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Comparative Evaluation of PCR and Imprint Smear Microscopy Analyses of Skin Biopsy Specimens in Diagnosis of Macular, Papular, and Mixed Papulo-Nodular Lesions of Post-Kala-Azar Dermal Leishmaniasis

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Diagnosis of post-kala-azar dermal leishmaniasis (PKDL), particularly the macular form, is difficult when based on microscopy. This study compared the results of nested PCR (91.9% positive samples) with imprint smear microscopy (70.9% positive samples) for 62 PKDL samples. We found that nested PCR, which indicated 87.5% positivity for the macular lesions, compared to 41.6% positivity by imprint smear microscopy, is an efficient method for early diagnosis of PKDL.

Post-kala-azar dermal leishmaniasis (PKDL), a chronic dermatosis, is a sequela of visceral leishmaniasis (VL), which is caused by *Leishmania donovani* infection. In India, it usually develops after 6 months to several years in 5 to 15% of cured VL cases. A past history of VL is absent in 15 to 20% of PKDL patients (1, 2).

PKDL, which is characterized by macular, papular, or papulonodular lesions on the face and other parts of the body, is often confused clinically and pathologically with leprosy, vitiligo, or fungal infection (1). In India, PKDL cases are the known reservoir of the leishmania parasite and have a major role in anthroponotic transmission of VL (3, 4). Therefore, early detection and management of PKDL is an essential strategy for the goals of elimination of VL from the Indian subcontinent by 2015 and of PKDL by 2018 (5, 6, 7).

Demonstration of leishmania parasites in skin biopsy specimen imprint/slit smear or culture from a PKDL lesion is considered the "gold standard" for diagnosis of PKDL. However, microscopy is less sensitive than molecular techniques, such as PCR, and requires prolonged searches, particularly in macular lesions with a very low parasite density. Cultures are often negative, prone to contamination, and this method is not feasible to perform in the field (1, 8).

Serological techniques do not provide direct evidence of parasite positivity, and they are not reliable in immunocompromised patients. Techniques involving use of monoclonal antibodies or isoenzyme or schizodeme analyses are tedious and require massive culturing of parasites. Histopathological diagnosis of PKDL is not very sensitive or specific, as visualization of intact parasites in tissue sections is difficult. Immunohistochemical staining is complex and has varied degrees of sensitivity (8, 9, 10).

In recent years, several studies have proved that PCR is a very sensitive and specific technique for detection of leishmania DNA (11, 12, 13, 14, 15). Few PCR methods have been developed for PKDL diagnosis, but its efficacy on biopsy specimens from various types of lesions has not been assessed properly (12, 15). This highlights an urgent need to develop a reliable and highly sensitive and specific technique to detect PKDL, especially for hypopigmented macular lesions (16).

In the present study, a nested PCR designed for the internal transcribed spacer (ITS) region of the rRNA gene of *L. donovani* was used on biopsy samples from macular, papular, and papulo-nodular lesions of PKDL, and the results were compared with those obtained from imprint smear microscopy. The study was approved by the Ethical Committee of Rajendra Memorial Research Institute of Medical Sciences (RMRIMS), Patna, Bihar, India, and written informed consent was obtained from all the subjects.

PKDL patients who attended the outpatient clinic of Rajendra Memorial Research Institute of Medical Sciences, Patna, Bihar, India, from nearby villages where kala-azar is endemic were selected on the basis of the appearance and distribution of skin lesions and the loss of sensation. Any history of kala-azar and its treatment were recorded. Skin biopsy samples were collected under aseptic conditions from 62 PKDL subjects (24 macular, 17 papular, and 21 papulo-nodular) and 30 control subjects (6 with confirmed PKDL [positive controls] and 6 with leprosy, 8 with a fungal disease, 5 normal skin samples from people cured of PKDL, and 5 healthy persons).

Collection of skin biopsy samples was performed, using a sterile surgical blade, by a trained pathologist of our institute. Multiple imprint smears of the inner surface of the biopsy specimen were prepared immediately on two clean grease-free glass slides and fixed with methanol. Giemsa-stained imprint smears were examined microscopically by two laboratory personnel for demonstration of leishmania parasites, and findings were confirmed by the pathologist. Any discrepancy in results was resolved by reexamination of the slides by both of the laboratory personnel and finally by the pathologist.

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Biopsy tissue was placed in sterile Tris-EDTA buffer (pH 8.0) and was sent to the Molecular Biology laboratory of the institute to be stored at 4°C until the extraction of nucleic acid. DNA from skin biopsy samples was extracted by using a QIAamp DNA mini-kit (Qiagen GmbH, Germany), following the manufacturer's instructions. DNA from *Leishmania* parasites and other organisms was extracted by using phenol-chloroform (8). PCR amplification was done using the ribosomal ITS region of *Leishmania* sense (forward) and antisense (reverse) primers designed for primary PCR (5′-ACACTCAGGTCTGTAAAC-3′ and 5′-CTGGATCATTTTC CGATGATTAC-3′) and nested PCR (5′-ACATAACGTGTCGC GATGGA-3′ and 5′-GAGAGAGAGCCACACACCA-3′) (17). Both primary and nested PCR assays were carried out in a 50-μl volume that contained 3 to 5 μg of DNA (for primary PCR) or 1 μl of primary PCR product (for nested PCR) in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate, 25 pM respective primer, and 1.25 U *Taq* polymerase enzyme (Qiagen GmbH, Germany) (17).

The amplification entailed initial denaturation at 94°C for 5 min and 35 cycles consisting of denaturation at 94°C for 1 min, annealing for 1 min at 48°C for primary and 58°C for nested PCR, and extension at 72°C for 2 min. A final extension cycle at 72°C for 10 min was included in the program. Amplified products were analyzed on 1.5% agarose gel; a 100-bp DNA ladder (Fermentas, Germany) was used as the marker, and gels were photographed by using the gel documentation system of Bio-Rad. The PCR results were considered positive when a band at 600 bp was visualized.

Negative and positive controls were included in each PCR test. DNA of three reference strains of *Leishmania donovani* parasites, obtained from the RMRIMS Leishmania Repository, were used as the positive control, whereas distilled water and DNA from other organisms were the negative controls. The other organisms, *viz.* the skin lesions of lepromatous leprosy (LL) patients for *Mycobacterium leprae* and the reference strain of *Mycobacterium tuberculosis* (H37Rv) were obtained from the Mycobacterium Repository Centre of the National JALMA Institute for Leprosy and Other Mycobacterial Diseases (NJIL OMD), Agra, India. Blood samples of malaria patients attending an outpatient clinic of RMRIMS, Patna, were the source of *Plasmodium vivax*.

Nested PCR was found highly sensitive and specific, as DNA from known reference leishmania isolates (MHOM/IN/80/DD8, MHOM/IN/83/AG83, and BI2303) showed positive amplification and none of the other organisms (*M. leprae*, *M. tuberculosis*, or *P. vivax*) demonstrated any PCR band.

Skin biopsy samples from parasitologically confirmed PKDL cases ($n = 6$) were PCR positive, whereas samples from patients with other diseases ($n = 14$) or from normal controls ($n = 10$) were both microscopically and nested PCR negative. The term "parasitologically confirmed PKDL" meant that the *Leishmania* parasites were observed microscopically in the imprint smears of skin biopsy samples from PKDL patients. Six randomly selected biopsy samples from PKDL cases, two each from macular, papular, and papulo-nodular lesions, along with positive controls, subjected to a nested ITS PCR-restriction fragment length polymorphism assay, were identified as *L. donovani*.

Out of 62 PKDL patients, the imprint smear microscopy showed 41.6%, 88.2%, and 90.4% positive results for macular, papular, and papulo-nodular lesions, respectively, whereas the PCR results were 87.5%, 94.1%, and 95.2% positive, respectively. The overall positivity of imprint smear microscopy was 70.9%, and for nested PCR it was 91.9% (Table 1). All PKDL patients who were identified positive by imprint smear microscopy were also positive by PCR, and none of the samples positive by microscopy was found negative by PCR.

TABLE 1 Comparative evaluation of PCR and imprint smear microscopy for detection of *Leishmania donovani* parasites in macular, papular, and papulo-nodular lesions of PKDL patients

Type of PKDL skin lesion	*n*	No. (%) positive by:	
		PCR	Imprint smear microscopy
Macular	24	21 (87.5)	10 (41.6)
Papular	17	16 (94.1)	15 (88.2)
Papulo-nodular	21	20 (95.2)	19 (90.4)
Total	62	57 (91.9)	44 (70.9)

The comparative analysis in our study revealed that nested PCR was 45.9% more sensitive for parasite detection in macular lesions, as it was overall 21% more sensitive than imprint smear microscopy in diagnosing all types of PKDL lesions. However, there was not much difference between papular and papulo-nodular lesions with the two techniques, because parasite density was higher in these lesions.

In a recent study conducted in Bangladesh, positivity by nested PCR in macular lesions was 93.2%, whereas microscopy had indicated only 2.7% positive samples, with overall positive results of 94.5% and 29.1%, respectively (11). Similar studies from India and Sudan reported positive PCR results in 93% and 82.7% of samples, whereas by microscopy the positive rates were 30.4% and 54%, respectively (12, 15). Our study revealed nearly similar results by nested PCR, whereas detection by imprint smear microscopy was much higher than reported in the other studies. We used the rRNA gene of the ITS region, whereas in the Bangladesh, India, and Sudan studies, a minicircle of kinetoplast DNA (kDNA) and the 18S rRNA gene were employed for the PCR (11, 12, 15).

The number of parasites in macular lesions is scanty in comparison to papular and papulo-nodular lesions, and so the chances of detection by microscopy are much lower for macular lesions, even by trained laboratory personnel. Since PCR amplifies multiple copies of a gene of the parasite in the thermal cycles, the possibility of missing a parasite is very rare. Hence, PCR is clearly superior to microscopy for the macular lesions, but it is only marginally better for papular and papulo-nodular lesions.

In conclusion, nested PCR was found highly efficient in comparison to imprint smear microscopy for PKDL diagnosis. Moreover, in hypopigmented macular forms of PKDL with very low parasite densities, nested PCR was almost twice as sensitive as imprint smear microscopy. Hence, nested PCR is suggested for early diagnosis of PKDL, particularly the macular forms, affording management of cases that may help in interrupting transmission of kala-azar infection.

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