mean duration between the past VL treatment and the present illness was 10.6 yr (range 8-15 yr).

It was observed that all the patients of kala-azar and PKDL gave a positive rK-39 ICT result, giving it a sensitivity of 100 per cent. The dipstick tests for rK-39 antibodies in immunocompetent patients have shown a sensitivity varying from 67 to 100 per cent in various studies. This variation in sensitivity may be due to the different antigen formulations, difference in antibody response elicited in different populations, a difference in circulating strains of Leishmania, or possible parasitic antigen alteration at high temperatures in tropical countries. In general, the sensitivity of the test in African, American and European countries was reported to be lower than the Indian subcontinent. In India, indigenously developed K-39 strip test and the earlier generation of rK-39 strips (Insure Leishmania), have been evaluated in endemic populations, and were found to be 100 per cent sensitive. Kala-azar Detect, a new generation of rK-39 dipstick test, available commercially was found to be highly sensitive in our study but the sensitivity of the same was comparatively lower (90%) in Brazilian patients. This could be because patients usually presented late in their disease to tertiary referral centres likes ours, after preliminary investigations failed to reveal any diagnosis at primary health care levels. However, in our experience the test was positive even in patients with a history of illness of less than one month’s duration. Eight patients in our study who had a microscopically negative bone marrow/splenic aspirate and a positive rK-39 strip test had a final clinical diagnosis of kala-azar.

### Table. Clinical and laboratory findings in cases of kala-azar, PKDL and controls

<table>
<thead>
<tr>
<th>Clinical/laboratory parameters</th>
<th>Kala-azar (n=51)</th>
<th>PKDL (n=6)</th>
<th>Control (n=116)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fever</td>
<td>51 (100)</td>
<td>0</td>
<td>69/69 (100)</td>
</tr>
<tr>
<td>Weight loss</td>
<td>47 (92)*</td>
<td>0</td>
<td>30/69 (43.4)</td>
</tr>
<tr>
<td>Splenomegaly</td>
<td>49 (96)*</td>
<td>0</td>
<td>43/69 (62.3)</td>
</tr>
<tr>
<td>Hepatomegaly</td>
<td>41 (80)*</td>
<td>0</td>
<td>34/69 (49)</td>
</tr>
<tr>
<td>Pancytopenia</td>
<td>45 (88)*</td>
<td>1</td>
<td>19/69 (27.5)</td>
</tr>
<tr>
<td>Reversal of albumin/globulin ratio</td>
<td>49 (96)*</td>
<td>0</td>
<td>4/69 (5.7)</td>
</tr>
<tr>
<td>Positive aldehyde test</td>
<td>40 (78)*</td>
<td>0</td>
<td>0/69</td>
</tr>
<tr>
<td>Positive ELISA*</td>
<td>24/27 (88)</td>
<td>2/3 (66)</td>
<td>5/32 (15)</td>
</tr>
<tr>
<td>Positive rK-39 strip test</td>
<td>51 (100)*</td>
<td>6 (100)</td>
<td>0/116</td>
</tr>
</tbody>
</table>

Values in parentheses are parentages

*Controls included 69 patients from endemic (50) and non endemic (19) areas, who were diagnosed to have malignancies (18), haemolytic disorders (15), chronic liver diseases (15), hypersplenism (7), portal hypertension (6), metabolic disorders (4), sarcoidosis (1) and fever of unknown etiology (3). The controls also included confirmed cases of other infections (34), patients of collagen vascular diseases (10) and patients having hypergamaglobulinemia (3)

*ELISA was done for 27 of the 51 cases of VL (which included eight bone marrow negative patients), 3 of 6 cases of PKDL and 32 of 69 controls

*P<0.05 compared to controls
Of the 69 patients with non infective aetiologies of PUO, 50 (72%) were from the VL endemic areas. Bone marrow aspirates of these patients were negative for LD bodies. The rK-39 strip test was also negative for all these patients. This was in contrast to two other Indian studies\cite{12,16}, where a major limitation of the test (using the earlier generations of rK-39 ICT) was the presence of antibodies in 10-12.5 per cent of endemic populations. The rK-39 ICT formats evaluated in earlier studies from the Indian subcontinent was a prototype version distributed in 2000 by the manufacturer for research purpose only, the commercial production of which was stopped for some unknown reason\cite{13}. This could be a reason for lower specificity in the earlier studies\cite{12,13,16}. All serum samples from confirmed cases of tuberculosis, malaria, typhoid, filariasis, leptospirosis, histoplasmosis, etc., gave a negative rK-39 dispstick test results in our study. Thus, the test had a specificity of 100 per cent, which was a strong positive point in favour of this test. Similar results of high specificity have been observed in earlier studies\cite{2,3,7,12,15,16,22}. Of the 221 malaria cases tested so far\cite{2,3,6,12-14,17,22,23}, only two have shown a false positive reaction\cite{2,12}, which deserves further evaluation.

Serum samples of the 10 patients who were followed up at the end of three, six and 12 months post-treatment gave a positive ICT result. None of them had fever although a palpable spleen was found in one of them (at the end of 3 months), three patients had a reversed A/G ratio (at three months), none had pancytopenia and all of them had a negative aldehyde test at follow up. The persistence of antibodies for prolonged periods after successful treatment of VL is a limitation of this test\cite{8,11,12,16}. The test would therefore be unsuitable for diagnosing relapse or reinfections\cite{12,16} and would be misleading in treated patients of VL who later develop an illness having clinical features like VL. Persistence of antibodies even up to three years after treatment of VL makes the interpretation of a positive rK-39 result difficult for diagnosis of PKDL\cite{11,23}, especially in regions where PKDL occurs early after treatment of VL\cite{11}. Since all patients in our study had a history of kala-azar 8-15 yr back, it seems likely that the observed positive result was due to the multiplication of parasites in the skin lesions during their current illness\cite{5}. However, studies involving a larger number of PKDL patients are needed to resolve this issue.

Of the patients who present with PUO in our study, weight loss, splenomegaly, hepatomegaly, pancytopenia and reversal of A/G ratio were significantly ($P<0.05$) more common in kala-azar cases than controls (Table). These clinic-epidemiological features of the disease could also be exploited for an early diagnosis. In our experience, the aldehyde test had a sensitivity of 78 per cent and specificity of 100 per cent. Of the 40 patients who had a positive aldehyde test, 38 (95%) gave a strongly positive reaction. All these 38 patients had a positive rK-39 ICT and bone marrow, a finding that can be utilized at the peripheral centres. ELISA, on the other hand was found to be less sensitive and specific (Table), apart from being technically demanding as compared to the strip test.

The rK-39 ICT was rapid and easy to perform even by the paramedical staff. Positive results could be read within one to three minutes and there was concordance between the observers in all readings. At a nominal cost of Rs.120/-, this format holds great promise for use under field conditions. Though there may be a regional limitation to the use of this test in other parts of the world\cite{12,22}, in Indian patients with a clinically consistent disease profile, this test appears to be highly sensitive, specific, rapid, field applicable and cost-effective for the diagnosis of VL and PKDL. However, more studies are needed before recommending replacement of invasive tests for diagnosis of VL and PKDL with this easy, non-invasive and rapid test.

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References


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