Development of a Species-Specific PCR Assay for Detection of *Leishmania donovani* in Clinical Samples from Patients with Kala-Azar and Post-Kala-Azar Dermal Leishmaniasis

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We have developed a PCR assay that is capable of amplifying kinetoplast DNA (kDNA) of *Leishmania donovani* in a species-specific manner among Old World leishmaniases. With Indian strains and isolates of *L. donovani* the assay was sensitive enough to detect kDNA in an amount equivalent to a single parasite or less. The extreme sensitivity of the assay was reflected in its ability to detect parasite DNA from small volumes of peripheral blood of patients with kala-azar (KA) and from skin lesions from patients with post-KA dermal leishmaniasis (PKDL). A total of 107 clinical leishmaniasis samples were analyzed. Of these 102 (95.3%) were positive by PCR. The test provided a diagnosis of KA with 96% sensitivity using patient whole-blood samples instead of bone marrow or spleen aspirates that are obtained by invasive procedures. The assay was also successful in the diagnosis of 45 of 48 PKDL cases (93.8%). Cross-reactions with pathogens prevalent in the area of endemicity, viz., *Mycobacterium tuberculosis*, *Mycobacterium leprae*, and *Plasmodium* spp., could be ruled out. Eighty-one control samples, including dermal scrapings from healthy portions of skin from patients with PKDL were all negative. Two of twenty controls from the area of endemicity were found positive by PCR assay; however, there was a good possibility that these two were asymptomatic carriers since they were serologically positive for KA. Thus, this PCR assay represents a tool for the diagnosis of KA and PKDL in Indian patients in a noninvasive manner, with simultaneous species identification of parasites in clinical samples.

The protozoan parasites of the genus *Leishmania* are the causative agents of a group of diseases called leishmaniases, endemic in more than 80 countries worldwide. Considerable morbidity and mortality occur in the visceral infections termed visceral leishmaniasis (VL) or kala-azar (KA), which is a significant infectious disease in the developing world and of late in the developed world because of increased international travel and human immunodeficiency virus (HIV) infection. KA is a symptomatic infection of the liver, spleen, and bone marrow caused by organisms of *Leishmania donovani* complex. The annual incidence and prevalence of VL cases worldwide are 0.5 million and 2.5 million respectively. Of these 90% of cases occur in India, Nepal, Bangladesh, and Sudan (9). The official estimate of 430,000 VL cases in Bihar State of India over the past 11 years may represent only a fraction of the real numbers. The actual number is believed to be at least five times as great (8). The causative organism in the Indian subcontinent and Africa is *L. donovani donovani*, while in the Mediterranean basin and South America it is *L. donovani infantum*.

Post-KA dermal leishmaniasis (PKDL) is an unusual dermatosis that develops as a sequel of KA, producing gross cutaneous lesions in the form of hypopigmented macules, erythemea, and nodules. The disease is relatively common in the Indian subcontinent and less frequent in East Africa but exceptional in the American and European continents (25). The need to search for cases of PKDL and treat them as a part of KA control programs has been emphasized since patients with PKDL provide the only known reservoir for the parasite in India (37). The cost and toxicity of current treatment regimens emphasize the importance of establishing control strategies and make diagnosis and typing of leishmaniasis critical (20). Detection and characterization of *Leishmania* from patients with KA or PKDL are important for deciding treatment regimens as well as for understanding the disease epidemiology. Current diagnostic methods based on parasite detection (stained smears, culture, and histopathology) and immunological methods (direct agglutination test, enzyme-linked immunosorbent assay [ELISA], etc.) have several limitations, including low sensitivity and specificity. Procedures for demonstration of the parasite in spleen or bone marrow in KA and in skin lesions in PKDL are invasive and often not sensitive enough. Immunological methods fail to distinguish between past and present infections and are not reliable in the case of immunocompromised patients (6, 11, 39). Furthermore, neither of these methods addresses the problem of species identification, which is important for determining appropriate treatment regimens and designing control measures. Procedures involving the use of monoclonal antibodies, isoenzyme and schizodeme analysis, and DNA hybridization have to be resorted to (12, 17, 29). Most of these procedures are tedious and require massive cultures of parasites. There is, therefore, an urgent need to
develop diagnostic procedures that are simple, sensitive, and specific. In recent years PCR-based diagnostic methods have been described for leishmaniasis, with a wide range of sensitivity and specificity. An excellent target for a sensitive and rapid detection method is the kinetoplast mini-circle DNA, which is present at thousands of copies per cell. The mini-circles have been used as targets for selective amplification of parasite DNA in various studies (5, 7, 21, 28, 35). The identification of conserved sequence elements represented within the kinetoplast DNA (kDNA) of a given species of *Leishmania* would allow the design of oligonucleotide primers to be used for species-specific identification of parasites in clinical samples. We have analyzed kDNA sequences from Old World leishmanias and designed primers specific for *L. donovani* species to detect kDNA from a single parasite in the presence of huge excesses of human DNA. The utility of the primers designed for *L. donovani* has been examined in clinical samples from patients with KA and PKDL in India. The PCR test was found to be sensitive enough to detect parasite DNA from peripheral blood of patients with KA and from skin lesions of patients with PKDL. Furthermore, the test was specific for *L. donovani* species of the parasite, leading to simultaneous species identification of the parasite.

**MATERIALS AND METHODS**

**Patients.** Fifty-one **KA patients** hailing from Bihar and reporting to Saifajung Hospital (SJH), New Delhi, India, were included in the study at the pretreatment stage. The patients presented with characteristic symptoms of KA such as fever, hepatosplenomegaly, anemia, and leukopenia. Only those patients for whom the diagnosis of KA was confirmed by demonstration of parasites in bone marrow aspirates were taken for the study. Blood was taken from all 51 patients. In addition bone marrow samples were obtained from 8 of those patients. Clinical samples were also taken from a total of 48 patients with PKDL that were originally from Bihar and reported to the Dermatology Department of SJH during the period from 1996 to 2000. Forty-five of these reported history of KA, while the remaining three were not aware of it. The time elapsed after cure from KA in the 45 patients ranged from 1 to 15 years. Clinical diagnosis in 36 patients was based on condition characterized by erythematous indurate areas and papulonodular and hypochromic macules in a bilateral distribution. The remaining 12 patients had a predominantly macular presentation, most of them being the patients with KA. Twenty healthy volunteers living in an area of endemicity (Muzaffarpur, Bihar) were also included in the control group.

**Parasites.** Ten World Health Organization reference strains of *Leishmania* originating from distinct geographic locations were used in the study. These included *L. donovani* DDB (MHOM/IN/80/DDB) from India, *L. donovani* AG83 (MHOM/IN/83/AG83) from India, *L. donovani* 15 (MHOM/SD/00/15-C12D) from Sudan, *L. donovani* WR 684 (MHOM/ET/67/82) from Ethiopia, *L. donovani* infantum (MCAN/SP/00/X5X) from Spain, *L. tropica* WR 683 (MHOM/SU/58/OD) from the Soviet Union, *L. tropica* WR 664 (MHOM/SU/74/K27) from the Soviet Union, *L. major* WR662 (MHOM/IL/67/Zericho IIWR662) from Israel, *L. major* LV39 (MHRO/SU/59/P/LV39) from the Soviet Union, and *L. major* ASKH (MHOM/SU/73/ASKH) from the Soviet Union. Three isolates of *L. donovani* (MHOM/IN/94/ICB8, MHOM/IN/94/ICB7, and MHOM/IN/94/ICB8) were kindly provided by D. Sarkar, Indian Institute of Chemical Biology, Calcutta, India. These were isolated from patients with VL (ICB8 and ICB8) and PKDL (ICB7) originating from Bihar and characterized as *L. donovani* (10). Ten parasite isolates were set up in culture in our laboratory over the last 2 years from patients with VL and PKDL reporting to SJH. All parasite cultures were set up and propagated in medium 199 supplemented with 25 mM HEPES, pH 7.5, and 10% fetal calf serum (30). Parasites were harvested in late log phase and washed in phosphate-buffered saline prior to DNA isolation.

**Sample collection and DNA isolation.** Bone marrow and skin scrapings were collected in NET buffer (150 mM NaCl, 15 mM Tris-HCl [pH 8.30], 1 mM EDTA). Blood was collected in heparinized tubes. Samples were transported to the laboratory at ambient temperature, except for blood collected in areas of endemicity, in which case they were brought on ice. Samples were transferred to 4°C and generally processed on the same day. Blood (0.2 to 1 ml) was treated with RBC lysis buffer (114 mM sodium phosphate [pH 8.0], 1 mM NH4Cl), and the buffycoat was isolated. DNA from parasite cultures as well as from clinical samples (bone marrow, or blood) was isolated by overnight lysis in NET buffer with proteinase-K (100 μg/ml) and 1% sodium dodecyl sulfate. DNA was extracted by phenol-chloroform extraction and ethanol precipitation. In a few samples DNA was isolated from 0.2 ml of blood using a QIAamp DNA blood mini kit (Qiagen) in order to determine if this method provided any advantage over the phenol-chloroform method for DNA extraction.

**Oligonucleotide primers.** The 792-bp *L. donovani* kinetoplast mini-circle sequence (accession no. Y11401) was analyzed using PC-Gene software, and appropriate primers were identified. The two primers used were 5'-AAATCCGCG TCCGAGGCGGGAAAC-3' and 5'-GGTACACTCTATGAGTACC-3', together designated the Ldl primers. These were synthesized using an Applied Biosystems DNA-RNA synthesizer (model 394). The Ldl primers amplify a fragment of approximately 600 bp that is seen on the gels.

**PCR amplification.** DNA from cultured parasites (1 ng) and from clinical samples (100 ng) was taken for amplification using the Ldl primers described above. The reaction mixture (50 μl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, a 200 μM concentration of each denucleoside triphosphate, 50 ng of each primer, and 1.25 U of Taq DNA polymerase (Gibco BRL). Each reaction mixture was overlaid with mineral oil, and amplification was performed in a thermal cycler (Perkin-Elmer, Warrington, Great Britain) programmed for 40 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for 2 min, preceded by an initial denaturation of 2 min at 94°C. Final extension was for 3 min at 72°C. Products were analyzed by electrophoresis in 1% agarose gel containing ethidium bromide (0.5 μg/ml) in TAE buffer (0.04 M Tris acetate, 0.001 M EDTA) and photographed under UV illumination.

**Southern blot analysis.** PCR products were analyzed in 1% agarose gel, and Southern blot analysis was done as described (14). Southern blots were hybridized with 32P-labeled cloned *L. donovani* kDNA fragments using the conditions described (14).

**Sequencing reaction.** The PCR amplification products from culture isolates and clinical samples from patients with KA and PKDL were cloned into the pGEM-T- Easy vector system (Promega). DNA sequencing was performed with the ABI PRISM Dye Terminator Cycle sequencing kit and an ABI PRISM automated sequencer (Model 377; Perkin-Elmer). Briefly, the sequencing reaction mixture contained terminator ready reaction mix, DNA template, primer and 5% dimethyl sulfoxide. Dimethyl sulfoxide was added to keep the DNA template denatured since leishmania DNA has a high GC content. The PCR was carried out in a DNA thermal cycler (model 4800). The PCR conditions in the Perkin-Elmer analytical manual were followed. Sequences were assembled and edited using Sequencer software (Gene Codes Corporation, Ann Arbor, Mich.) and analyzed with Mac Vector DNA and protein sequence analysis software (Genetics Computer Group Inc., Madison, Wis.).

**RESULTS**

The initial aim of the study was to define a set of PCR primers based on kDNA sequences which would allow sensitive and specific detection of *L. donovani*. For this purpose, we analyzed kDNA mini-circle sequences from an *L. donovani* DDB strain of Indian origin and designed oligonucleotide primers that showed lack of cross-reactivity with organisms phylogenetically or geographically related (G. P. Pogue and H. L. Nakhasi, unpublished data). The sensitivity and effectiveness of the PCR-based detection system was seen in its ability to amplify kDNA fragments from as little as 1 fg of DNA of *L. donovani* (Fig. 1). When the amplification properties of PCR were combined with the specificity and sensitivity of Southern blot-based DNA hybridization, kDNA fragments could be detected by probes generated from the parasite.
kDNA sequences in PCRs containing as little as 10 fg of *Leishmania* DNA diluted in a 10 million-fold excess of human DNA (Fig. 2).

Initially the primers were evaluated with various strains of Old World *Leishmania* as described under Materials and Methods. Both strains of *L. donovani* of Indian origin gave positive results by PCR, as did the three isolates from Indian patients (Fig. 3, lanes 1 to 5). These three isolates of *L. donovani* were isolated 6 years prior to the present study from patients with KA and PKDL and were preserved in the Parasite Bank at the IICB. Strains of *L. donovani* from Sudan and Ethiopia as well as *L. donovani infantum* from Spain were positive by PCR, though the bands were of significantly lower intensity (Fig. 3, lanes 6 to 8). DNA from *L. major* and *L. tropica* was not amplified, indicating the species specificity of primers (Fig. 3, lanes 9 and 10). Specificity of the primers was also evaluated using DNA (10 ng) from microorganisms causative of the common infectious diseases prevalent in India, such as *Plasmodium* spp., *Mycobacterium leprae*, *Mycobacterium tuberculosis*; there was no amplification with DNA from any of these organisms using our primers (Fig. 3, lanes 11 to 13).

In order to establish the clinical utility of the assay, PCR amplification was evaluated with DNA from several recent isolates of the parasite. Parasite cultures were set up from bone marrow aspirates of five patients with KA that reported to SJH over the last 2 years (designated KA1 to KA5). DNA isolated as *L. donovani infantum* from Spain were positive by PCR, though the bands were of significantly lower intensity (Fig. 3, lanes 6 to 8). DNA from *L. major* and *L. tropica* was not amplified, indicating the species specificity of primers (Fig. 3, lanes 9 and 10). Specificity of the primers was further established, since the use of DNA up to 10 ng from three different strains of *L. major* and two strains of *L. tropica* as described in Materials and Methods did not give any amplification. Specificity of the primers was also evaluated using DNA (10 ng) from microorganisms causative of the common infectious diseases prevalent in India, such as *Plasmodium* spp., *Mycobacterium leprae*, *Mycobacterium tuberculosis*; there was no amplification with DNA from any of these organisms using our primers (Fig. 3, lanes 11 to 13).

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![FIG. 1. Sensitivity of the PCR assay. Shown are the results of PCR amplification of the serially diluted *L. donovani* (DD8) DNA analyzed on agarose gels. DNA was extracted from parasite cultures and amplified as described in Materials and Methods. Lane M, 1 kb Ladder (Gibco BRL); lane 1, 10 ng of DNA; lane 2, 1 ng of DNA; lane 3, 10 pg of DNA; lane 4, 1 pg of DNA; lane 5, 10 fg of DNA; lane 6, 1 fg of DNA.](image1)

![FIG. 2. Sensitivity of PCR amplification of *Leishmania* kDNA followed by Southern blot analysis. The PCR contained 100 ng of human genomic DNA and the indicated amount of total DNA from *L. donovani* DD8. The PCR product was probed with parasite kDNA and exposed for about 1 h. Lane 4 represents a PCR containing only human DNA as a control.](image2)

![FIG. 3. Amplification of parasite DNA from various strains and isolates of *Leishmania*. DNA (1 ng) isolated from parasite cultures was subjected to PCR and analyzed. Lane 1, *L. donovani* AG83; lane 2, *L. donovani* DD8; lane 3, *L. donovani* IICB8; lane 4, *L. donovani* IICB6; lane 5, *L. donovani* IICB7 (PKDL origin); lane 6, *L. donovani* IICB6; lane 7, *L. donovani* WR684; lane 8, *L. donovani infantum*; lane 9, *L. tropica* WR683; lane 10, *L. major* LV39; lane M, 1-kb ladder, lane 11, *Plasmodium*; lane 12, *M. leprae*; lane 13, *M. tuberculosis*.](image3)

![FIG. 4. DNA amplification from recent field isolates of KA and PKDL. DNA (1 ng) extracted from cultures of parasite isolates was used for PCR amplification. Lanes: M, 1-kb ladder; 1, KA-1; 2, KA-2; 3, KA-3; 4, KA-4; 5, KA-5; 6, PK-1; 7, PK-2; 8, PK-3; 9, PK-4; 10, PK-5; 11, isolate from a patient with cutaneous leishmaniasis.](image4)
from each of these cultures was observed to be amplified by PCR (Fig. 4, lanes 1 to 5). The assay was also positive with a number of cultures isolated from dermal lesions of patients with PKDL (PK1 to PK5) (Fig. 4, lanes 6 to 10), while the parasite culture isolated from a patient with cutaneous leishmaniasis hailing from Afghanistan gave no amplification in the PCR test (Fig. 4, lane 11). The sensitivity of the assay with the isolates of KA and PKDL was found to be 1 fg of total DNA.

A clinical study was undertaken with Indian patients with KA and PKDL using PCR based on LdI primers. The PCR assay was evaluated with clinical samples from patients with KA and PKDL along with samples from suitable controls. PCR analysis of representative sample from each of test materials—i.e., bone marrow and whole blood from patients with KA, blood from patients with malaria and tuberculosis, blood from control subjects from areas of endemicity, and skin lesion samples from patients with PKDL and leprosy—is shown in Fig. 5. Only samples of bone marrow and blood from patients with KA and from PKDL skin lesion specimens were PCR positive (Fig. 5, lanes 1, 2, and 6). The rest of the samples were negative (Fig. 5, lanes 3 to 5 and 7). All eight samples of bone marrow aspirates of patients with KA gave positive results when subjected to PCR amplification (Table 1). Our results showed that the primers could specifically amplify DNA from peripheral blood the parasites are relatively scarce. Studies

![FIG. 5. PCR assay with clinical samples of KA and PKDL. DNA (100 ng) isolated from clinical samples was used for PCR amplification. Lane M, 1-kb ladder; lane 1, KA (bone marrow); lane 2, KA (blood); lane 3, malaria (blood); lane 4, tuberculosis (blood); lane 5, control from the area of endemicity (blood); lane 6, PKDL (skin lesion); lane 7, leprosy (lesion).](http://jcm.asm.org/)

TABLE 1. Results of PCR assay with clinical samples from patients with KA and PKDL and controls

<table>
<thead>
<tr>
<th>Source of DNA</th>
<th>Total no.</th>
<th>No. PCR positive</th>
<th>% PCR positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>KA (bone marrow)</td>
<td>8</td>
<td>8</td>
<td>100</td>
</tr>
<tr>
<td>KA (blood)</td>
<td>51</td>
<td>49</td>
<td>96</td>
</tr>
<tr>
<td>Malaria (blood)</td>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tuberculosis (blood)</td>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Controls (blood)</td>
<td>20</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>PKDL (lesions)</td>
<td>48</td>
<td>45</td>
<td>93.8</td>
</tr>
<tr>
<td>Leprosy (lesions)</td>
<td>32</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PKDL (Healthy tissue)</td>
<td>19</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*The disease and sample type (in parentheses) are given. Controls, control subjects from areas of endemicity.*
reporting PCRs with detection sensitivities comparable to ours (less than a single parasite) did not obtain sensitivities as high as that of our assay when using blood samples of patients with KA (15, 35). With clinical samples the sensitivity in practice may be affected by factors such as accessibility of the DNA in parasite-containing biopsy samples and the conditions used in the PCR amplification.

DNA isolated from the pathogens causative of common coendemic diseases (M. leprae, M. tuberculosis, and Plasmodium) was not amplified. Blood from patients with malaria and tuberculosis were PCR negative in all cases (30 of 30), while two of the blood samples from control subjects from areas of endemicity were PCR positive, giving an overall specificity of 96% in the control blood samples examined. The two positive controls from areas of endemicity were relatives of patients with KA and possibly asymptomatic carriers since both samples were positive by ELISA with recombinant antigen k39 and in a dipstick test using immunochromatographic strips coated with rk39 antigen (P. Salotra and G. Sreenivas, unpublished data), tests reported to be specific for KA (34, 36). A recent study has reported a PCR assay that could often detect parasites in clinical samples (two each of KA blood and PKDL tissue) were subjected to sequence analysis. Identical sequences were obtained for the PCR products obtained in each case, which matched exactly with the published sequence of a 792-bp kDNA mini-circle segment of the DD8 strain of L. donovani (GenBank accession no. Y11401). The positions of primers are indicated in boldface type.

FIG. 6. Sequence of PCR product with DNA isolated from L. donovani DD8 strain and isolates and clinical samples of patients with KA and PKDL. PCR products obtained from DNA isolated from L. donovani DD8 strain, parasite isolates from patients with KA and PKDL (two each) and clinical samples (two each of KA blood and PKDL tissue) were subjected to sequence analysis. Identical sequences were obtained for the PCR products in each case, which matched exactly with the published sequence of a 792-bp kDNA mini-circle segment of the DD8 strain of L. donovani (GenBank accession no. Y11401). The positions of primers are indicated in boldface type.
sensitivity, and our assay would provide a rapid detection as well as species identification of Leishmania. Since this method is rapid and reproducible, we believe that it can be used for the reliable identification and characterization of cultured parasites. The test has other potential values in detecting and typing parasites in vectors for epidemiological surveys and in retrospective studies of archival material.

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