Evaluation of Enzyme-Linked Immunosorbent Assay for Diagnosis of Post-Kala-Azar Dermal Leishmaniasis with Crude or Recombinant k39 Antigen

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The diagnosis of post-kala-azar dermal leishmaniasis (PKDL), a dermatosis that provides the only known reservoir for the parasite Leishmania donovani in India, remains a problem. Timely recognition and treatment of PKDL would contribute significantly to the control of kala-azar. We evaluated here the potential of the enzyme-linked immunosorbent assay (ELISA) as a diagnostic tool for PKDL. Antigen prepared from promastigotes and axenic amastigotes with parasite isolates that were derived from skin lesions of a PKDL patient gave sensitivities of 86.36 and 92%, respectively, in the 88 PKDL cases examined. The specificity of the ELISA test was examined by testing groups of patients with other skin disorders (leprosy and vitiligo) or coendemic infections (malaria and tuberculosis), as well as healthy controls from areas where this disease is endemic or is not endemic. A false-positive reaction was obtained in 14 of 144 (9.8%) of the controls with the promastigote antigen and in 14 of 145 (9.7%) of the controls with the amastigote antigen. Evaluation of the serodiagnostic potential of recombinant k39 by ELISA revealed a higher sensitivity (94.5%) and specificity (93.7%) compared to the other two antigens used. The data demonstrate that ELISA with crude or recombinant antigen k39 provides a relatively simple and less-invasive test for the reliable diagnosis of PKDL.

MATERIALS AND METHODS

Individuals infected with the protozoan parasite Leishmania donovani present with the clinical disease visceral leishmaniasis (VL) or kala-azar (KA), which is fatal if left untreated. The annual incidence and prevalence levels of VL are 0.5 and 2.5 million, respectively, of which 90% of cases occur in the Indian subcontinent and Sudan (3). Post-KA dermal leishmaniasis (PKDL) is a dermatotropic form of disease caused by L. donovani that develops as a sequela in 10 to 20% of VL cases in India and in >50% of VL cases in Sudan (18, 30). PKDL is characterized by hypopigmented macules and erythematous eruptions leading to the formation of papules and nodules (13, 18). In India, PKDL occurs several months to as many as 35 years after KA is cured and is considered to be the main reservoir for transmission of the visceral disease in the absence of a zoonotic host (1, 27).

Definitive diagnosis of PKDL by demonstration of L. donovani parasites (LD bodies) in skin biopsies has a sensitivity of only 58% (23), since parasites are scanty in the lesions. The disease is therefore often misdiagnosed as leprosy, a coendemic disease that resembles PKDL both clinically and pathologically (17). Serodiagnosis has been used as an important alternative for the diagnosis of KA, although its value is often limited for specificity and reproducibility when crude parasite antigen is used (6, 12, 24, 25). The use of recombinant k39 (rk39) has been shown to overcome these limitations to a considerable extent (2, 16, 22, 26, 29). Antileishmanial antibodies of the immunoglobulin G (IgG) and IgM classes have been demonstrated in the sera from PKDL patients (8, 20); however, limited studies have been conducted to develop serological methods for the diagnosis of PKDL (9). Increased sensitivity has been reported when the immunoperoxidase technique and PCR are used (10, 15, 21). We evaluate here the utility of the enzyme-linked immunosorbent assay (ELISA) in diagnosing PKDL with total antigen extract and rk39. Antigen extracts were prepared from indigenous parasites at two different developmental stages—promastigotes and amastigotes—isolated from PKDL lesions.

**Patients.** Blood samples were collected by venipuncture for sera from individuals in the following clinical categories.

PKDL. A group of 88 patients from Bihar, where PKDL is endemic, and reporting to Safdarjang Hospital, New Delhi, India, over a period of 4 years were included in this category. PKDL was diagnosed clinically and confirmed by the demonstration of parasites in skin lesions or by histopathologic findings (18). All patients included in this category were found to respond to therapy with sodium antimony gluconate.

KA. Thirty patients reporting to the Department of Medicine, Safdarjang Hospital, with fever and splenomegaly and parasitologically confirmed to have leishmania parasites in bone marrow aspirates were categorized as having KA.

Tuberculosis and malaria. A total of 22 patients with confirmed pulmonary tuberculosis and another 19 with malaria (peripheral blood smear positive) were included in this group.

Leprosy and vitiligo. This group included 30 patients confirmed to have lepromatous leprosy and 20 vitiligo patients (confirmed by histopathology) who reported to the Department of Dermatology, Safdarjang Hospital.

**Healthy controls.** Healthy controls (*n* = 32) were subjects living in Delhi, India, an area where KA is not endemic.

**Endemic controls.** “Endemic controls” (*n* = 22) were the first-degree healthy relatives of patients living in Muzaffarpur, Bihar, an area known for its KA endemicity.

**Parasite cultures.** Parasites isolated from lesions of PKDL patients propagated as promastigotes in M99 supplemented with 25 mM HEPES (pH 7.5) and 10% fetal calf serum as described earlier (19). Axenically grown amastigotes were cultured by the gradual adaptation of promastigotes to growth at pH 5.5 and 37°C in a 6 to 7% CO₂ atmosphere as described by Joshi et al. (11).
FIG. 1. Comparison of ELISA with rk39 antigen and antigens derived from promastigotes and amastigotes of *L. donovani* isolated from the dermal lesions of a PKDL patient. Sera were used in a 1:200 dilution with rk39 and a 1:100 dilution for promastigote and amastigote antigens in all of the samples. The mean OD values at 492 nm as determined by ELISA for patient and control sera were plotted.

**RESULTS**

A total of 263 serum samples were collected and tested by ELISA using three different antigens: the promastigote antigen, the amastigote antigen, and the rk39 antigen. Initially, we compared results with antigen derived from promastigotes of reference strain AG83 (MHOM/IN/83/AG83) and those derived from dermal lesions and found that the latter gave higher absorbance values upon ELISA with PKDL sera (data not shown). Subsequently, the promastigotes and amastigotes used for antigen preparation were those cultured from parasites isolated from PKDL lesions. The test samples included 88 PKDL patients and 146 controls, along with 30 KA patients to serve as positive control samples. In PKDL samples, the mean OD was highest with rk39 antigen, even though the serum dilution was double (1:200) compared to that used for amastigote and promastigote antigens (1:100; Fig. 1). The titer for PKDL cases was up to 10^5 with rk39 (data not shown). The controls, on the other hand, gave lowest mean OD with rk39 antigen, making it the most suitable antigen to use as a diagnostic agent. The amastigote antigen gave a significantly higher mean OD (*P* < 0.05) compared to the promastigote antigen in PKDL patients, but the OD was not significantly different in the control group. In general, KA samples gave higher OD values than the PKDL samples with all three antigens, but the difference in the mean OD was statistically significant (*P* < 0.05) only with the crude antigens and not with the rk39 antigen.

![Graph](http://cvi.asm.org/)

**TABLE 1.** Comparison of the results of ELISA for the three serological assays—performed with the rk39 antigen, the amastigote antigen, and the promastigote antigen—used to diagnose PKDL in Indian patients

<table>
<thead>
<tr>
<th>Group</th>
<th>ELISA result (no. of sera) with:</th>
<th>Positive</th>
<th>Negative</th>
<th>Positive</th>
<th>Negative</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKDL</td>
<td>rk39 Ag Amastigote Ag Promastigote Ag</td>
<td>84 4 81 7 76 12</td>
<td>30 0 30 0 30 0</td>
<td>0 20 1 19 1 19</td>
<td>3 19 4 18 4 18</td>
<td>1 18 2 17 2 17</td>
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</table>

*a* An OD of 0.45 was used as a cutoff value in all cases. Ag, antigen.

![Graph](http://cvi.asm.org/)

**TABLE 2.** ELISA values obtained with the three antigens in 88 PKDL cases grouped according to KA history

<table>
<thead>
<tr>
<th>Group</th>
<th>KA history (yr)</th>
<th>No. of cases</th>
<th>ELISA result (Mean OD ± SD) with:</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td>rk39 Ag Amastigote Ag Promastigote Ag</td>
</tr>
<tr>
<td>1</td>
<td>&lt;5</td>
<td>17</td>
<td>1.306 ± 0.217 1.19 ± 0.38 1.057 ± 0.469</td>
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<tr>
<td>2</td>
<td>5–10</td>
<td>43</td>
<td>1.087 ± 0.424 1.036 ± 0.435 0.805 ± 0.392</td>
</tr>
<tr>
<td>3</td>
<td>10–15</td>
<td>19</td>
<td>1.27 ± 0.40 1.29 ± 0.524 0.836 ± 0.33</td>
</tr>
<tr>
<td>4</td>
<td>≥15</td>
<td>9</td>
<td>1.31 ± 0.324 1.061 ± 0.489 0.977 ± 0.377</td>
</tr>
</tbody>
</table>

*a* Ag, antigen.

*b* no history of KA.
DISCUSSION

In India, PKDL occurs in 10 to 20% of KA cases months to as many as 35 years after patients are cured of KA. This is quite different from the situation with PKDL in Sudan, since PKDL occurs in >50% of cured KA cases usually weeks or months after recovery from the visceral disease (30). Diagnosis of PKDL is a problem since it is often confused with other dermatological conditions such as leprosy. It is important to identify and treat PKDL patients, since they constitute the only known reservoir for *L. donovani* in India (27). We recently described a sensitive and species-specific PCR assay for the diagnosis of PKDL (21); however, PCR is expensive and requires sophisticated facilities and trained personnel. Each of the ELISA tests described in the present study, although not as sensitive or as specific as a PCR assay, would provide a more economical and practical assay for the diagnosis of PKDL.

The serodiagnostic potential of rk39 for VL has been shown with subjects from various parts of the world, including the Indian subcontinent, Brazil, and Sudan, establishing conservation of the k39 epitope among visceralizing species of *Leishmania* (2, 16, 22, 29). In a study with a limited number of PKDL patients in Sudan, an rk39 ELISA was found to be sensitive and specific as a diagnostic test (29). In the present study we used a large number of serum samples to demonstrate that PKDL patients in India have high titers of anti-k39 IgG and that ELISA with rk39 antigen provides a highly sensitive (95.45%) and specific (93.5%) tool for diagnosing PKDL.

Several studies have shown serological tests to be useful in the diagnosis of KA; however, methods for the diagnosis of KA often lack sensitivity for the diagnosis of PKDL. In order to improve the sensitivity of the ELISA, we used promastigote antigen prepared with parasite isolates from dermal lesions of a PKDL patient. The indigenous PKDL antigen was found to give generally higher titers than those obtained with the reference strain (AG83), which is similar to the observation made during a direct agglutination test for PKDL in Sudan (9). Since *Leishmania* parasites are present in the amastigote form in the human host, the humoral immune response would be directed against antigens of the amastigote form. Antigen prepared from amastigotes has indeed been shown to be superior to the promastigote-derived antigen (25); however, amastigotes are generally difficult to isolate in large quantity and in pure form. To overcome this problem, we set up an axenic amastigote culture from parasites isolated from dermal lesions and used it for antigen preparation. The amastigote antigen was found to give significantly higher absorbance values and better sensitivity compared to the promastigote antigen, although the specificity was similar in both the cases. Hence, the amastigote antigen is recommended for use when recombinant K39 is not available or affordable. Use of crude antigen is known to lead to false-positive results with samples of other diseases due to certain common antigenic epitopes. Sera from patients suffering from other common skin disorders in India, namely, leprosy and vitiligo, have been examined, since PKDL is most frequently misdiagnosed as one of these. Among this group use of the promastigote and amastigote antigens gave false-positive results in only 1 of 50 cases. False-positive results were more frequent in patients with malaria (2 of 19) and tuberculosis (4 of 22). The overall specificity of detection was ca. 90% with either promastigote or amastigote antigens.

The causative organism in PKDL has been characterized as *L. donovani* by methods such as isoenzyme typing (5), reaction with monoclonal antibodies (P. Salotra et al., unpublished data), and species-specific PCR (21). It has also been demonstrated that there are indeed molecular differences between PKDL isolates and KA isolates of *L. donovani* (7). Gene expression in the parasite is also expected to be different in PKDL since not only the site of infection but also the host humoral immune response is distinct compared to KA (20). The antibody titer to leishmanial antigens is known to be much lower in PKDL patients than in KA patients (8). We observed here high anti-k39 titers of up to 10^−5 in PKDL patients in a large number of serum samples, indicating that k39 is abundantly expressed in parasites causing PKDL.

Antileishmanial antibody titers measured by direct agglutination test have been reported to remain positive for up to 5 years after recovery in >50% of VL patients examined (14). In our study only 17 of 88 (19.3%) patients had a history of KA of <5 years. The remaining 80.7% had either no history of KA or a history of KA exceeding 5 years for periods of even up to 15 years. Therefore, it would be reasonable to conclude that the antibodies detected in ELISA were largely due to PKDL and not those persisting due to a history of KA.

ACKNOWLEDGMENTS

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REFERENCES