

Review Article

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Challenges in the diagnosis of post kala-azar dermal leishmaniasis

Poonam Salotra & Ruchi Singh

Institute of Pathology (ICMR), Safdarjung Hospital Campus, New Delhi, India

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Post kala-azar dermal leishmaniasis (PKDL) is a dermatosis that occurs as a sequel of visceral leishmaniasis (VL). Elimination of VL requires detection and treatment of PKDL, necessarily because of its capacity to serve as a reservoir for the causative parasite, *Leishmania donovani*. Diagnosis of PKDL presents a challenge due to low parasite burden in the lesions. In this article we have reviewed the recent advances in the development of immunological and molecular methods for diagnosis of PKDL.

Key words Diagnosis - *Leishmania* - post kala-azar dermal leishmaniasis (PKDL) - visceral leishmaniasis

Leishmaniasis is a group of diseases caused by various species of the protozoan parasites of the genus *Leishmania*. Leishmaniasis can be categorized in 3 major forms ranging in severity from spontaneously healing skin ulcers in cutaneous leishmaniasis (CL), destructive mucocutaneous leishmaniasis (MCL) to fatal visceral leishmaniasis (VL). The disease remains a major health problem, being currently prevalent in 88 countries, infecting 12 million individuals and threatening 350 million people. The geographical distribution of leishmaniasis is restricted to tropical and temperate regions, the living area of the sand fly. Ninety per cent of cases with cutaneous forms of leishmaniasis occur in Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Syria, while ninety per cent of visceral leishmaniasis cases are found in Bangladesh, Brazil, India, Nepal and Sudan¹. In India, VL is

endemic in the eastern regions of the country and often becomes epidemic, claiming the lives of thousands and causing severe morbidity to hundreds of thousands². *Leishmania donovani*, the causal organism, is a dimorphic parasite: the flagellated promastigote form is found in the insect vector whereas the non motile intracellular amastigote stage resides within macrophages of human host. VL is clinically characterized by fever, weight loss, hepatosplenomegaly, and pancytopenia and has a high mortality rate in untreated cases. Post kala-azar dermal leishmaniasis (PKDL) is a dermal complication, caused as a sequel to VL. In India, it manifests in 5-15 per cent of VL cases after months or several years of remission from infection, while in Sudan, it develops within weeks or months in 50-60 per cent of cured VL cases^{3,4}. The disease develops in a variety of clinical forms from hypopigmented

macules to infiltrated papules or nodules. High interleukin-10 (IL-10) levels in the skin and peripheral blood as well as high level of C reactive protein in plasma of patients with VL are predictive of the subsequent development of PKDL^{5,6}.

PKDL was first described by Brahmchari in 1922 in cured VL patients with eruption and plaque in the skin, confirmed by demonstration of Leishman-Donovan bodies (LDB) in slit skin smear and termed as dermal leishmanoid⁷. Later the disease was renamed as PKDL since eruptions follow the visceral disease, commonly called as kala-azar. There are distinct features in PKDL in Sudan, and in the Indian subcontinent. In India, PKDL develops as a dermatosis in a small percentage of treated VL patients with a usual interval of 2-3 yr but it may occur much earlier (*i.e.*, after 6 months) or much later (up to 32 yr). In 15-20 per cent of PKDL cases no preceding history of VL is available, suggestive of subclinical infection³. On the contrary, in Sudan the disease manifests in more than 50 per cent of VL patients, usually soon after, or sometimes even during the treatment. The interval between VL and PKDL is short, with all cases occurring 0-13 months after treatment, usually within first 6 months. About 8 per cent of cases in Sudan have no previous VL history while parallel VL and PKDL is reported in 18 per cent cases⁸.

In Indian subcontinent, untreated cases of VL and PKDL are considered to be the sole reservoir to house and disseminate the causative parasite in the absence of zoonotic transmission^{9,10}. *Phlebotomous argentipes*, the vector transmitting VL in India, when allowed to feed on PKDL patients, became infected and developed promastigotes in the midgut, seeming capable of transmitting the parasite^{11,12}. In VL, the parasite is predominantly found in deep seated organs and not accessible to vector, though the presence of parasite in skin of VL patients is documented¹³. On the other hand, LD bodies rich nodular skin lesions in PKDL may be the main source of vector infection in the community particularly in the absence of animal reservoir, and play an important role in the disease transmission from humans to the insect vector.

PKDL manifests in a variety of clinical forms from hypopigmented macules to infiltrated plaques and nodules (Fig.). The three major representation of skin lesion are described of which one or two forms predominate and two forms generally co-exist in the same patient. (i) Erythematous indurated lesions on the butterfly area of face; (ii) multiple symmetrical hypopigmented macules with irregular margins that may coalesce, having generalized distribution to the extremities and trunk; and (iii) combination of papules, nodules and plaques.

PKDL persists as a chronic dermatosis without complication in most cases. In Indian PKDL the nodules enlarge with time and form big plaques but rarely show ulceration in contrast to African PKDL where ulceration is common in advanced cases. The other unusual variants of PKDL include the annular, warty, papillomatous growth, fibroid with erythematous plaques and xanthomatous growth and presence of lesions in uncommon site such as eyelids, palms and the perionychium. The complications can result when mucous membranes are affected, the most serious being blindness due to corneal involvement³. Recently, nerve involvement in Indian PKDL has been documented which is common in Sudan PKDL, showing that PKDL may simulate leprosy both clinically and pathologically^{14,15}. PKDL is now being detected and reported in individuals infected with HIV from South America, Europe¹⁶⁻¹⁸. In India, there are documented and undocumented reports of PKDL patients with HIV infection, one such patient suffered from recurrence of VL following PKDL¹⁹. In another study, Nandy *et al*²⁰ reported recurrence of VL after episodes of PKDL, due to immunosuppression caused by measles or repeated attacks of malaria and tuberculosis.

The incidence of PKDL may have important implications in transmission of VL, as PKDL provides the only known reservoir of the parasite in India⁹. It is important to understand the pathogenesis of the disease and control it. So far, little is known about the factors of parasite/host origin that drive the parasite to cause a shift in the site of infection from viscera to dermis and thereby the clinical manifestation of the disease. It is not known whether the parasite in PKDL lesions is the residual parasite

Clinical presentation in PKDL



Fig. Clinical presentation in PKDL (A) PKDL nodular and papular lesions on face and hypopigmented macules on neck in a patient with polymorphic presentation (B) hypopigmented macules on trunk in a patient with macular PKDL. (C) Erythematous and indurated lesions over the butterfly area of face.

after VL infection or is introduced upon re-infection by sandfly vector. In the later event, the cause of change in site of predilection of the parasite could be attributed to altered immune status of the human host. It is also not clear why antimony therapy for PKDL patients needs to be continued for much longer duration than for VL patients (4 months instead of 4 wk for VL)^{3,8}.

Diagnosis

Presently, the diagnosis of PKDL is based on clinical and epidemiological parameters. Demonstration of parasite in the slit smear or by culture of the dermal tissue is considered to be the gold standard but the methods involved are invasive, less sensitive (58%) and difficult to perform in field conditions²¹.

Commonly used methods for diagnosis of leishmaniasis, including VL and CL, include (i) demonstration of parasite in tissues of relevance by light microscopic examination of the stained specimen, *in vitro* culture, or animal inoculation; (ii) immunodiagnosis by detection of parasite antigen in tissue, blood, or urine samples, by detection of nonspecific or specific antileishmanial antibodies, or by assay for *Leishmania*-specific cell-mediated immunity; or (iii) detection of parasite DNA in tissue samples.

Methods for the diagnosis of VL often lack sensitivity or specificity for the diagnosis of PKDL as (i) the number of parasites in skin smears and biopsy specimens is often low, thus requiring prolonged searches by routine microscopy^{14,21,22};

(ii) serological tests may be positive due to the past occurrence of VL^{23,24}; (iii) the leishmanin skin test (LST) may or may not be positive³; and (iv) cultures are often not positive and, in the rural region where the disease is endemic, cultures are prone to contamination²⁵. In our hands, nearly 54 per cent sensitivity was obtained for culture isolation from skin lesions of PKDL patients²⁶. In particular, the hypopigmented form of PKDL has been often misdiagnosed as vitiligo since the parasite load is scanty and not always proportional to the extent of dermal lesions while its nodular form may be easily confused with a number of dermatological conditions among which leprosy is the most important^{3,14}. When the parasite is not demonstrated in skin biopsies, the diagnosis of the PKDL hinges on the endemicity of VL in the area and previous history of infection by the parasite. Absence of VL history in 15-20 per cent of PKDL patients suggests subclinical infection and poses difficulty for diagnosis.

Histopathology

Conventional diagnosis using histopathology in PKDL tissue sections using haematoxylin & eosin (H & E) staining shows a variable degree of positivity for LD bodies ranging from 67-100 per cent in nodular lesions, 36-69 per cent in papular lesions and 7-33 per cent in macular lesions^{3,27}. Histopathological observations in PKDL revealed that the granuloma comprises of mainly lymphocytes, macrophages, and plasma cells in varying proportions²⁸. Macular form of PKDL shows a normal epidermal layer with a scattered perivascular lymphocyte and plasma cell infiltrate in superficial dermis. LDBs are rare and difficult to demonstrate in macular PKDL. The histopathological features are non specific at this stage. Nodular lesions show an atrophied epidermis with a narrow Grenz zone. The dermis shows a widespread infiltration of the superficial and mid dermis by a polymorphic infiltrate of lymphocytes, macrophages and plasma cells. LDBs are numerous and can be detected with ease in these lesions. Follicular plugging and hyalinization of dermal blood vessels are additional features²⁹. Generally, the histopathological features are specific enough to make a diagnosis of PKDL, even if LDBs cannot be identified. Even in ideal situations the success rate

for LDB demonstration is about 58 per cent²¹. In attempts to enhance the detection of *Leishmania* parasite in tissue sections, alternative methods have been introduced. Recently, it has been shown that sensitivity of parasite identification can be increased by using *Leishmania* specific monoclonal antibodies in an immunohistochemistry (IHC) assay, as IHC showed a higher percentage of LDB localization (80%) than detected by H&E staining (50%)^{26,30}.

Immunological methods

The potential of serological tests, such as direct agglutination test (DAT) and ELISA for diagnosis of PKDL has been evaluated and these show promise as rapid and non-invasive tests. The antileishmanial antibodies may persist for years as a result of previous VL infection, and titres measured by DAT have been reported to remain positive for up to 5 yr after recovery in >50 per cent of VL patients examined. As the interval between VL and PKDL in India is often long, it is argued that this immune response is likely to be the results of PKDL occurrence rather than persistence of antibodies of earlier VL infection³¹⁻³³.

Immunoblotting and ELISA: The Western blot technique is among the most sensitive and specific serological methods that provides information about the parasite's antigenic profile. Differences in VL and PKDL patients' antibody profile have been documented. Only 1 out of 10 *i.e.*, 10 per cent of PKDL patients showed antibody response to a 200 kDa axenic amastigote soluble antigen compared to 97 per cent VL patients³⁴. In an attempt to analyze the humoral immune response to *L. donovani* antigens in PKDL patients, we had earlier reported that two parasite antigens (of 110 and 65 kDa) elicit an antibody response in 97-100 per cent PKDL patients and in none of the control patients including leprosy and vitiligo³⁵. Absence of antibody for a 31 kDa band of promastigote membrane antigen in PKDL sera in comparison to majority of the VL sera, both before and after treatment, was reported indicating the potential of this polypeptide for the differentiation of PKDL from past and ongoing VL infection³³. Adaptation of antigen may be of help as humoral immune responses are quite distinct in VL and PKDL³⁵.

The use of recombinant DNA technology to produce serodiagnostic antigens has resulted in reagents of diagnostic promise for leishmaniasis. In recent times several *Leishmania* genes have been cloned and characterized as a result of extensive scientific activity aimed at improving the serological methods for diagnosis of leishmaniasis. The examples include rK39, A2, ORF F, rH2A, rH2B, rGBP, rLACK, rgp63, rP20, rPSA, and purified LPG, etc³⁶. Several studies have evaluated the potential of ELISA as a diagnostic test for VL and a few of them have included PKDL patients in their study.

K39 and A2 are abundantly expressed in the amastigote stage and are composed of repetitive units of amino acids which represent amplified targets for serological diagnosis. K39 is an antigen coded by a kinesin related gene and contains a repetitive epitope of 39 amino acid residues. The recombinant product rK39 was shown to be an early surrogate marker for disease progression in VL, with the seroactivity correlating with the active disease; 98 per cent of active disease cases were detectable with this marker^{37,38}. In India, rK39 has been widely used for the diagnosis of VL as well as PKDL, with sensitivity of the test ranging from 95 to 100 per cent^{31,32}. rK39 ELISA has been used as a prognostic test for monitoring VL patients undergoing drug therapy and is also useful in predicting clinical relapse^{32,39}. rK39 has been shown to have high predictive value for detecting VL in immunocompromised patients with VL-HIV co-infection⁴⁰. In a comprehensive study with 88 Indian patients of PKDL, the potential of crude antigen derived from indigenous parasite at two different developmental stages- promastigote and amastigote isolated from indigenous PKDL cultures, was compared with the rK39 antigen³¹. While the recombinant antigen, rK39, gave 94.5 per cent sensitivity, the amastigote antigen gave better sensitivity (92%) compared to the promastigote antigen (86%). Evaluation in 114 control subjects comprising of leprosy, vitiligo, malaria, tuberculosis revealed that the specificity of rK39 was 94 per cent while that of promastigote and amastigote antigens was 90 per cent. In comparison to crude *Leishmania* antigen, rK39 has been proved to be better diagnostic antigen for PKDL, with high sensitivity and specificity³¹.

A2 antigen has been used to detect antileishmanial antibody in Indian and Sudanese VL patients with the sensitivity of 60 and 82 per cent, respectively. The sensitivity of detection was improved by using co-immunoprecipitation assay to determine reactivity against conformational epitopes of A2 up to 92 per cent in acute VL patients' sera from Indian patients while at post treatment stage the assay was positive in 67 per cent of cases³⁸. The prospective value of A2 in diagnosis of PKDL is yet to be explored. The repetitive sequence of *L. major* gene B protein (GBP) has previously been shown to be a useful tool in the diagnosis of CL. Using recombinant *L. donovani* GBP (rGBP) in ELISA the diagnosis of *L. donovani* infections in Sudan was reported with the sensitivity of 93 per cent for PKDL and 92 per cent for VL⁴¹. A glucose regulated protein 78 (GRP78) of *L. donovani* has been used for serological evaluation of plasma samples in Sudan and antibody reactivity to this *Leishmania* antigen was revealed in 89 per cent of VL cases, 78 per cent PKDL patients, and 85 per cent patients of CL⁴². Other molecules like rPSA, rgp63, LPG, H2A, H2B and ORF F have not been evaluated for their capability of diagnosing PKDL, but their potential is well proven for the diagnosis of VL^{36,43}.

Recently, Saha *et al* (2005) have evaluated the utility of leishmanial membrane antigen specific Ig isotypes and IgG subclasses for the specific diagnosis of PKDL³³. Most of the PKDL patients exhibited elevated levels of antileishmanial IgG, IgM, IgA, and IgG subclass (IgG1, IgG2, and IgG3) antibodies, on the other hand, the absence of IgE and low levels of IgG4 were documented³³. The sensitivity and specificity of the assay of the IgG ELISA using membrane proteins for the diagnosis of PKDL was 100 and 96.7 per cent respectively, higher than the specificities of ELISAs with promastigote and amastigote antigen extracts or rK39. The specificity needs to be established with reasonable number of endemic controls since this study reported evaluation with very few (5) controls³³. The applicability of *L. donovani* species-specific monoclonal antibody D2 for sensitive and specific serodiagnosis by C-ELISA in sera from Indian patients with VL and PKDL was proven with sensitivity of 90.9 and 100 per cent, respectively. The C-ELISA can be used to evaluate the success of drug treatment⁴⁴. Various antigens used

Table I. Antigens used in ELISA for detection of antileishmanial antibody in PKDL patients sera

Antigen	Sensitivity (%)	Specificity (%)	References
CLA	86-100	90-100	31, 44
SLA	83	90-100	24, 34, 45
MP	100	96.7	33
rK39	94.5-100	93.7-100	31, 32
GBP	93-100	83	41
GRP78	78	90	42
C-ELISA (D2)	100	100	44

CLA, Crude *Leishmania* antigen; SLA, soluble *Leishmania* antigen; MP, membrane protein; rK39, recombinant K39; GBP, gene binding protein; GRP78, glucose related protein 78; C-ELISA(D2), competitive ELISA based on D2 (*L. donovani* specific monoclonal antibody)

in ELISA for diagnosis of antileishmanial antibody in PKDL patients are summarized in Table I.

Direct agglutination test (DAT)

DAT is a simple technique with high specificity and sensitivity making it suitable for both field and laboratory use. The test can be carried out using plasma or serum. The method uses whole, trypsinized, Coomassie stained promastigotes either as a suspension or in a freeze-dried form. The freeze-dried form is heat stable and facilitates the use of DAT in the field. The major disadvantage of DAT is the relative long incubation time of 18 h and the need for serial dilutions of blood or serum. Also DAT has no prognostic value for evaluating the parasitological cure of the disease as the test may remain positive for several years after cure. DAT remains the first line diagnostic tool for VL in many developing countries. The potential of DAT in the diagnosis of PKDL was first demonstrated by El Harith *et al*²³ using antigen prepared from indigenous isolates. With the use of indigenous isolates, the DAT was found capable of distinguishing CL, MCL and other skin disease conditions like leprosy, vitiligo, psoriasis, cutaneous tuberculosis, *etc.* DAT had been used in diagnosis of VL in India with high specificity (100%) and sensitivity (96-98%)^{45,46}. With reference to its use for PKDL diagnosis, DAT has not been used very widely, in a study with four PKDL cases of macular type, DAT was negative⁴⁷. We have developed DAT based on axenic amastigotes with about 95 per cent sensitivity and 100 per cent

specificity of diagnosing PKDL cases (unpublished data). Moreover, the test has high sensitivity for PKDL cases with macular presentation, which are often difficult to diagnose. The data suggest that DAT with amastigote antigen may be advantageous for monitoring subclinical infections and all forms of PKDL.

A fast agglutination screening test (FAST) for the rapid detection (<3h) of anti-leishmania antibodies in serum samples, has been developed. The FAST utilizes only one serum dilution leading to qualitative results⁴⁸. The FAST claims advantages over the DAT as it uses freeze-dried antigen which gives more antigen stability, reproducibility, specificity and sensitivity.

Rapid antibody detection methods

Strip test: A simple, rapid, easy to perform dipstick test using rK39 is available (Corixa Corp[®], InBios Inc.[®], DiaMed IT[®]) in the form of antigen impregnated nitrocellulose paper strips adapted for use under field conditions. In India, the rK39 strip test has been found to be highly sensitive and reliable for both VL and PKDL diagnosis. The sensitivity of the strip test for VL diagnosis, in India, has been reported to be 100 per cent, this could correctly diagnose 95 per cent of cases of PKDL with polymorphic presentation while with macular PKDL cases the sensitivity was 73 per cent^{49,50}. The specificity of the test is reported to be 100 per cent in laboratory studies^{50,51}. However, recent reports

reveal that this antigen shows decreased sensitivity and specificity in some countries endemic for VL, such as Sudan where it is reported to miss 7 per cent parasitologically proven cases⁵². The rK39 strip test showed a sensitivity of 97 per cent and a specificity of 71 per cent in Nepal⁵³. Similar reports are available from Southern Europe and Brazil^{54,55}.

Latex agglutination test: Latex agglutination tests based on crude *Leishmania* antigen, soluble *Leishmania* antigen or recombinant antigen have been developed for diagnosis of VL^{45,51}. Recently, latex agglutination test, using recombinant antigen, rK39, has been reported for the diagnosis of VL and PKDL⁵¹. The sensitivity of latex agglutination test was found to be 80 per cent as compared to ELISA but it did not miss any clinically active VL case. About 20 per cent cases were missed due to low antibody levels. The antibody titre was reported to vary from 10^{-3} to $>10^{-6}$ and titre increased for 6 to 9 months after development of symptoms. The test could detect the antibodies only if the titres were 10^{-4} or more in ELISA. Latex agglutination test may not be suitable for PKDL as generally the antibody titres are found to be low in PKDL.

Antigen detection/KATEX: To diagnose the active *Leishmania* infection antigen detection tests are important especially in immunocompromised patients, where antibody response is poor. The antigen levels are expected to theoretically correlate with the parasite load. The detection of antigen in the serum is complicated by the presence of high level of antibodies, circulating immune complexes, serum amyloid, rheumatoid factor and autoantibodies, all of which may mask immunologically important antigenic determinants or competitively inhibit the binding of free antigen. Recently, a latex agglutination test (KATEX) for the detection of leishmanial antigens in the urine of VL patients has been developed^{56,57}. This is a unique test that detects a stable, non protein, disease specific antigen in the urine of patients with an active infection. It gives result in two minutes and is both simple to use and diagnostically accurate. The results obtained with KATEX using samples collected from different foci of VL indicate that the test works well regardless of the geographical origin of samples. The test had

100 per cent specificity and sensitivity between 68-100 per cent. Whether the test has applications for the diagnosis of PKDL, detection of asymptomatic cases of VL and monitoring therapy remains to be confirmed.

There are a number of other antibody and antigen detection methods existing with variable sensitivity and specificity. These tests include indirect haemagglutination (IHA), countercurrent immuno electrophoresis (CCIEP), immunodiffusion (ID) and several others. However, these tests are cumbersome and lack sensitivity and specificity and most of them have been developed for diagnosis of VL. IHA when used for PKDL was positive in only one third of chronic PKDL cases, while all fresh cases were positive indicating antibody titre reduced during the chronicity²⁴. Heamagglutination assay had been developed for diagnosis of Indian VL using Achatinin H, a lectin isolated from the haemolymph of the snail *Achatina fulica*, that binds exclusively to 9-O-acetylated derivatives of sialic acid (9-O-AcSA). The test exploits the fact that 9-O-AcSA is present on erythrocytes of VL patients and absent on the erythrocytes of patients with malaria, tuberculosis, as well as on those of healthy controls from endemic and nonendemic areas⁵⁸. This is a rapid, non invasive assay that is adaptable to most clinical settings for VL but has not been tested for PKDL.

Assay for cell mediated immunity (CMI) and skin test

There is accruing evidence indicating that immune responses developed during and after cure in VL patients play a major role in the development of PKDL. Earlier reports from India showed that in VL, *Leishmania*-specific as well as generalized CMI responses were absent. These responses were restored in VL patients after successful treatment, while in PKDL, suppression of the CMI response was found to be associated only at the specific level. With administration of the drug (sodium antimony gluconate), the immunosuppression was gradually eliminated with concomitant clinical improvement in both VL and PKDL patients⁵⁹. In a recent study, *Leishmania* specific CMI using membrane and soluble proteins have been shown in cured VL

patients⁶⁰. Studies from Sudan also showed immunosuppression in VL patients but in some cases it was skewed towards Th2 type and in PKDL patients *Leishmania* specific CMI as well as interleukin-10 production was documented⁶¹.

The leishmanin skin test (LST) measures delayed type hypersensitivity reactions to intradermal injection of leishmanin antigen (killed *L. donovani* parasite) in patients' fore arm. After 48-72 h the induration is measured in mm; a reaction of 5 mm or more is considered positive. However, several problems have been associated with skin tests like reference standards for skin test antigens and for performance, including reading, of the tests have not been developed. Most antigens are crude extracts of parasites and are neither sensitive nor specific. In India, skin test positivity to whole promastigote antigen was not demonstrable until 5 months after the acute phase of VL. At this time the positivity rate was 20 per cent, and it increased to 86.6 per cent after 8 months⁶². The test may or may not be positive for PKDL as reported from India. In Sudan, 11 per cent of the PKDL patients with concomitant VL were LST positive while LST positivity was more (37%) in patients with PKDL only. Persistence of the lesions has been frequently associated with non reactivity in the LST and high levels of anti-leishmanial antibodies⁶³.

Most serological tests for the diagnosis of PKDL are of limited value in areas of endemicity, as these are also positive for VL patients with active disease or past history. Even rK39 ELISA titres remain positive for up to 2 yr after treatment, with no decline in OD values⁶⁴. Identification of *Leishmania* infection, for laboratory and clinical diagnosis, by culture or microscopic techniques, is tedious and time consuming and has poor sensitivity. Most of these limitations can be overcome with the application of DNA based methods.

Molecular methods

Molecular biology is increasingly becoming relevant to the diagnosis and control of infectious diseases. A variety of nucleic acid detection methods targeting DNA and RNA genes have been developed for leishmaniasis^{65,66}. However, amongst all the

molecular advances gene amplification techniques have been most rewarding as far as diagnosis and disease management are concerned. In addition to diagnosis, PCR may be used in prognosis of disease, strain identification of parasite and molecular epidemiology, detecting HIV-*Leishmania* co-infection and can also be utilized for determining the drug resistance⁶⁵.

Polymerase chain reaction (PCR): Amongst the molecular methods used for clinical diagnosis, PCR has proved to be the most promising technique. The specificity of the PCR can be adapted to specific needs by targeting conserved region of the gene. Gene amplification through the PCR has several advantages compared to traditional techniques, because of its extremely high sensitivity, rapidity and the ability to be performed with a broad range of clinical specimens. Also, the detection or identification of the causative agent is possible directly in the clinical specimens. Maximum sensitivity can be achieved by using multicopy sequences as the PCR target⁶⁷. Several target sequences have been used for the PCR like ribosomal RNA genes, kinetoplast DNA (kDNA), mini-exon-derived RNA (med RNA) genes and genomic repeats, the β -tubulin gene region, glycoprotein 63 (gp63) gene locus, internal transcribed spacer (ITS) regions⁶⁸. By targeting conserved or variable regions the specificity of the PCR may be adapted to specific needs to characterize the parasite to the level of the genus complex, species or even the individual isolate.

Reports on evaluation of PCR based diagnostic assays for PKDL are limited and are adapted from the approaches developed for VL diagnosis^{25,69-71}. For diagnosis of VL, bone marrow and lymph node aspirates as well as blood samples have been evaluated. Bone marrow aspirates from parasitologically confirmed VL patients were always PCR positive in several studies^{69,71,72}. Since collection of lymph node, bone marrow or splenic aspirates involves painful procedures that can even be dangerous for the patient, peripheral blood, which is easy to obtain, is often used for the initial PCR screening of people suspected of VL. The sensitivity of PCR for the detection of *Leishmania* DNA in blood samples ranges from 70-95 per cent^{68,71-73}.

The major advantages of using PCR based techniques are the ability to detect *Leishmania* parasite in patients with low levels of parasitaemia and identify them to the species level. Several studies have reported that PCR assay could detect parasitaemia a few weeks before the appearance of any clinical signs or symptoms. ELISA based detection of PCR products further increases sensitivity. Martin-Sanchez and coworkers⁷⁴ reported a highly sensitive (83%) PCR-ELISA in the diagnosis of cutaneous and visceral leishmaniasis caused by *L. infantum*. A PCR-ELISA, for blood samples from Nepal, was more sensitive (83.9%) than conventional PCR (73.2%), and demonstrated 100 and 87.2 per cent specificity respectively, when using healthy controls who had never travelled to a VL endemic area and those from a VL endemic area as references⁷⁵. PCR may also be useful for the confirmation of the diagnosis in HIV/*Leishmania* co-infected patients. Pizzuto *et al*⁷⁶ showed that all 76 HIV/*Leishmania* co-infected patients were parasitaemic by PCR on peripheral blood before therapy. In another study, 15 of 20 (75%) such patients were PCR positive⁷⁷.

A species-specific PCR assay, based on kDNA was developed and tested in Indian patients of VL and PKDL. The test yielded a detection limit of 1.0 fg of purified *L. donovani* DNA (equivalent to less than one parasite) or 10 fg of parasite DNA (equivalent to single parasite) in presence of 10 million fold excess of human DNA⁷¹. Using samples of peripheral blood the test enabled the diagnosis in 49 of 51 parasitologically confirmed VL patients, corresponding to a sensitivity of 96 per cent. The sensitivity was 100 per cent for VL diagnosis when bone marrow samples were used. The preferable samples for PKDL diagnosis are skin biopsy of lesions, slit aspirates or lymph node aspirates. The kDNA based PCR assay detected parasite's presence in 45 of 48 PKDL skin biopsies giving the sensitivity of 93.8 per cent. The specificity of the test was 100 per cent for PKDL as leprosy and healthy region of skin of PKDL patients did not show PCR positivity⁷¹. In a study from Sudan, the sensitivity of the detection of parasite from slit aspirate and lymph node aspirate was reported to be 82 and 83 per cent, respectively²⁵. The slit aspirates samples are preferred over invasive

lesion biopsy that may leave disfiguring marks on face. Recently, a nested PCR method was developed for less invasive diagnosis of PKDL. The assay successfully identified parasite DNA in slit aspirates of 27 of 29 cases with 93 per cent sensitivity in comparison to the primary PCR which could detect 20 of 29 cases (69%)⁷⁸.

Yazdi *et al*⁷⁹ employed laser capture microdissection to microdissect lymphocytic histiocytes infiltrates from CL patients dermal lesions and amplified the kDNA of parasites from extracted DNA. The lesion though not showing any evidence of intrahistiocytic *Leishmania* organism on Giemsa staining, was positive by PCR for *Leishmania* parasite⁷⁹. Similarly, this technique can be adapted for PKDL with macular presentation which have low load of parasite and at times only histopathological features are indicative of PKDL. Microdissection of infected cells from the tissue and subsequent analysis by PCR would provide proven diagnosis in such cases. PCR has caused a revolution in the diagnosis of leishmaniasis, since it is capable of detecting the *Leishmania* parasite in a variety of clinical samples and for all clinical manifestations of the disease. However, the execution of this very sensitive technique requires precautions; care should be taken for the risk of contamination, false positive results, and inclusion of appropriate and sufficient positive and negative controls.

Real time PCR: Recent developments in PCR technology such as fluorogenic probes and automation take care of non specific amplification, speed and inter-operator variability as well as allow to monitor the amplification in real time. Quantitative real-time PCR allows the continuous monitoring of the accumulation of PCR products during the amplification reaction. This allows the identification of the cycle of near-logarithmic PCR product generation (threshold cycle) and, by inference, the relative quantification of the template DNA present at the start of the reaction. Since the amplification products are monitored in "real-time" as they form cycle-by-cycle, no post-amplification handling is required. The absolute quantification is performed according to either an internal standard co-amplified with the sample DNA, or to an external standard

curve obtained by parallel amplification of serial known concentrations of a reference DNA sequence. From the quantification of the template DNA, an estimation of the relative load of parasites in the different samples can be obtained. Real time PCR assay is a quantitative PCR proved to be a reliable and non invasive tool for diagnosis of VL in HIV infected patients. In recent years, quantitative PCR methods based either on SYBR Green or TaqMan technology have been set up for the quantification of *Leishmania* in mouse liver, skin and human peripheral blood, targeting nucleotide sequences used to detect parasite are same as are being used for PCR *i.e.*, repetitive sequences such as SSU rRNA, mini-exon-derived RNA (med RNA) and kDNA minicircles⁸⁰⁻⁸².

A recently developed real time PCR assay based on 18S rRNA sequences simultaneously detects, quantitates, and distinguishes *Leishmania* organisms at species level into *L. donovani* complex, the *L. brasiliensis* complex, and species other than these by means of distinct melting temperatures obtained with fluorescence resonance energy transfer probes in different clinical samples (blood, bone marrow, skin, and liver)⁸⁰. Nicolas and colleagues⁸¹ have developed an assay based on kDNA sequences for monitoring *L. major* load in mice tissue with the sensitivity equivalent to 0.1 parasite per reaction. The assay had the capability to detect *L. donovani* and *L. amazonensis* infections also⁸¹. Bossolasco and co-workers⁸² used the primers and probes from SSU rRNA gene in the assay for detection and clinical management of the infection in *Leishmania*-HIV co-infected patients obtaining the sensitivity of detection as less than 1 (0.625) parasite/ml of blood. Recently, Mary *et al*⁸³ have developed a real time PCR assay to quantify kDNA of *Leishmania* and optimized the sensitivity to detect 0.0125 parasite/ml of blood. Comparative analysis of classical diagnostic test reveals that the detection range is 18 to 42, 0.7 to 42 and 0.12 to 22.5 parasites/ml for microscopic examinations, culture and conventional PCR, respectively⁸³. A multiplex real time PCR assay was recently developed by our group for synchronized detection of *Bacillus anthracis*, *Yersinia pestis* and *L. donovani* in blood. A limited clinical study established that this multiplex fluorescence PCR

assay works efficiently in the detection of *Leishmania* in blood samples⁸⁴. The assay revealed 50-1000 parasites per ml blood in Indian VL patients, while samples from cured VL patients showed no amplification for the presence of the parasite. It is evident that the real time PCR assay would be useful for epidemiologic and diagnostic purposes and to assess parasite burden in symptomatic patients, cured patients, asymptomatic carriers as well as PKDL patients. These studies are under evaluation in our laboratory.

Nucleic acid sequence-based amplification (NASBA): PCR is generally based on the detection of parasite DNA, which may be present even after the parasite has been cleared. Therefore, a test based on RNA detection would be useful to detect live parasites. NASBA technology, for the amplification of specific RNA sequences, has been proved to be a very sensitive and specific assay in diagnostic microbiology⁸⁵. Tests have been developed for HIV, human papillomavirus, *Mycobacteria* and *Plasmodium falciparum*. NASBA detects RNA in a background of DNA and may thus serve to measure viable parasites and is specific and sensitive to amplify as little as 10-100 target molecules in a sample⁸⁶. The technique is not reported for leishmaniasis but is under investigation. Quantitative analysis of RNA levels after drug treatment could be a useful method to assess the efficacy of anti-*Leishmania* treatment.

Genetic polymorphism in PKDL isolates

Biological or genetic markers for PKDL, that can determine relative taxonomic position of *L. donovani* isolates from PKDL lesions within the genus *Leishmania*, are currently under investigation. Molecular techniques such as polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), random amplification of polymorphic DNA (RAPD) and single strand conformational polymorphism (SSCP) have been used to demonstrate the genetic variability within and between different *Leishmania* species including *L. donovani* complex⁸⁷. Earlier studies to differentiate between VL and PKDL isolates in Sudan did not reveal any correlation between sequence polymorphisms and clinical

manifestation of human disease⁸⁸. Among Indian isolates, Das Gupta *et al*⁸⁹ cloned a minicircle from *Leishmania* strain UR6 which hybridized to parasite isolated from PKDL patient but not to an isolate from VL patients. This probe was also found weakly reactive in hybridization with kDNAs from *L. mexicana*, *L. braziliensis*, *L. aethiopica* and *L. major*. Recently we have reported a DNA polymorphism assay that distinguishes *L. donovani* isolates of PKDL from VL using the nuclear gene probe LdP-13 identical to the τ and ϵ subunit regions of 28S ribosomal RNA gene of *L. donovani* MHOM/SD/00/Khartoum⁹⁰.

Insights in search for new *Leishmania* diagnostic molecules

Interactions between host and parasite, responsible for outcome of infection are governed by the genomes of the host and parasite. With the availability of whole genome sequence of both human as well as *Leishmania* (*L. major*) parasite, the understanding of interaction between parasite virulence and host response factors is within reach. Analysis of the *L. major* genome sequence of size 33.6 MB, using several algorithms, predicts 911

rRNA genes, 39 pseudogenes, and 8272 protein coding genes⁹¹. So far, nearly 4 per cent of the genes are experimentally characterized while the function of about 32 per cent of genes is inferred from homology. Conserved hypothetical proteins coding genes along with sequence orphan with no predicted function contribute 64 per cent of the genes. Without doubt the availability of genome information will have a major impact on basic research in *Leishmania*, particularly in molecular science and biochemistry. This would not only contribute to the understanding of the basis of drug resistance, intergenic diversity, infectivity and pathology but would also facilitate the identification of new targets for drugs, vaccine and diagnostics.

The information from *Leishmania* genome and advent of new tools like microarray technology is being applied to the identification of functionally important *Leishmania* genes and proteins. The high throughput technologies like DNA and protein microarrays have the promised potential of identification of novel molecules which can not only emerge as more sensitive and specific diagnostic markers but may also provide newer therapeutic and vaccine targets. In recent times, two techniques - laser

Table II. Comparison of important features and sensitivity of diagnostic methods for polymorphic and macular PKDL in Indian patients

	Polymorphic PKDL	Macular PKDL
Clinical presentation	mix of papular, nodular erythematous lesions	hypopigmented macules
Histopathological features	specific features for diagnosis even in the absence of LDB atrophied epidermis	non specific features normal epidermis
Cellular infiltration LDB	dense in superficial and mid dermis adequate	scattered in perivascular areas in superficial dermis rare and difficult to demonstrate
Diagnosis		
H&E	up to 85%	low positivity
IHC	100%	72%
ELISA	100%	90%
rK39 strip test	94%-100%	73%
DAT		
Amastigote	100%	90-98%
Promastigote	90-100%	0 - 80%
PCR	100%	90-100%

LBD, Leishman-Donovan bodies; H&E, haematoxylin & eosin; IHC, immuno histochemistry; DAT, direct agglutination test; PCR, polymerase chain reaction

capture microdissection and single cell cDNA amplification have greatly improved the prospects of studying gene expression in single cells and in pathological specimens. The different states of cellular machinery in any altered form, like a disease situation can now be successfully profiled and the difference in the expression of protein profile is now considered to be a most promising area dubbed as functional genomics. At any stage of high throughput biological world, genomics now is always complemented by proteomics. Proteomic technology has the potential to determine all the *Leishmania* proteins that are recognized by the patients' immune system. The proteome serological approach can be used to generate total antibody profile of patient suffering from leishmaniasis/PKDL in order to determine the quantitative and qualitative phenomenon of parasite components that are reactive against the host immune system.

Conclusion

VL transmission in India is thought to be anthroponotic and in the absence of animal reservoirs, PKDL patients are deemed singular source of the parasite *L. donovani*. Therefore, rapid, sensitive and specific tools for identifying PKDL cases are highly desirable, because it would allow control interventions in endemic area of VL, a prerequisite for successful elimination of VL. Parasite detection in PKDL cases, particularly those with macular presentation, presents a challenge because of low parasite load. The comparison of various important features of the two major forms of PKDL, polymorphic and macular, and the diagnostic value of different tests in identifying these cases, are reviewed in Table II. Conventional HE staining yields limited success and use of *Leishmania* specific monoclonal antibodies vastly improves the sensitivity of detection even in macular cases. Immunological tests provide useful alternatives as rapid and sensitive measures for PKDL diagnosis; however, the question whether antileishmanial antibodies are those persisting due to VL or elicited due to PKDL remains unanswered. Diagnosis when augmented with PCR provides exquisite sensitivity and specificity. Cases of PKDL infection in HIV patients are being reported and since incidence of

HIV infection is on the increase in India, cases of co-infection with *Leishmania* are likely to go up in future. In such cases the molecular methods described here would have potential to provide a rapid and reliable detection of *L. donovani* in PKDL.

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Reprint requests: Poonam Salotra, Assistant Director, Institute of Pathology (ICMR), Safdarjung Hospital Campus
New Delhi 110029, India
e-mail: salotra@vsnl.com