Abstract. Identification of new foci of cutaneous leishmaniasis (CL), along with reports of *Leishmania donovani* causing the disease, is an issue of concern. Clinico-epidemiologic analysis of 98 cases in the endemic regions of Rajasthan state, India, suggested the preponderance of infection in men (62.24%) compared with women (37.75%). Species characterization by internal transcribed spacer 1 (ITS1) polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP), kDNA-PCR, and immunofluorescence assay established *L. tropica* as the causative organism. When applied directly to 32 clinical samples, kDNA PCR had a sensitivity of 96.6%, whereas ITS1 PCR had a sensitivity of 82.75%, thus facilitating diagnosis and species identification. Either parasite culture or direct microscopy alone detected 48.2% and 65.5% of the positive samples, respectively, whereas culture and microscopy together improved overall sensitivity to 89.3% (25/28). Except for the kDNA PCR, all other assays were 100% specific. This study provides the first comprehensive molecular and immunologic studies of CL in India.

INTRODUCTION

Leishmaniasis is a parasitic disease affecting >12 million people in 88 countries (66 Old World and 22 New World countries), with 350 million more at risk. The clinical picture of leishmaniasis is heterogeneous and can be classified into three forms: cutaneous, mucocutaneous, and visceral leishmaniasis. According to the World Health Organization (WHO), the annual incidence is estimated at 1–1.5 million cases of cutaneous leishmaniasis (CL) and 500,000 cases of the visceral form. About 90% of the CL cases occur in only seven countries (Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia, and Syria), whereas ~90% of visceral leishmaniasis (VL) cases occur in rural and suburban areas of five countries (Bangladesh, India, Nepal, Sudan, and Brazil).

CL is a dermal manifestation commonly caused by various species of *Leishmania*, namely *Leishmania major*, *L. tropica*, *L. aethiopica*, *L. mexicana*, *L. amazonensis*, or *L. braziliensis*. The clinical manifestation includes spontaneously healing lesions to chronic and mutilating cutaneous or mucocutaneous ulcers and rarely a chronic diffuse cutaneous disease. *L. major* and *L. tropica* are the prevalent species in semiarid subtropical regions, important foci being in the Middle East, Middle Asia, Transcaucasia, and North Africa.

In India, CL is reported primarily in some pockets in the Thar Desert of Rajasthan state, located in the Western part of the country and bordering Pakistan. The first evidence for the existence of a CL was based on clinico-epidemiologic analysis of cases in 1973 during a large-scale outbreak of the disease in Bikaner. More than 2,000 people suffered from this infection during this time, sporadic cases were detected in villages in the vicinity of the Rajasthan canals. It was reported that Indian desert gerbils, *Meriones hurrianae*, and dogs were found to be the reservoirs of this infection. There is no authentic information regarding the vector species transmitting CL infection, but a few studies concluded that, in this region, one or both species of the two species of sandfly, *Phlebotomus papatasi* and *P. sergenti*, are the vectors. A recent report from Himachal Pradesh, an emerging foci for CL, situated in the western Himalayas in northern India, has provided concrete evidence of CL cases in India caused by *L. tropica* and *L. donovani*. This report and established endemicity of CL in Bikaner prompted us to study cases of CL in this area, which is comprised of a 37.95-km² area in the northwestern region of Rajasthan, which has a hot and dry climate.

CL exhibits symptoms that are very similar to those seen in several other skin diseases; thus, confirmation of parasites is mandatory when the diagnosis is suspected. The diagnosis of CL in regional clinics relies on demonstration of parasite in the smears or skin biopsy specimens by direct microscopic examination. These classic methods lack high sensitivity and specificity and do not provide any clues regarding the species involved in the disease pathogenesis. The species characterization in Indian CL is an important aspect, particularly in view of the recent identification of *L. donovani* causing CL in some parts of India. Polymerase chain reaction (PCR) has been applied successfully in recent years to detect *Leishmania* spp. in cases with any of the clinical manifestation of leishmaniasis. Several PCR assays for combined detection and differentiation of parasites exist, including species-specific PCR, single-strand conformation polymorphisms (SSCP), and restriction fragment length polymorphism (RFLP) analysis. Here, we present the first comprehensive study of identification and characterization of species causing CL in Rajasthan, India, confirming *L. tropica* as the prevalent species in the endemic area, which has immense importance from an epidemiologic, transmission, and treatment point of view.

MATERIALS AND METHODS

**Patients and samples.** Ninety-eight patients of suspected CL were recorded between November 2004 and April 2006 in the Department of Skin, V. D. & Leprosy, S. P. Medical College, Bikaner (Rajasthan), India, and were carefully examined. The study was approved by and carried out under the guidelines of the Ethical Committee of the S. P. Medical College and A. G. of Hospitals, Bikaner, (Rajasthan). In an en-
indemic area, suspected CL is largely diagnosed clinically by the appearance of erythematous nodules or ulcerated lesions, which present on an exposed area of the body for > 2 months. Thirty-two of the 98 patients were selected at random for a detailed analysis. The patients presented with ulcers that were up to 2–3 cm in diameter, consisting of nodular, ulcerated, or nodulo-ulcerative and non-ulcerated diffuse infiltrative type, with or without crusting. Skin biopsies of 5–10 mm in diameter were taken under sterile conditions from the border of the ulcers.

**Parasite culture.** Tissue material from the border of the ulcers were collected and inoculated into medium M199 supplemented with 25 mmol/L HEPES (pH 7.5) and 20% fetal calf serum (FCS) followed by incubation at 24°C. Cultures were checked microscopically for promastigotes for up to 2 weeks. Once the culture was positive, they were propagated in the same medium. Parasites were harvested in late log phase and washed with ice-cold phosphate buffered saline (PBS) before DNA isolation. Six WHO reference strains of Leishmania originating from distinct geographic locations were also used in the study. These included L. donovani DD8 (MHOM/IN/80/DD8) and L. donovani AG83 (MHOM/IN/83/AG83) from India, L. tropica WR683 (MHOM/SU/58/OD), L. tropica WR664 (MHOM/SU/58/K27), and L. major LV39 (MHOM/IN/80/DD8) and L. major WR662 (MHOM/IL/67/JerichoII, WR662. JerichoII) from Israel.

**Immunofluorescence assay.** An immunofluorescence assay (IFA) was performed with clinical isolates and WHO reference standards as described earlier.23 Genus-specific (G2D10 for Leishmania) and species-specific monoclonal antibodies (T1 for L. major, D2 for L. donovani, T10 for L. tropica) were used (obtained as a kind gift from TDR, World Health Organization, Geneva, Switzerland) for assay.23,24 Negative controls for IFA were comprised of omission of the primary antibody and its replacement with PBS. Suitable reference strains of Leishmania were used as positive controls.

**DNA isolation.** Biopsy tissue was collected directly in NET buffer (150 mmol/L NaCl, 15 mmol/L Tris-HCl, pH 8.3, 1 mmol/L EDTA) for isolation of DNA. DNA from parasite cultures and from lesion biopsies was isolated by overnight lysis in NET buffer containing Proteinase K (Sigma) and 1% sodium dodecyl sulphate as described previously.18

**PCR amplification.** The internal transcribed spacer 1 (ITS1) PCR assay was undertaken with primers LITSR and L5.8S to amplify the ribosomal ITS1 region as described by Schonian and others.25 Amplification of the DNA was performed in a 50-μL reaction comprised of 200 μmol/L dNTPs mix, 2 mmol/L MgCl2, 1 U of Taq polymerase (Invitrogen, Carlsbad, CA), 25 pmol of each primer, and 10 ng of DNA from culture isolates or 200 ng DNA from clinical samples. PCR was carried out with initial denaturation at 95°C for 2 minutes, followed by 35 cycles of denaturation at 95°C for 20 seconds, annealing at 53°C for 30 seconds, and extension at 72°C for 1 minute. This was followed by a final extension at 72°C for 6 minutes. Nested ITS1 PCR was performed on the 2 μL of the previous ITS1 PCR products using the same primer combination and PCR conditions as for the first round of amplification.

Kinetoplast DNA (kDNA) PCR assays were based on published sequences from the L. major kDNA minicircle.25,26 The primers Uni21 designed on a sequence within the conserved region and Lmj4 based on a variable region of the same strain were used. PCR was performed in a 50-μL reaction consisting of 1.5 mmol/L MgCl2, 200 μmol/L dNTPs mix, 50 ng of each primer, 1 U of Taq polymerase (Invitrogen), and 100–200 ng of DNA from clinical samples. PCR was programmed for 30 cycles with initial denaturation at 94°C for 6 minutes, followed by denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds, and final extension at 72°C for 5 minutes.

PCR assays were performed in a DNA thermal cycler (Perkin Elmer, Warrington, UK), and the products were analyzed by electrophoresis on 1% agarose gels in Tris-acetate EDTA (TAE) buffer. Ten nanograms of DNA isolated from cultured promastigotes of different geographical strains of Leishmania were also subjected to PCR experiments as positive controls.

**Restriction analysis.** The nested ITS1 PCR product was subjected to RFLP analysis by digesting the PCR amplified product with 1 U HaeIII enzyme (Genei, Bangalore, India) at 37°C for 2 hours. The restriction fragments were analyzed on 2% agarose gel.22

**Sequence analysis.** The nested ITS1 PCR products (340 bp) obtained with DNA from clinical isolates, lesion tissues, and reference strains were cloned in pGEM-T easy TA cloning vector (Promega, Madison, WI). Ligated product was transformed in E. coli cells. Colonies were screened with EcoR1, and the insert was sequenced (ABI prism automated sequencer). Sequences were subjected to NCBI BLAST analysis for the homology.

**Statistical analysis.** Specimens were considered confirmed positives (C-Pos) when cultures or stained tissue smears were positive for parasites or both PCR assays were positive for leishmanial DNA.27 When all four assays were negative or only one PCR was positive for parasite DNA, specimens were considered confirmed negatives (C-Neg). These values were used as the “consensus standards” against which each individual diagnostic assay was compared. Sensitivity, specificity, positive predictive values (PPVs), and negative predictive values (NPVs) were determined. Cohen κ coefficient was also calculated, which is a measure of the agreement between two tests beyond that expected by chance, where 0 is chance agreement and 1 is perfect agreement.28

**RESULTS**

**Demographic features and clinical profile.** Of the 98 CL cases, 61 (62.24%) were men and 37 (37.75%) were women. Most of the cases were in the age range of 5–30 years, with the oldest patient being a 70-year-old man, and the average age was 22.14 ± 15.45 (SD) years. None of the patients examined had been out of the state or district during 1 year preceding the onset of cutaneous lesions. In most (73.3%) cases, there was a history of 2–6 months of onset of lesions at the time of diagnosis. Fifty-two patients had a single lesion, 24 patients had double lesions, 5 patients had three lesions, and 14 patients had four to nine lesions. The lesions were primarily on the extremities, particularly the upper and lower limbs. In some cases, the lesions were found on the facial region, involving the nose, lips, neck, and angle of mouth.

**IFA.** IFA analysis was performed with clinical isolates (N = 7) and WHO standard reference strains of L. donovani, L. major, and L. tropica using genus- and species-specific monoclonal antibodies. The clinical isolates and references
strains from *L. tropica*, *L. major*, and *L. donovani* displayed the typical morphology when incubated with genus-specific monoclonal antibody G2D10 (data not shown). The clinical isolates examined from patients with CL and reference *L. tropica* strains reacted positively when subjected to IFA with *L. tropica*–specific antibody T-10 (Figure 1) and did not show any reaction with *L. donovani*– or *L. major*–specific antibodies (D-2 and T-1, respectively). Only 50% randomly selected clinical isolates were examined by this assay. On the other hand, *L. donovani* and *L. major* references strains did not show any reaction with *L. tropica*–specific antibody T-10 but reacted positively with D-2 and T-1, respectively. The assay provided confidence that the isolates from patients with CL belonged to the species *L. tropica*.

**Analysis of diagnostic methods.** Thirty-two of 98 suspected CL cases, with an average age of 23.19 ± 10.97 years, were randomly selected and examined by four diagnostic methods. Results obtained with each assay were compared for sensitivity and specificity (Table 1). Of 32 specimens received for analysis, 29 (90.63%) samples were C-Pos and 3 (9.38%) were C-Neg by the consensus criteria.

**Tissue smears and culture studies.** As defined by the consensus standards, both parasite culture and microscopic examination of smears were specific (100%) for diagnosis of CL, and when analyzed together, they correctly identified 25/28 (89.3%) of the C-Pos samples, showing that, for greater efficacy and specificity, they should be used together. The individual sensitivity of each assay was lower, 65.5% and 48.2% for smear and culture examination, respectively. The NPV of smears (23%) and culture (16.7%) was low. The specificity and PPV of smears and culture was 100%, because a positive result for either was always considered to be a true positive. The level of agreement between Cohen’s κ coefficient (κ ± SE) and diagnosis by culture and smear, 0.56 ± 0.21, was moderate, and levels of agreement between culture or smear and the confirmed results (C-Pos and C-Neg) were 0.06 ± 0.88 (slight) and −0.08 ± 0.21 (poor), respectively.

**ITS1 PCR-RFLP.** DNA from culture isolates (*N = 14*) were subjected to ITS1 PCR-RFLP for confirmation of *Leishmania* species. Standards isolates of *L. donovani*, *L. tropica*, and *L. major* (two strains of each) were used as controls. PCR to amplify the ITS1 region gave an amplified fragment of ~300–350 bp with all *Leishmania* species, and its subsequent digestion with the restriction enzyme *Hae*III revealed three bands for *L. donovani* (50, 80, and 190 bp), two bands for *L. tropica* (50 and 190 bp), and two for *L. major* (160 and 210 bp), clearly differentiating the *Leishmania* species (Figure 2A). Consistent band patterns corresponding to *L. tropica* were noticed in all 14 isolates examined (Figure 2B). The assay was further extended to DNA isolated from dermal lesions; the pattern corresponding to *L. tropica* was obtained in all cases (data not shown), and no amplification was observed using DNA from healthy skin tissues (*N = 5*). The assay correctly identified 24/29 of the C-Pos specimens, and the sensitivity and specificity of the assay was 82.75% and 100%, respectively, No false positives were seen. The PPV and NPV for the ITS1 assay were 100% and 37.5%, respectively. Agreement between the confirmed results and the ITS1 PCR was 0.47 ± 0.22 (moderate).

**Sequence analysis.** To confirm authenticity of the amplification products, the 340-bp ITS1 PCR product obtained with DNA from clinical tissue samples of patients with CL (*N = 5*) and from parasites isolates (*N = 2*) were subjected to sequence analysis. NCBI BLAST analysis revealed 99% homology with corresponding *L. tropica* sequence in the database (accession no. AJ000301).

**kDNA PCR.** We also exploited primers (Uni21/Lmj4) suitable for amplification and identification of parasite DNA of *Leishmania* species. Standard WHO reference strains of all three species were used as positive controls. PCR with all the culture isolates (*N = 14*) gave a consistent pattern of *L. tropica* (850 bp), clearly differentiated from *L. major* (650 bp; Figure 3). The assay was extended to DNA isolated directly from dermal lesions of patients with CL to determine its sensitivity and specificity for clinical use. Most of the CL cases (29/32) gave positive results, whereas the DNA from healthy skin tissue (*N = 5*) were all negative. The kDNA PCR had the highest sensitivity (96.6%) of any individual assay, correctly diagnosing 28/29 of the C-Pos. False positives, positive by kDNA PCR but negative by all the remaining assays, were observed for one sample. The PPV and NPV for this assay were 96.6% and 66.7%, respectively. The measure of agreement, 0.63 ± 0.26, indicates substantial agreement between the confirmed results and the kDNA PCR.

**DISCUSSION**

In this study, we reported molecular and immunologic characterization of *Leishmania* species that cause CL in Bikaner, Rajasthan, which is the major focus of CL in India. Sporadic cases of CL are reported in other parts of India such as Himachal Pradesh, where both *L. tropica* and *L. donovani* were found as the causative agents in the four cases examined. The presence of multiple species of *Leishmania* in a region, with overlapping clinical pictures, demands the development of sensitive laboratory tests with simultaneous species identification. Our study revealed *L. tropica* as the causative

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**Figure 1.** IFA of *Leishmania* parasites isolated from tissue biopsies using *L. tropica*–specific monoclonal antibodies, T10. The immune complexes showing the *L. tropica* promastigotes were visualized at a magnification of ×100.
organism in all cases examined in the endemic region in Rajasthan state in India.

Most of the 98 cases studied belonged to a low socioeconomic class, living in overcrowded conditions. The infection was more prevalent in men than in women, which is similar to previous reports. Diagnosis and characterization of *Leishmania* species in clinical infections in endemic areas are important for both clinical and epidemiologic reasons because of similar symptoms caused by other dermal manifestations (such as sarcoidosis and lupus vulgaris) and overlapping clinical presentation caused by different *Leishmania* species. Species and subspecies of *Leishmania* can be distinguished by reaction with species-specific monoclonal antibodies. IFA with species-specific antibodies revealed the presence of *L. tropica* species in all the isolates examined from patients with CL in the endemic region but needs culture of the parasites and cannot be applied directly to the clinical samples.

Molecular characterization of *Leishmania* species in clinical isolates of patients with CL was carried out by ITS1 PCR-RFLP analysis. Previous reports divulged an 0.9- to 1.2-kb non-coding spacer region present between Ssu and Lsu rRNA lying between the genes coding for 18S and 5.8S rRNA, which are highly repetitive variable regions that distinguish old world and new world *Leishmania* species by amplifying the internal spacer region and digesting with specific restriction enzymes such as *Hae* III. To appraise ITS1 PCR-RFLP genotyping, the amplicon was subjected to restricted digestion with *Hae* III, which produced three different patterns that unambiguously differentiated *L. tropica* from *L. donovani* and *L. major*. All the parasites isolates from patients with CL in Bikaner displayed similar patterns, identical to *L. tropica* WHO standard isolates. Furthermore, for clinical use, the assay was applied to DNA isolated directly from dermal lesions, which confirmed the presence of *L. tropica* in all the clinical samples examined. The ITS1 PCR products amplified were subjected to sequencing before digestion, and the sequence of products with parasite isolates and with clinical specimens showed sequence identical to that obtained with standard *L. tropica* species.

Diagnostic methods such as *in vitro* culture and direct microscopy require the presence of a relatively high number of viable or morphologically intact micro-organisms, but in the chronic phase of CL, the number of viable and morphologically intact parasites in skin lesions is very low. In laboratories in endemic regions, culture is often not available, and diagnosis is based on clinical features and microscopy, which have low sensitivity. In this study, direct microscopy with smear biopsies showed 65.5% sensitivity, whereas culture was 48.2% sensitive. Similar results have been reported for the sensitivity of tissue smears (37–60%) and culture (38%) in CL in India and in the neighboring country of Pakistan. Furthermore, our study showed that smear and culture taken together gave an overall sensitivity of 89.3% (25/28); however, species identification was not possible by these classic methods.

PCR has shown promise as a sensitive tool for diagnosis of

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<th>Table 1</th>
<th>Comparative analysis of diagnostic methods in samples obtained from patients with CL in the endemic area</th>
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<td>Assay</td>
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<td>Smear</td>
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<td>Culture</td>
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<td>ITS1 PCR</td>
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<td>kDNA PCR</td>
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**Figure 2.** The restriction analysis pattern of nested ITS1 PCR product digested with *Hae* III. (A) DNA (10 ng) isolated from culture of different *Leishmania* species was subjected to PCR-RFLP and analyzed on 2% agarose gels. Lane 1, *L. donovani* DD8 (MHOM/IN/80/DD8); lane 2, *L. donovani* AG83 (MHOM/IN/83/AG83); lane 3, *L. tropica* WR683 (MHOM/SU/58/OD); lane 4, *L. tropica* WR 664 (MHOM/SU/74/K27); lane 5, *L. major* WR662 (MHOM/IL/67/Jericho II, WR662. JerichoII); lane 6, *L. major* LV39 (MHOM/SU/59/P/LV39); lane 7, no DNA. Lane M, 100-bp ladder as molecular size marker. (B) Characterization of *Leishmania* species in clinical isolates of patients with CL using nested ITS1 PCR and restriction enzyme (*Hae* III) analysis. Lane 1, *L.tropica* WR 664 (MHOM/SU/74/K27); lanes 1–11, cultures from tissue biopsies of different patients with CL.
Various parasitic diseases, and in this report, we evaluated its potential in the diagnosis and characterization of Leishmania species in Indian CL in comparison with conventional direct microscopy and culture. The sensitivity of the ITS1 PCR assay was 82.75% when used directly in samples, similar to earlier reports.22,31 For further improvement in sensitivity, we exploited the kDNA PCR for diagnosis of CL in Rajasthan, using DNA obtained directly from clinical samples. The mini-circles have been reported as an excellent target for selective amplification of parasite DNA because of the thousands of copies per cell.16,18,25,32 As reported earlier, this assay amplifies parasite DNA with variability in amplicon size and can detect < 1 pg of DNA or as few as 10 leishmanial parasites.25 We found this assay to be sensitive enough for diagnosis of Leishmaniasis, and it could discriminate species such as L. tropica or L. donovani with L. major. Furthermore, the kDNA PCR had the highest sensitivity (96.6%) of any individual assay, suggesting its suitability as a tool for detection of Leishmania parasites directly in samples from CL lesions from an epidemiologic and treatment point of view. No amplification was observed in any of the healthy skin tissues from the endemic area with either ITS1 or kDNA PCR. Other groups have reported sensitivities of 85–92% for diagnosing CL using PCR based on kDNA.33–35

This study was focused on diagnosis and species identification in a large number of patients with CL in endemic regions of Rajasthan, India, and it was confirmed that CL infections in the endemic area are caused by L. tropica. The kDNA PCR was the most sensitive diagnostic assay and was established as a valuable tool in the diagnosis of CL in India. The test may be used as a new standard for the detection of parasite in patients suspected of CL with negative microscopic examination and/or culture results. The kDNA PCR may also help in regular randomized prospective screening in epidemiologic surveillance studies in CL in India. The species characterization in Indian CL is an important aspect, particularly in view of the recent identification of L. donovani causing CL in some parts of India. The ITS1 PCR-RFLP assay is recommended for reliable characterization of Leishmania species. Species identification is a vital part of the diagnostic procedure, especially in areas where more than one species of Leishmania occurs. This impinges on control strategy, therapy, and determining the epidemiology and dynamics of the disease.

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