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Detection of viable *Mycobacterium leprae* in soil samples: Insights into possible sources of transmission of leprosy

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1. Introduction

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae*, and it primarily affects the skin and nerves. The disease has been described to be present since ages. Due to the resulting deformities caused by the infection, it has been a dreaded disease and had a social stigma attached to it till recently. With the advent and usage of multi drug therapy (MDT), it is totally curable and is now associated with low frequency of deformities if treated early. However, several aspects of the disease are still unknown, and the organism cannot be grown in any acceptable culture medium and besides the wild armadillos, there is no known animal reservoir for leprosy. The transmission of leprosy is believed to be due to a large extent by droplet discharge of bacilli through nose and mouth and to a lesser extent by direct contact susceptible host with a patient for long duration. The exact role of the environment in the transmission dynamics is still speculative. In the present study, we have tried to detect viable *M. leprae* from soil samples in endemic areas by using molecular methods. Eighty soil samples were collected from villages of this area, DNA and RNA of *M. leprae* extracted and identified using specific *M. leprae* primers. PCR amplification was done and real-time RT-PCR was used to detect viable *M. leprae*. DNA targeting the 16S region of *M. leprae* was detected in 37.5%, whereas *M. leprae* RNA targeting the same region was detected in 35% of these samples. Of the total 80 samples, 40 were collected from residential areas of leprosy patients whereas 40 samples were from no-patient areas. Fifty-five percent positivity for 16S rRNA of *M. leprae* was observed from the "patient" area in comparison to 15% positivity from the "no-patient" area (*p* < 0.001). This study thus provides valuable information of presence of viable *M. leprae* in soil specimens, which would be of use in investigating the transmission dynamics in leprosy.

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described by Miskin et al. (1999) with slight modification. The concentration of lysozyme used for cell lysis was increased from 5 mg to 10 mg/ml to break the cell wall of M. leprae and potassium acetate was used for the removal of humic acid (which interfere with the PCR amplification) from the soil specimens. The other major difficulty encountered is that the processes is lengthy and requires expensive equipments (such as Hybaid Ribolyzer) or commercial kits. Also, some methods do not allow isolation of RNA of sufficient purity for RT-PCR and may require long treatment to purify the RNA (Miskin et al., 1999). We have optimized a procedure that allows rapid and easy extraction of M. leprae RNA from soil samples. The RNA extracted with the modified protocol was amplified by RT-PCR and real-time RT-PCR.

2. Materials and methods

2.1. Collection of samples

Ghatampur, situated 40 km from Kanpur has been known to be endemic for leprosy. During a house to house survey of the area it was observed that certain villages had a high prevalence of the disease whereas there were areas where no patients of leprosy were observed. Also, in the same village (same environmental conditions) there were areas where many patients were clustered whereas in other areas there were no patients. The area was stratified and patients with more than 3 patients in the street were labeled as “patient area”. Streets with no patients residing along it were labeled “no-patient” areas. Forty soil samples were collected from around the houses of “patient area” (within 5 m) and the same a number of samples were collected from the vicinity of houses from the “no-patient” area of the same village. Soil was dug (4’ deep) and collected in clean plastic containers (10 g each) with a help of “khupri” and labeled bearing the village name. The person who collected the soil entered the soil collected in a register with the names of the patients residing in the vicinity in his record. The collected samples were transported to the Institute at room temperature (within 2 days) for the study and thereafter stored at 4–8 °C till further processing. The scientist and working processes the samples were not aware if these samples were from a “patient” area or “no-patient” area till the compilation of results and their analysis. All the isolations as well as estimations for RNA and DNA were done twice for confirmation.

2.2. DNA extraction

These soil samples were processed for DNA extraction using method described earlier (Lavania et al., 2006). 100 mg of dried soil was weighed in a 1.5 ml micro-centrifuge tube and 500 µl of 0.125 M EDTA pH 8.0 was added to it. Soil samples were lysed in 100 µl of lysis buffer (Proteinase K and 0.05% Tween 20) and incubated at 60 °C overnight in a water bath. The reaction was terminated by inactivating Proteinase K enzyme at 95 °C for 10 min. Thirty microlitres of 10% SDS was added and incubated at 100 °C for 10 min,三十 microlitres of 10% SDS was added and incubated at 100 °C for 10 min, supernatant was collected and DNA was precipitated by adding 0.6 volume of isopropanol and then washing with 70% ethanol; pH 8.0). This was agitated for 20 s. Homogenate was transferred to new vials containing 500 µl of 10% (w/v) SDS, vortexed and incubated at 80 °C for 30 min with vigorous shaking every 10 min. Repeat centrifugation was done at 2800 × g for 15 min at 4 °C. Supernatant was decanted and pellet was re-extracted with 2.5 ml extraction buffer and again centrifuged. Supernatant was collected and double volume of PEG 6000 (Merck, USA) was added and kept for 2 h at room temperature for the precipitation of RNA. Pellet was re-suspended in 1 ml DEPC-treated water.

Hundred microlitres of 7.5 M potassium acetate was added to make a final concentration of 0.5 M. This was done for the precipitation of humic acids present in the sample (which interfere with the PCR) and centrifuged at 8000 × g for 5 min. RNA was again precipitated with double volume of chilled ethanol and kept at –20 °C overnight. RNA was recovered by centrifugation at 8000 × g for 15 min and dissolved in 20 µl of DEPC-treated water. RNA preparations were treated with DNasel (Ambion, USA) for removing any traces of contaminating DNA present in the soil samples. This was stored at –70 °C until use.

2.4. Reverse transcription-PCR of M. leprae 16S rRNA gene

cDNA was synthesized from 2 to 4 µl of purified RNA (50 ng) using the AMV Reverse Transcriptase (Bangalore Genei, India). Control reactions were also performed simultaneously with each experiment M. leprae 16S rRNA gene region was amplified using the primers 5’-TCGACGGAAGGCTCTCATAAAAATC-3’ (forward) and 5’-CCTGACCGCAGAATCCTCC-3’ (reverse), with 2–4 µl of cDNA as template (Jadhav et al., 2005). The total volume (50 µl) of PCR amplification mixture contained 10 mM Tris–HCl (pH 9.0 at room temperature), 60 mM KCl, 1.5 mM MgCl2, 200 µM dNTPs, 50 ng of each primer P(Forward) and P(Reverse), 1.5 units of Taq DNA polymerase and 10 µl of sample (template). Thirty-seven PCR amplification cycles were carried out in Gene Amp9 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA). Denaturation was done at 94 °C for 2 min, annealing was performed at 60 °C for 2 min followed by extension at 72 °C for 3 min. This was followed by final extension at 72 °C for 10 min. Reaction was maintained at 4 °C till used for gel electrophoresis. PCR products were run on 1.5% (w/v) agarose gel (Bangalore Genei, India), stained with ethidium bromide and scanned in Gel Documentation System (Bio Rad Laboratories, USA).

2.5. Real-time RT-PCR of 16S rRNA gene

M. leprae RNA in soil samples was also detected and quantified by amplification of 16S rRNA by real-time reverse transcription PCR using RNA amplification SYBR Green I kit (Roche Diagnostics, Germany), according to the standard protocols of the manufacturer. Briefly, master mix containing SYBR Green I reaction mix + RT-PCR enzyme mix + MgCl2 + 0.5 µM primers (described by Jadhav et al., 2005) was prepared, and poured into PCR tubes. Two tubes were run for each sample. Four microlitres of RNA template was added to each tube. Total reaction volume (20 µl) was transferred to Light Cycler capillaries, soft spun and loaded
to Light Cycler sample carousel. Positive control used in the experiment was plasmid DNA having *M. leprae* 16S rRNA gene to check for positive amplification. Negative control with no nucleic acid in the tube was run along with the reactions to check for contamination.

Amplifications of these samples were further quantified by real-time RT-PCR using the fluorescence dye SYBR Green I, targeting the same region of 16S rRNA. Standard curve was plotted by crossing over point against the known log serial dilution (3.7 × 10⁶ to 3.7) copy number of template (amplification efficiency 95.2%). The load of viable bacilli was calculated in the samples by importing the standard curve and measuring the load by relative quantification method. Relative quantification was performed to compute the mRNA copies/specimen by using standard curve.

3. Results

In the 80 soil samples collected, DNA of *M. leprae* using the described protocol was extracted and found to be positive in 30 samples (37.5%). However, *M. leprae* RNA was amplified in 28/80 (35%) of these soil samples targeting the same gene region (Table 1).

Data was further classified village wise from a “patient” area or a “no-patient” area and tabulated accordingly. It was observed that of the 40 samples collected from residential area of leprosy patients, 22/40 (55%) showed positive amplification by both RT-PCR and real-time RT-PCR. However, in the “no-patient” area only 6/40 (15%) samples were found positive (Table 1 and Figs. 1 and 2). Remaining fifty-two soil specimens from both “patient” area and “no-patient” area were found negative. This difference in “patient” area and “no-patient” area was statistically significant with $X^2 = 14.07, p < 0.001$.

In the present study no RNA was detected from 18 of the 40 samples from the “patient area” showing the absence of live *M. leprae* using these methods.

Relative quantification for the load of the viable bacilli was done by importing the standard curve. Only 6 out 40 (15%) samples form “no-patient area” were positive (mean copy number $1.8 ± 0.035 × 10^4 /100$ mg soil) and 22 out 40 (55%) samples from “patients area” were positive (mean copy number $4.3 ± 0.027 × 10^4 /100$ mg soil) by real-time RT-PCR (Table 2). Mean copy number in both patient and no-patient area were compared using student’s t-test and it was found that the mean copy numbers were statistically significantly higher in samples collected from “patient area” in comparison of “no-patient area” ($p < 0.00001$).

### Table 1

<table>
<thead>
<tr>
<th>Source</th>
<th>PCR positive (<em>M. leprae</em> DNA)</th>
<th>Reverse transcription-PCR positive</th>
<th>Total samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>“Patient” area</td>
<td>24</td>
<td>22 (55%)</td>
<td>40</td>
</tr>
<tr>
<td>“No-patient” area</td>
<td>6</td>
<td>6 (15%)</td>
<td>40</td>
</tr>
<tr>
<td>Total</td>
<td>30 (37.5%)</td>
<td>28 (35%)</td>
<td>80</td>
</tr>
</tbody>
</table>

Difference in the positive results from “patient” and “no-patient” area: $X^2 = 14.07, p < 0.001$.

* Two more samples from this group showed the presence of *M. leprae* DNA in the soil.

**Fig. 1.** Electrophoresis of RT-PCR performed using primers targeted for *M. leprae* specific region in 16S rRNA region from the RNA extracted from soil samples. Lanes 1–4: Soil samples from endemic area, Lane 5: Positive control (*M. leprae* DNA).

**Fig. 2.** Amplification of *M. leprae* RNA from soil samples by real-time RT-PCR.

4. Discussion

We used an extraction procedure not requiring any expensive reagent (such as Trizol/Tri reagent) to obtain \textit{M. lepraes} RNA from the soil samples. In this method we increased the concentration of lysozyme from 5 mg to 10 mg to break the cell wall of \textit{M. lepraes}. Precipitation of RNA was increased from 1 h to overnight for obtaining the maximum yield. DNA contamination was taken care of by treating RNA preparations with DNase 1 enzyme to completely degrade DNA which might interfere with the results. Hurt et al. (2001) extracted mRNA from soil sediments by using liquid nitrogen and Qagen resin column. They detected the RNA by reverse transcriptase PCR. Somerville et al. (1989) used single cylindrical filter membrane (type SVGS01015; Millipore Corp., Bedford, MA) cell lysis, and then proteolysis for nucleic acid extraction. All the above described protocols are expensive.

Degradation of the RNA by RNases was prevented by treating and washing all glass ware by DEPC. Humic acid present in different proportions in the soil may interfere with the PCR results and the humic acid was precipitated by the addition of potassium acetate.

As \textit{M. lepraes} is not cultivable in any known artificial media and the non-availability of suitable animal models for experimental studies, the use of molecular tools to demonstrate its presence have been used in the present study. Demonstration of \textit{M. lepraes} from clinical specimens has been reported earlier by Sharma et al. (1996) and Donoghue et al. (2001) using DNA amplification methods. In our study using PCR targeting 16S rRNA gene region \textit{M. lepraes} DNA could be demonstrated in 30 of the 80 (37.5%) of the soil samples collected from villages of Ghataumpur, India. Matsuoka et al. (1999) demonstrated the presence of \textit{M. lepraes} DNA in 47% of water samples in highly endemic villages in Indonesia. This thus shows that the technique can be employed for detection of \textit{M. lepraes} from the soil samples. However, an important limitation of using DNA amplification methods is its inability to distinguish between viable and dead organisms.

Detection of viable \textit{M. lepraes} in the environment is possible using molecular methods. Miskin et al. (1999) extracted the nucleic acid from aquatic environments and amplified the bacterial 16S rRNA gene region. 16S rRNA gene region as target has been successfully used in several studies for assessing the viability of mycobacteria such as \textit{M. tuberculosis}, \textit{M. lepraes}, etc. (Hellyer et al., 1999; Kurabachew et al., 1998; Phetsukiri et al., 2006) from clinical specimens. In the present study using reverse transcription PCR targeting 16S rRNA gene region viable \textit{M. lepraes} could be demonstrated in 28 of the 80 (35%) of the soil samples collected from villages of endemic area in Ghataumpur, India. These studies therefore show that \textit{M. lepraes} is present in the soil and this should be investigated as a potential source of continued transmission of the disease.

The earlier studies by Desikan (1977) using the mouse foot pad technique demonstrated that \textit{M. lepraes} could remain viable in the soil up to 45 days. Furthermore, Jadin (1975) have also reported that Acanthamoeba spp may be playing a role as an environmental reservoir for the \textit{M. lepraes}. Pathogenic mycobacteria including \textit{M. tuberculosis} and \textit{M. lepraes} can survive in the environment but these aspects have not received adequate attention as focus has been mainly on patients.

This study also shows that there is a statistically significant difference in the positivity rates and amount of viable bacilli detected in the soil from near the patient’s residences as compared to other areas of the village which were relatively away from patient areas. It is well known that \textit{M. lepraes} are shed by the patients in the environment during coughing, sneezing and can survive for varying periods depending on the environmental conditions like sunlight, temperature, moisture, etc. Detection of \textit{M. lepraes} DNA by molecular methods is considered as a potential tool for rapid detection and measurement of the viability of \textit{M. lepraes} (Kurabachew et al., 1998). The recovery of RNA sequences from an RNA template using RT-PCR implies that the source organisms were live at the time of soil sample collection and these live bacilli may be playing a role in the continued transmission of the disease. Our preliminary data shows that both the molecular techniques RT-PCR and real-time PCR can be used successfully for detection of viable \textit{M. lepraes} in environment and can be used as a tool for studying the transmission dynamics of the disease. More studies are required to establish the extent of role of survival of \textit{M. lepraes} in environment or different reservoirs in the transmission dynamics in leprosy.

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