Visceral leishmaniasis (VL) is a vector-borne disease caused by an obligate intramacrophage protozoan parasite. Diagnosis of VL is complex because its clinical manifestations mimic other microbial diseases. Confirmatory diagnosis relies upon demonstration of the parasite in tissue samples either directly in or after culture. Although microscopic examination of splenic aspirates and/or bone marrow smears offer the higher diagnostic efficacy, these are associated with risk because they are invasive procedures. This limits their use in field or in peripheral health centers. Immunodiagnostic techniques like the direct agglutination test (DAT), ELISA, immunochromatographic strip test, and indirect hemagglutination test have been extensively used. Various reports have indicated 70–100% sensitivity and 90–100% specificity with the use of trypsinized, formalin-fixed, stained whole promastigotes to detect parasite-specific IgG in the serum or plasma by DAT. In several studies, detection of antibody response to *Leishmania donovani* amastigote antigen was reported as a better marker for diagnosis than antibody response to promastigote antigen. Because the parasite exists in the amastigote form in the human host, we evaluated the efficacy of the use of amastigote and promastigote antigens in the DAT and compared it with ELISA, the rk39 strip test for detection of antibodies, and bone marrow findings.

Nehru Hospital is associated with the Postgraduate Institute of Medical Education and Research, Chandigarh, as a tertiary-care referral hospital. Serum samples were received in the Department of Parasitology for the laboratory diagnosis of VL from 94 clinically suspected VL cases and 1 case of post–kala-azar dermal leishmaniasis (PKDL) during the 2-year period from January 2005 to December 2006, and bone marrow samples were received in the Department of Haematology. Serum samples from 40 controls (10 cases each suffering from malaria and toxoplasmosis and 20 from apparently healthy age-matched subjects) were also assessed for antibody detection by the same techniques. The healthy controls were serologically negative for VL and recruited from the Chandigarh area where there are no indigenous cases of VL, and these subjects had not visited *Leishmania* endemic areas in the past 5 years.

Serum samples were assessed for antibody detection by an in-house developed DAT using the amastigote/promastigote antigen. The antigen for DAT was prepared as described earlier, from the Indian strain of *L. donovani* (MHOM/IN/80/DD8), and DAT was performed as detailed out previously, except that the volume of the diluted serum and antigen were 30 μL/well,respectively (as determined by checkerboard titration) instead of 50 μg/well, with the parasite concentration remaining the same. All sera were analyzed in duplicate and in doubling dilutions (1:100–1:3200). Agglutination of more than 50% was considered as positive for DAT using both amastigotes and promastigotes. Antibody response by the rk39 immunochromatographic strip test (InBios, Seattle, WA) and the ELISA kit (Vircell, Granada, Spain) was determined by as per the manufacturers’ instructions.

Serological techniques and direct smear examination of the bone marrow samples were carried out in separate departments by different laboratory personnel. The mean age of the patients and the controls was 20 years (15–25 years), and the male-to-female ratio was approximately 2:1 for both the cases and controls. Out of the 94 suspected VL cases, 16 (17%) of the cases had parasite-specific IgG detectable by all 4 techniques, i.e., DAT with promastigote or the amastigote antigen, the rk39 strip test, and the ELISA. Twenty-two (23.4%) samples were seropositive with the rk39 strip test and the ELISA, while 16 (17%) were immunoreactive in the DAT with either the promastigote or the amastigote antigen. Notably, the antibody response to amastigote antigen in 3 cases (2 VL and 1 PKDL) in the DAT indicated 1- to 2-fold higher titers than with the promastigote antigen (Table 1). Two samples from malaria cases and 1 from a toxoplasmosis case had a titer of ≤ 200 with the use of amastigote antigen, while 3 samples from malaria cases and 1 sample from a toxoplasmosis case had titers of ≤ 400 with the use of promastigote antigen. A serum sample from a patient with PKDL had a titer of ≥ 3200 in DAT and was positive in all of the other serological tests. Two patients with toxoplasmosis had DAT titers of ≤ 200 when the amastigote/promastigote antigen was used (Table 1).

On retrospective analysis, out of the 16 serologically positive cases by all the techniques, 11 (69%) had demonstrable *Leishmania donovani* (LD) bodies in their bone marrow specimens, while in the other 5, the bone marrow was not examined. Six (0.06%) cases were positive by ELISA and the...
r k39 strip test and negative by DAT (at cut-off titer ≥ 800), and out of these 6 cases, 2 had myelofibrosis while 4 had chronic myeloid leukemia, as confirmed with the bone marrow examinations. Serum samples from these 6 cases had a titer of ≤ 400 only (Table 2). Bone marrow aspirates in these 6 cases were found negative for Leishmania parasites by microscopic examination and culture. The cut-off titer of 1:800 has been reported in other studies from India, too.8,15 Because demonstration of the amastigote is confirmative, the sensitivity and the specificity were calculated13 on the basis of bone marrow reports and samples seropositive by all techniques. Sensitivity and specificity of the DAT using the promastigote or the amastigote antigen were 100%, indicating perfect correlation. All samples that were detected as positive in the DAT using the promastigote antigen also tested positive in the DAT using the amastigote antigen. The sensitivities of the ELISA and the rk39 strip test were both 100%, and the specificity was 87%.

The present study demonstrated a high diagnostic yield of DAT with the use of either amastigote or promastigote antigen. In contrast, the rk39 strip test and ELISA falsely detected 6 cases, 2 with myelofibrosis and 4 with chronic myeloid leukemia. A study from Nepal14 demonstrated a sensitivity of 87% and specificity of 77%, while a similar study to L. donovani DAT with the use of amastigote or promastigote antigen also tested positive. All samples that were detected as positive in the DAT using the amastigote is confirmative, the sensitivities of the ELISA and the rk39 strip test were both 100%, and the specificity was 87%.

In 16†, 11 had demonstrable LD bodies in their bone marrow, while in the remaining 5 cases, bone marrow was not examined.

TABLE 1

Comparison of the DAT with the use of amastigote and promastigote antigens

<table>
<thead>
<tr>
<th>Disease groups</th>
<th>Antigen used</th>
<th>100</th>
<th>200</th>
<th>400</th>
<th>800</th>
<th>1600</th>
<th>≥ 3200</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>True VL, N = 16*</td>
<td>Amastigote</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3 (19)</td>
<td>4 (25)</td>
<td>9 (56)</td>
<td>16</td>
</tr>
<tr>
<td>PKDL, N = 1</td>
<td>Amastigote</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5 (31)</td>
<td>4 (25)</td>
<td>7 (44)</td>
<td>16</td>
</tr>
<tr>
<td>Malaria, N = 10</td>
<td>Amastigote</td>
<td>1 (10)</td>
<td>1 (10)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Healthy controls, N = 20</td>
<td>Amastigote</td>
<td>1 (10)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

* Of 16, 11 had demonstrable LD bodies in their bone marrow, while in the remaining 5 cases, bone marrow was not examined.

** Of 22 positives, 16 were positive by DAT at a titer of ≥ 800, while 6 samples were non-reactive beyond 1:800 serum dilution.

TABLE 2

Performance of the DAT versus rk39 strip test and ELISA (number positive)

<table>
<thead>
<tr>
<th>Disease groups</th>
<th>rk39 strip test</th>
<th>ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>N = 94*</td>
<td>16†</td>
<td>16†</td>
</tr>
<tr>
<td>PKDL, N = 1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Malaria, N = 10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Healthy controls, N = 20</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Of 94 suspected VL cases, only 16 were seropositive, 11 of which had demonstrable LD bodies in their bone marrow.
† Of 16 positives, 11 had demonstrable LD bodies, while in the remaining 5 cases, bone marrow was not examined.
‡ Of 22 positives, 16 were positive by DAT at a titer of ≥ 800, while 6 samples were non-reactive beyond 1:800 serum dilution.

However, the number of samples in our study was small, and it should be confirmed by testing on a large number of confirmed VL samples. DAT is a simple, inexpensive technique with high specificity and sensitivity. It uses very little serum, can use plasma as well, and is performed at room temperature. Moreover, the antigen is reported to have a shelf life of more than 6 months. The only disadvantage is that it takes 12–18 hours. As the merits outweigh the disadvantages, it is suggested that performance of DAT on a larger number of patients may verify the present findings to improve diagnosis of VL in endemic areas.

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