Comparative Evaluation of PCR and Imprint Smear Microscopy of Skin Biopsy in Diagnosis of Macular, Papular and Mixed Papulo-nodular Lesions of PKDL. (Manuscript # JCM01482-13R2)

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Title: Comparative Evaluation of PCR and Imprint Smear Microscopy of Skin Biopsy in Diagnosis of Macular, Papular and Mixed Papulo-nodular Lesions of PKDL.

Abstract:
Diagnosis of Post Kala-azar dermal leishmaniasis (PKDL), particularly the macular form is difficult by microscopy. This study compared the positivity of nested polymerase chain reaction (91.9%) with imprint smear microscopy (70.9%) in 62 PKDL samples. Nested PCR, showing 87.5% positivity in macular lesions compared to 41.6% by imprint smear microscopy, is an efficient method for early diagnosis of PKDL.

Running Title: PCR vs microscopy in PKDL diagnosis.

Keywords: PKDL, PCR, Imprint smear microscopy, Kala-azar Elimination Program.

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

PKDL, characterized by macular, papular or papulo-nodular lesions on face and other parts of body, are often confused clinically and pathologically with leprosy, vitiligo and fungal infections (1). In India, PKDL cases are the known reservoir of leishmania parasite having major role in anthroponotic transmission of VL (3, 4). So, early detection and management of PKDL is an essential strategy for elimination of VL from the Indian subcontinent by 2015 and PKDL by 2018 (5, 6, 7).
Demonstration of leishmania parasites in skin biopsy imprint / slit smear or culture from PKDL lesions is considered “gold standard” for diagnosis of PKDL. However microscopy is less sensitive than the molecular technique such as polymerase chain reaction (PCR) and requires prolonged searches particularly in macular lesions having very low parasite density. Cultures are often negative, prone to contamination and not feasible to perform in the field (1, 8).

Serological techniques are neither the direct evidence of parasite positivity, nor reliable in immunocompromised patients. Techniques involving use of monoclonal antibodies, isoenzyme and schizodeme analysis are tedious and require massive culture of parasites. Histopathological diagnosis of PKDL is not very sensitive and specific with difficult visualization of intact parasite in tissue sections. Immuno-histochemical staining is complex with varying 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 from various types of lesions has not been assessed properly (12, 15). This highlights an urgent need to develop a reliable, highly sensitive and specific technique to detect PKDL, especially with hypopigmented macular lesions (16).

In the present study, a nested PCR designed from Internal Transcribed Spacer (ITS) region of ribosomal RNA gene of *L. donovani* was used on biopsy samples from macular, papular and papulo-nodular lesions of PKDL and the results were compared with imprint smear microscopy. The study was approved by 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 cases attending the out-patient-clinic of Rajendra Memorial Research Institute of Medical Sciences, Patna, Bihar from nearby endemic villages were selected on the basis of skin lesion’s appearance, distribution and loss of sensation. Any past history of kala-azar and its treatment were recorded. Skin biopsies were collected under aseptic condition from 62 PKDL (24 macular, 17 papular and 21 papulo-nodular) and 30 control subjects (6 confirmed PKDL, 6 leprosy, 8 fungal diseases, 5 normal skin from cured PKDL and 5 healthy persons).

Collection of skin biopsy was performed using sterile surgical blade by trained pathologist of the institute. Multiple imprint smears of the inner surface of biopsy 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 confirmed by the pathologist. Any discrepancy in result was resolved by re-examination of the slides by both laboratory personnel and finally by the pathologist.

Biopsy tissue was placed in sterile Tris-EDTA buffer (pH-8.0) and was sent to 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 minikit (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 ribosomal ITS region of Leishmania “Sense (Forward)” and “Antisense (Reverse)” primers, designed for primary PCR (5’-ACACTCAGGTCTGAAAC-3’ and 5’-CTGGATCATTTTCCGATGATTAC-3’) and nested PCR (5’-ACATAACGTGTCGCGATGGA-3’ and 5’-GAGAGAGAGCCACACACCA-3’) (17). Both primary and nested PCR were carried out in 50μl volume: 3-5μg of DNA (for primary PCR) and 1μl of primary PCR (for nested PCR) product was used for PCR mix containing 10mM Tris-HCl (pH-8.3), 50mM KCl, 1.5mM MgCl2, 0.2mM of each dNTP, 25pM of respective primers.
and 1.25U Taq polymerase enzyme (QIAGEN, GmbH Germany) (17).

The amplification was done by initial denaturation at 94°C for 5 minutes and for 35 cycles consisting of denaturation at 94°C for 1 minute, annealing for 1 minute 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 marker and photographed by gel documentation system (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 RMRIMS Leishmania Repository, were used as the positive control whereas distilled water and DNA from other organisms were the negative control. The source of 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 Mycobacterium Repository Centre of National JALMA Institute for Leprosy & Other Mycobacterial Diseases (NJIL & OMD), Agra, India. Blood samples of malaria patients attending out-patient-clinic of RMRIMS, Patna was 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* and *P. vivax*) demonstrated any PCR band.

Skin biopsies from parasitologically confirmed PKDL (n=6) were PCR positive whereas other diseases (n=14) and normal controls (n=10) were both microscopically and nested PCR negative. The “parasitologically confirmed PKDL” meant that the Leishmania parasites were demonstrated under microscope in the imprint smears of skin biopsies from PKDL patients. Six
randomly selected biopsies from PKDL cases, two each from macular, papular and papulo-nodular lesions, along with positive controls, subjected to nested ITS PCR-RFLP assay, were identified as *L. donovani*.

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

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

In a recent study conducted in Bangladesh, positivity by nested PCR in macular lesion was 93.2% whereas by microscopy it was only 2.7%, with overall positivity of 94.5% and 29.1% respectively (11). Similar studies from India and Sudan, reported PCR positivity 93% and 82.7% whereas by microscopy 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 other studies. We used ribosomal RNA gene of ITS region whereas in Bangladesh, India and Sudan studies, minicircle of kinetoplast DNA (kDNA) and 18S ribosomal RNA gene was employed for PCR (11, 12, 15).

The number of parasites in macular lesions is scanty in comparison to papular and papulo-nodular lesions, so the chances of detection by microscopy are very less in macular lesions even by
the trained laboratory personnel. Since PCR amplifies multiple copies of a gene of the parasite in
thermal cycler, possibility of missing parasite is very rare. Hence, PCR is clearly superior to
microscopy for the macular lesions but 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 density, nested PCR was almost twice sensitive than imprint smear microscopy. Hence,
nested PCR is suggestive for early diagnosis of PKDL, particularly the macular forms, with
management of the cases that may help in interrupting transmission of kala-azar infection.

ACKNOWLEDGMENTS

There are no conflicts of interest reported by any of the authors. We thank Mr. Manohar Kumar
(Laboratory Technician), Mrs. Rakhi Kumari (Technical Assistant), Sri Naresh Kumar Sinha
(Technical Assistant) and Devendra Prasad (Laboratory Assistant) for the help rendered by them in
the technical work.

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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.
<table>
<thead>
<tr>
<th>Types of PKDL Skin Lesions</th>
<th>N</th>
<th>PCR +Ve (%)</th>
<th>Imprint smear microscopy +Ve (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macular</td>
<td>24</td>
<td>21 (87.5%)</td>
<td>10 (41.6%)</td>
</tr>
<tr>
<td>Papular</td>
<td>17</td>
<td>16 (94.1%)</td>
<td>15 (88.2%)</td>
</tr>
<tr>
<td>Papulo-nodular</td>
<td>21</td>
<td>20 (95.2%)</td>
<td>19 (90.4%)</td>
</tr>
<tr>
<td>Total</td>
<td>62</td>
<td>57 (91.9%)</td>
<td>44 (70.9%)</td>
</tr>
</tbody>
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