

ORIGINAL ARTICLE

Parasite detection in patients with post kala-azar dermal leishmaniasis in India: a comparison between molecular and immunological methods

P Salotra, G Sreenivas, K R Beena, A Mukherjee, V Ramesh

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See end of article for authors' affiliations

Correspondence to:
Dr P Salotra, Molecular
Biology Laboratory,
Institute of Pathology
(ICMR), Safdarjung
Hospital Campus, New
Delhi-110 029, India;
salotra@bol.net.in

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Aims: To evaluate the sensitivity and specificity of serological, immunohistochemical, and molecular methods in the diagnosis of post kala-azar dermal leishmaniasis (PKDL).

Methods: Twenty five patients with confirmed PKDL and 25 controls were included in the study. G2D10, a monoclonal antibody against *Leishmania*, was used for the immunohistochemical (IHC) staining of lesion sections to visualise anti-*Leishmania donovani* antibodies. The diagnostic usefulness of IHC was compared with enzyme linked immunosorbent assay (ELISA) with a recombinant (rk39) antigen, and a species specific polymerase chain reaction (PCR) assay, amplifying a kinetoplast minicircle DNA sequence.

Results: IHC detected 22 of 25 PKDL cases, giving a sensitivity of 88%. The diagnostic sensitivity of both the ELISA and PCR tests was higher (96%). All of the 25 controls examined were negative in PCR, indicating 100% specificity of the test, whereas ELISA showed 96% specificity.

Conclusions: IHC with G2D10 significantly enhances the sensitivity of detection of PKDL over routine haematoxylin and eosin staining. ELISA with a recombinant antigen is an economical and practical assay. PCR is the most sensitive and specific diagnostic method for PKDL. The tests described would facilitate the recognition of patients with PKDL, enabling timely treatment, which would contribute greatly to the control of kala-azar.

The protozoan parasites of the genus *leishmania* cause a diverse group of diseases, known as leishmaniasis, which involve visceral reticuloendothelial organs, skin, and mucosal surfaces. *Leishmania* currently infects about 12 million people in 88 countries, with 600 000 new clinical cases reported annually and many more unreported. Globally, leishmaniasis is responsible for approximately 57 000 deaths annually, with 350 million individuals at risk.¹

“Reliable diagnostic tests are urgently required for the detection of post kala-azar dermal leishmaniasis to control the spread of visceral leishmaniasis”

Post kala-azar dermal leishmaniasis (PKDL), caused by *Leishmania donovani*, occurs in nearly 10–20% of patients cured of visceral leishmaniasis (VL) or kala-azar (KA) in India and in about 50% of patients cured of VL in the Sudan.^{2–4} In India, the disease occurs between one and 20 years after recovery from VL. In contrast, in the Sudan PKDL most often develops during or within months after treatment of VL, and in some patients the symptoms may persist for decades. Clinically, the condition is characterised by the appearance of macules, papules, or nodules in the skin. Patients developing chronic PKDL in India require long and expensive treatment. The cost and toxicity of current treatment regimens highlight the importance of establishing control strategies and make the diagnosis and typing of leishmaniasis crucial. In the absence of animal hosts, patients with PKDL are deemed the singular source of *L. donovani* in India.⁵ Reliable diagnostic tests are urgently required for the detection of PKDL to control the spread of VL.

Current methods to demonstrate the parasite in PKDL skin lesions are invasive and are often not sensitive enough, particularly in macular cases where parasites are scanty. As

a result, PKDL cases are often confused with several dermatological conditions, such as leprosy.⁶ In recent years, great advances have been made in the development of serological tests, including direct agglutination tests and enzyme linked immunosorbent assays (ELISAs) based on crude or recombinant antigens.^{7–8} The recombinant antigen rk39 has proved to be highly sensitive and specific for KA and PKDL.^{8–10} In addition, DNA based molecular methods such as the polymerase chain reaction (PCR) appear to be very promising tools for the diagnosis of KA^{11–14} and PKDL.^{14–15}

The aim of our present study was to develop and compare the usefulness of different immunological and molecular methods for the diagnosis of PKDL, and to analyse their respective advantages for routine diagnosis or epidemiological use.

METHODS

Patients

Our study comprised a total of 25 patients with PKDL reporting to Safdarjung Hospital (SJH), New Delhi, India, hailing from the eastern state of Bihar, where the disease is endemic. Consent was obtained from patients before collecting the biopsy material, according to the guidelines of the ethical committee, SJH. The patients comprised 19 men and six women, aged between 18 and 35 years. All patients presented with clinical symptoms of PKDL and features suggestive of PKDL on routine haematoxylin and eosin (H&E) staining. Ten patients had a history of KA of one to six

Abbreviations: ELISA, enzyme linked immunosorbent assay; H&E, haematoxylin and eosin; IFA, immunofluorescence assay; LD, *Leishmania donovani*; KA, kala-azar; OD, optical density; PKDL, post kala-azar dermal leishmaniasis; PCR, polymerase chain reaction; SJH, Safdarjung Hospital; VL, visceral leishmaniasis

years, 12 had a history of six to 14 years, and the remaining three were not aware of a history of KA. The histopathological findings were similar to those reported previously.¹⁶ Fourteen patients showed a generalised distribution of papules, nodules, and hypochromic macules, indicating a polymorphic presentation, whereas the remaining 11 patients showed a predominantly macular presentation. Nodular lesions showed a dense infiltrate occupying > 70% of the dermis, comprising lymphocytes, histiocytes, and plasma cells. Macular lesions showed a sparse inflammation (inflammatory infiltrate occupying < 20% of the dermis). Epithelioid cell granuloma was seen in one case. *Leishmania donovani* (LD) bodies were identified in 12 of 25 cases (< 50%) by means of H&E staining, and were seen within histiocytes and sometimes outside them. The diagnosis in the remaining cases was mainly by exclusion of other disorders and therapeutic response to parenteral sodium antimony gluconate.

Ten patients with lepromatous leprosy (confirmed by histopathology) reporting to the department of dermatology, SJH were included in our study as controls.

Culture

The skin biopsy samples were collected under aseptic conditions. The epidermis was carefully dislodged and only the dermal portion of the biopsy material was placed in culture medium comprising M199 and 25mM Hepes (pH 7.4), supplemented with a vitamin and amino acid mixture (Sigma, Poole, Dorset, UK) and 10% heat inactivated fetal calf serum. Antibiotics including streptomycin (100 µg/ml) and penicillin (100 U/ml) were added to the medium, and the samples were incubated at 26°C in a BOD incubator.

IHC and IFA

Unstained sections of PKDL or lepromatous leprosy skin were taken on poly-L-lysine coated slides for immunohistochemistry (IHC). A monoclonal antibody, G2D10, raised against a promastigote membrane antigen of *Leishmania gerbelli*, was used. This antibody, obtained as a kind gift from Tropical Disease Research, World Health Organisation, is known to recognise all species of *Leishmania*.¹⁷ Staining was by means of an avidin–biotin–peroxidase complex (ABC) method, using the Dako StreptABCComplex/HRP duet kit (Dako, Glostrup, Denmark). An immunofluorescence assay (IFA) with the axenic amastigotes was performed as described previously,^{18,19} using G2D10 as the primary antibody and antimouse IgG conjugated with fluorescein isothiocyanate (Dako) as the secondary antibody. Negative controls for both IHC and IFA comprised omission of the primary antibody and its replacement with Tris buffered saline.

ELISA

Serum samples, collected by venupuncture from 25 patients with PKDL, 10 patients with leprosy, and 15 healthy volunteers, were tested by ELISA using a leishmania specific recombinant antigen (rk39) obtained as a kind gift from S Reed, InBios, Seattle, USA.^{8,9} In brief, 96 well microtitre plates (Corning, New York, USA) coated with 10 ng of rk39 were blocked with 5% fat free milk, washed three times with phosphate buffered saline/Tween 20, and incubated for two hours with serum at a 1/200 dilution. Wells were washed and incubated for two hours with goat antihuman IgG conjugated with horseradish peroxidase (a gift from National Institute of Immunology, New Delhi, India). This step was followed by thorough rinsing with phosphate buffered saline/Tween 20 and the addition of *o*-phenylenediamine with hydrogen peroxide. The optical density (OD) of each well was measured at 492 nm in an ELISA reader (Titertek Multiskan Plus; Titertek, Finland). Each sample was assayed in triplicate or

more, along with appropriate controls. The cut off value was derived on the basis of the mean absorbance obtained with control sera.

DNA isolation and PCR amplification

DNA was isolated from PKDL lesions (n = 25) and leprosy lesions (n = 10) according to a method described previously.¹⁵ Briefly, excised tissue was frozen in liquid nitrogen, pulverised with a pestle and mortar into powder, and genomic DNA was extracted with extraction buffer (15mM Tris, pH 8.30, 150mM NaCl, 1mM EDTA) in the presence of 1% sodium dodecyl sulfate and proteinase K (100 µg/ml). DNA was extracted by phenol/chloroform extraction and ethanol precipitation. DNA (100 ng) was amplified using primers of sequences 5'-AAATCGCTCCGAGGCGGAAAC-3' and 5'-GTACTCTATCAGTAGCAC-3'. The reaction mixture (50 µl) contained 200 mM of each dNTP, 50 ng of each primer, 1.5mM MgCl₂, and 1.25 units Taq DNA polymerase in PCR buffer (Gibco BRL, New York, USA). Amplification was carried out as before using appropriate controls each time.¹⁵ Amplification products were analysed by electrophoresis in a 1% agarose gel and samples producing a single band of size 600 bp were recorded as positive.

RESULTS

Culture and IFA

In total, 24 skin biopsies were taken for parasite culture using an enriched medium. *Leishmania* parasites were evident as flagellated promastigotes in samples from 13 patients with PKDL. Five samples showed contamination and in the remaining samples parasites were not detectable.

IHC

Immunohistochemical analysis of the skin sections revealed LD bodies in 22 of the 25 cases, which appeared as large (3–5 µm) dark brown spherules (fig 1). The cell membrane and the nucleus were clearly visible and the kinetoplast could be detected occasionally. In contrast, in leprosy skin sections no LD bodies were seen in the 10 samples tested.

ELISA

In total, 50 serum samples (25 patients with PKDL and 25 controls) were tested by ELISA using the rk39 antigen. The mean (SD) absorbance in PKDL and control samples was 1.35 (0.21) and 0.20 (0.09) OD units, respectively. The cut off value was calculated as 0.40 OD units; that is, twice that of

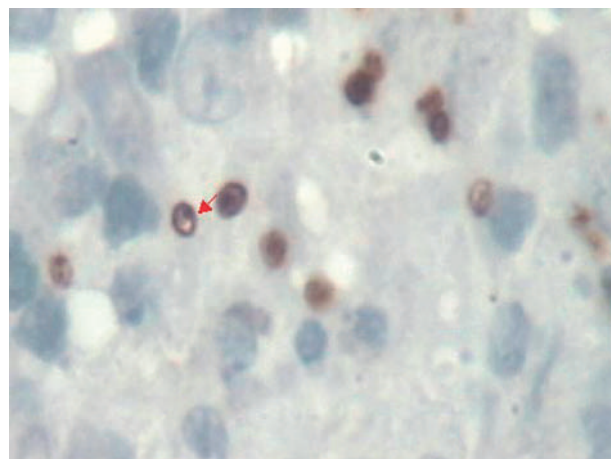


Figure 1 Immunohistochemical staining of tissue from a post kala-azar dermal leishmaniasis lesion with the G2D10 monoclonal antibody. The immune complexes showing the leishmania amastigotes (LD bodies) were visualised at a magnification of $\times 100$.

Table 1 Results of parasite identification in patients with post kala-azar dermal leishmaniasis by immunological and molecular methods

	Culture	H&E	IHC	ELISA	PCR
Presentation					
Polymorphic	11/14	12/14	14/14	14/14	14/14
Macular	2/10	0/11	8/11	10/11	10/11
Total	13/24	12/25	22/25	24/25	24/25
Sensitivity (%)	54	48	88	96	96

The number of positive cases out of the total number tested by each method is shown.
ELISA, enzyme linked immunosorbent assay; H&E, haematoxylin and eosin; IHC, immunohistochemistry; PCR, polymerase chain reaction.

the mean absorbance obtained with controls. The sensitivity for detection of PKDL was found to be 24 of 25 (96%). Among the controls, the use of rk39 led to a correct diagnosis in 24 of 25 cases, whereas one case gave a false positive result (table 1).

PCR

The PCR assay was evaluated with clinical samples from patients with PKDL and in suitable controls (fig 2). DNA from the dermal lesions of patients with PKDL and leprosy were tested by PCR. Most PKDL cases (24 of 25) gave a positive result, whereas all the leprosy cases (10 of 10) were negative. Samples of normal dermal tissue (n = 3) from unaffected parts of the skin of patients with PKDL were also negative.

DISCUSSION

The need to search for cases of PKDL and treat them as a part of kala-azar control programmes is well recognised, because PKDL provides the only known reservoir for the parasite in India.⁵ In our present study, different molecular and immunological methods were developed for an accurate diagnosis of PKDL and compared for their relative merits.

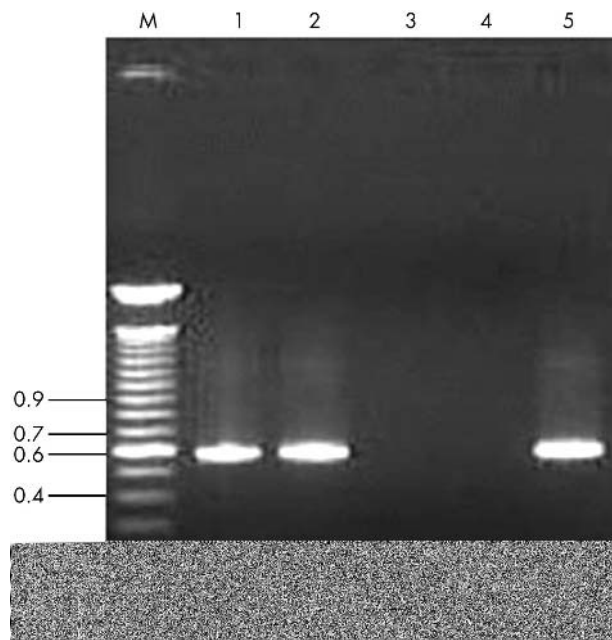


Figure 2 Polymerase chain reaction (PCR) assay with clinical samples of post kala-azar dermal leishmaniasis (PKDL); 100 ng of DNA isolated from clinical samples was used for PCR amplification. Lane M, 1 kb ladder; lane 1, PKDL nodular lesion; lane 2, PKDL macular lesion; lane 3, leprosy lesion; lane 4, normal skin; lane 5, parasite culture DNA.

Patients with PKDL with a polymorphic presentation could be readily diagnosed by each of the methods. Cases with macular presentation generally need rigorous assessment because leishmania parasites are scanty and difficult to demonstrate in skin lesions.²⁰ The immunological and molecular methods used in our present study showed a high degree of reliability even in macular cases. Considerable success was achieved in setting up cultures from PKDL lesions, a task that has met with limited success in the past. Although culture has limited usefulness as a diagnostic procedure, it provides an unlimited source of parasite material, giving an opportunity to investigate fundamental questions about important issues, such as drug sensitivity, gene expression, and so on, in leishmania parasites of PKDL origin.

The sensitivity of parasite detection (88%) using leishmania specific monoclonal antibodies provided a definite improvement over conventional H&E staining, where the detection rate was less than 50%. Increased parasite detection rates by IHC were also obtained in a study conducted on patients with PKDL in the Sudan using a leishmania specific monoclonal antibody.²¹ Thus, IHC provides a very useful adjunct to the histological diagnosis of PKDL.

“Even the macular cases that present a considerable challenge in diagnosis could be resolved with the polymerase chain reaction, testifying to its advantage over immunohistochemistry”

Although limited by its inability to discriminate between antibodies resulting from past or current infection, the serodiagnostic method based on the detection of antibody to a recombinant antigen provided a more economical and practical assay for PKDL diagnosis. In our present study, ELISA could identify leishmania specific antibodies in 96% of the cases with high specificity. Serological testing has the added advantage that it avoids the invasive procedure of biopsy collection. However, it is difficult to ascertain whether the immune response is specific for the PKDL antigen or is persisting after recovery from KA, particularly in cases appearing soon after the original KA episode. The long history of KA in most of the patients with PKDL investigated in our present study suggests that the immune response is possibly specific for PKDL, as was observed in an earlier study.²² An important limitation of any serodiagnostic test is its low sensitivity in immunocompromised cases. The incidence of leishmania/human immunodeficiency virus co-infection is increasing, and PKDL has been reported in patients infected with human immunodeficiency virus.^{23–25}

Although not standardised universally or used widely, PCR appears to be the most sensitive and specific assay for the diagnosis of PKDL, according to studies conducted in India and the Sudan.^{14, 15} Several DNA targets have been described for leishmania, such as ribosomal RNA genes, kinetoplast DNA, minicircle derived genes, and genomic repeats.^{11–15} Maximum sensitivity can be achieved by using multicopy sequences as the PCR target.²⁶ In our present study, the primers used were based on kinetoplast minicircle DNA, which is present in thousands of copies in each cell. Therefore, even the macular cases that present a considerable challenge in diagnosis could be resolved with PCR, testifying to its advantage over IHC. Furthermore, PCR would be useful for parasite detection in immunocompromised patients, where serodiagnostic methods often fail. Because PCR directly detects parasite DNA, it could also provide a useful tool as a prognostic indicator.

In conclusion, PCR was superior to IHC and serodiagnosis for the diagnosis of PKDL. Nevertheless, in the absence of

Take home messages

- Immunohistochemistry (IHC) was a significantly more sensitive method for the detection of post kala-azar dermal leishmaniasis (PKDL) than routine haematoxylin and eosin staining
- Enzyme linked immunosorbent assay was an economical and practical assay, although less specific
- The polymerase chain reaction was the most sensitive and specific diagnostic method for PKDL, but IHC or serodiagnosis offer useful alternatives because they are easier to perform
- All three tests would facilitate the recognition of patients with PKDL, enabling timely treatment, which would contribute greatly to the control of kala-azar

adequate training and a sophisticated laboratory in which to perform PCR on a routine basis, IHC or serodiagnosis offer useful alternatives because of the relative ease of the processes.

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Authors' affiliations

P Salotra, G Sreenivas, Molecular Biology Laboratory, Institute of Pathology (ICMR), Safdarjung Hospital Campus, New Delhi-110 029, India

K R Beena, A Mukherjee, Dermatopathology Laboratory, Institute of Pathology (ICMR)

V Ramesh, Department of Dermatology, Safdarjung Hospital Campus

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