Detection of \textit{M.leprae} by reverse transcription- PCR in biopsy specimens from leprosy cases: A preliminary study

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**ABSTRACT**

A reverse transcription (RT)-PCR assay targeting 16S rRNA of \textit{Mycobacterium leprae} has been used to detect \textit{M.leprae} specific nucleic acids. This study has been initiated to gain experience about detection of RNA from seven biopsy specimens by RT-PCR assay using species- specific primers described earlier. These biopsy specimens were from clinically confirmed and untreated leprosy cases belonging to BB and BL types. The earlier reported method was established in our laboratory. 171 bp fragment by RT-PCR was amplified from 4/7 cases. The positives results by RT-PCR were from the biopsies from fresh or short term treated cases whereas negative results were from specimens from long term treated cases showing clinical features of relapse. DNA targeting PCR (36 KDa) showed positivity in both groups. These results suggest that RT-PCR positivity possibly reflect the presence of viable organisms. Thus as earlier predicted RT-PCR assay may be useful for viability determinations for assessing the response to chemotherapy as well as presence of persisters in relapse cases.

**Key Words:** \textit{M.leprae}, reverse-transcription PCR, 16S rRNA, viability.

**INTRODUCTION**

Leprosy is a chronic granulomatous disease with a long incubation period (between 9 months to 20 years) after infection and can affect all age groups. The signs or symptoms vary depending upon the degree of immunological status of patient\(^1\).

Although the prevalence of leprosy has been reduced dramatically by the WHO multi-drug therapy regimens, yet the new case detection rates are declining slowly and continue to be high in several areas. Some patients who had been released from treatment (RFT) also relapse\(^2,5\) and it becomes difficult to
distinguish them from the late reaction. Knowledge about persister drug sensitive *M. leprae* or the multi-drug resistant *M. leprae* is very limited. The “resisters” escape or nullify the effect of drugs, whereas the “persisters” are sensitive to the drugs, but they reduce their metabolism to the minimum and are not acted upon the drugs thus living in a dormant stage. Significant number of patients with leprosy have nerve damage and disabilities at the time of diagnosis. Although the sub-clinical infection is quite common, the studies on epidemiology of leprosy are incomplete due to lack of reliable methods for the identification of potential reservoirs, which may be contributing to the spread of the disease.

For patients which are difficult to diagnose clinically, skin or nerve biopsy specimens are obtained and diagnosis is made on the basis of characteristic histological findings and the presence of AFB within the biopsy specimen. Because acid fast staining requires at least 10^4 organisms per grams of tissue for reliable detection, sensitivity is low, particularly for patients at the tuberculoid end of the leprosy spectrum where AFB are rare or absent. Development of molecular tools-probes and gene amplification methods has been considered to be promising for the purpose. A number of investigators have used PCR to amplify various genomic sequences of *M. leprae* in order to improve the sensitivity of diagnosis when low number of bacteria is present. It is known that DNA fragments may be present for a long time after bacterial death, hence RNA (rRNA or mRNA) detection and or estimation by RNA targeting probe and gene amplification methods would be more suitable for this purpose.

This study has been initiated to gain experience about detection of RNA from clinical specimens by RT-PCR. In this preliminary study a reverse-transcription PCR targeting the 16S rRNA gene of *M. leprae*, an earlier described technique to detect viable *M. leprae* in the tissue biopsy specimens has been restandardized and established.

**MATERIAL AND METHODS**

Biopsy specimens from clinically confirmed and untreated cases (untreated or <3 months treatment) as well as treated and relapse patients attending the Medical Unit I of OPD of National JALMA Institute for Leprosy and Other Mycobacterial Diseases (Agra) were included in the study. Number of cases were seven which belonged to BB and BL types (Table-1).

**HOMOGENIZATION**

Biopsies were properly minced to a uniform consistency with sterile scissors and homogenized in Trizol solution (Invitrogen, USA) thoroughly in the pestle mortar.

Smear Preparation for Acid Fast Bacilli: All samples were processed for Ziehl Neelsen staining for AFB confirmation. Bacteriological index (BI) was determined on Ridley scale.

**DNA Isolation:** DNA was isolated from the homogenates by Lysozyme,
Proteinase-K treatment method. 

PCR: Initially the cases were confirmed positive by using diagnostic 36KDa PCR targeting 36KDa gene, which amplifies a fragment of 531 bp.

**RNA Isolation**

Total RNA including rRNA was extracted from skin biopsy homogenates using the protocol described by Trizol reagent (Invitrogen, Carlsbad, Calif, USA). RNA was dissolved in DEPC (Diethyl-pyrocarbonate) treated distilled water. The isolated RNA was treated earlier by DNase using DNA free™ kit (Ambion Inc., USA) for the removal of DNA contamination and stored at -70°C until use.

**RT-PCR**

RT-PCR was done by the procedure described by the manufacturer (Promega Access RT-PCR kit, USA) using the species-specific primers developed by the Arnoldi et al. and Cox et al.

**Primers**

P1 (M. leprae 16S rRNA, position 69 to 91, DNA)-CGG AAA GGT CTC TAA AAA ATC TT

P2 (M. leprae 16S rRNA position 218 to 239, DNA) - CAT CCT GCA CCG CAA AAA GCT T

RT-PCR was performed in a total volume of 20μl which included 1x RT-PCR buffer, 250μM dNTPs, 20μM MgSO4, 2.5 U AMV Reverse Transcriptase, 2.5 U Taq DNA polymerase. Primers were added at a final concentration of 1μM and finally 3μl of template RNA (10-100ng) was added. The cycling profile for the reverse transcription was 1 cycle at 48°C for 45 min followed by denaturation at 94°C for 2 min., annealing at 60°C for 1.30 min., extension at 68°C for 2 min followed by the final extension of 68°C for 7 min. for 40 cycles.

**Agarose gel electrophoresis**

PCR products were electrophoresed through 2% agarose gel at 1.2 V/cm for 3 hrs.

**RESULTS AND DISCUSSION**

Out of the 7 patients, 3 were new cases, 2 were old (were treated earlier) and other two were relapsed cases (Table 1).

1. **Bacterial Index (BI):** BI of patients included in the study ranged from 1+ to 5+ (Table 1).

2. **RT-PCR**

Amplification of a 171 bp fragment was observed (Table 1 and Figure 1). Out of seven patients, biopsies of 4 patients were positive. The 3 patients whose biopsies were negative had history of leprosy treatment completed and were relapse cases. Out of 4 positive samples, 3 were fresh cases and one of them had taken PB-MDT for six months. This finding is likely to correlate with the presence of viable M. leprae. Negative results by RT-PCR in three treated cases of which two turn out to be positive by DNA targeting PCR also shows the potential of this approach for differentiation of live from dead organisms and would be relevant for monitoring the impact of treatment.

RNA targeting systems including probe and gene amplification assays for the detection of M. leprae, have been
method of grading the positivity levels of *M. leprae* specific rRNA in the tissues has been proved useful for monitoring therapeutic responses. Techniques such as RT-PCR targeting 16S rRNA and nucleic acid sequence based amplification (NASBA) targeting 16S developed but their application potential is limited. The molecular techniques for the viability estimation of *M. leprae* are based on a quantitative estimation of RNA levels by direct hybridization with specific probes or by amplification by PCR or isothermal reactions. The method of grading the positivity levels of *M. leprae* specific rRNA in the tissues has been proved useful for monitoring therapeutic responses. Techniques such as RT-PCR targeting 16S rRNA and nucleic acid sequence based amplification (NASBA) targeting 16S
rRNA gene have been reported\textsuperscript{21} to be useful for the determination of viability of \textit{M.leprae}. The 16S rRNA has been targeted in many RT-PCR assays, but mRNA of the 18 KDa protein has also been used as a target sequence\textsuperscript{25}.

Detection of rRNA targeting 16S rRNA gene is likely to be more sensitive than targeting DNA as \textit{M.leprae} has one copy of 16S rRNA gene\textsuperscript{28} but an estimated 4000 molecules of 16S rRNA per cell\textsuperscript{27}.

Detection of RNA rather than DNA is expected to be useful for the detection of viable organisms. The half life of mRNA may be as little as 2 min. following cell death. Several investigators have shown that mRNA detection is a reliable indicator of cell viability. Patel et al\textsuperscript{28} showed that heat treatment of \textit{M.leprae} reversed their ability to detect \textit{M.leprae} by an RT-PCR assay that detected a 71kDa Heat Shock Protein. Bej et al\textsuperscript{29} showed that the viability of \textit{Legionella} was related to the levels of mRNA for the macrophage infectivity potentiator protein. However, the half life of rRNA is less certain. The fact that ribosomes rapidly disappear when mycobacterial cells are degraded\textsuperscript{30} suggests that the rRNA found within these structures might also be degraded shortly after cell demise. Vliet et al\textsuperscript{31} demonstrated a strong co-relation between the isothermic RNA amplification product of the 16S rRNA of \textit{M.smegmatis} and the number of colony forming units (CFU) after treatment of cultures with various doses of rifampicin and ofloxacin. In contrast DNA amplification of genomic DNA encoding the 16S rRNA gene did not co-relate with viability assessed by in-vitro cultures.

RT-PCR assay may be useful in a number of clinical situations. In addition, the method helps in the diagnosis of difficult case of paucibacillary leprosy and facilitates the epidemiological studies. This assay can also be useful for determining the persisters i.e. those viable organisms present at the completion of the 2 or more years of multi-drug therapy\textsuperscript{32,33} and therefore may be potential source of relapse, especially in multibacillary leprosy\textsuperscript{34}.

Our earlier used systems like mouse foot pad and ATP bioluminescence assays could be used as complementary methods in assessing the efficacy of chemotherapy in patients. RT-PCR needs to be compared with these techniques and has greater potential application because of its sensitivity and rapidity.

**ACKNOWLEDGEMENT**

This work was supported by grant from ICMR (Genomics project, Project No. 63/132/2001-BMS) India and CSIR-UGC fellowship. Suggestions from Dr. Deepti Parashar and Ms. Aradhana Chauhan are gratefully acknowledged.

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