Presence of viable *Mycobacterium leprae* in environmental specimens around houses of leprosy patients


**Abstract**

**Purpose:** Leprosy is a chronic systemic infectious disease caused by *Mycobacterium leprae*, one of the first organisms to be established as the cause for disease in humans. Because of high prevalence pockets of leprosy in the endemic regions, it is necessary to identify the possible sources of *M. leprae* in the environment and its mode of transmission.

**Materials and Methods:** Slit skin smears (SSSs) from lesions were collected in 70% ethanol from 50 leprosy cases staying in the leprosy resettlement village and hospital from a high endemic area. One hundred and sixty soil samples were collected from different areas around the leprosy hospital and from the resettlement village of cured leprosy patients where active cases also resided at the time of sample collection. *M. leprae* specific gene region (RLEP 129 bp) and 16S rRNA targets were used for polymerase chain reaction (PCR) based detection for the presence and viability of *M. leprae*. An rpoT region was also amplified to determine presence of numbers of 6 bp tandem repeats.

**Results:** All the SSS samples collected from patients showed three copies of rpoT region (6 bp tandem repeat, an ancient Indian type). Fifty-two soil samples showed presence of *M. leprae* DNA whereas *M. leprae* specific 16S rRNA gene was amplified in sixteen of these samples. PCR amplification and fragment length analysis showed 91 bp, i.e., three copies of the rpoT 6 bp tandem repeats from soil samples and similar three copies observed in patient samples.

**Conclusion:** Presence of viable *M. leprae* in the soil having same rpoT genotype of *M. leprae* noted in patients suggests that it could be the same strain of *M. leprae*. *M. leprae* found in the soil could be the one that is excreted out by the patient. Significance of its viability in the environment and its pathogenicity with respect to transmission needs to be further explored. Findings of this study might provide possible insights for further exploration into understanding transmission patterns in leprosy and also will throw light on identifying potential for existence of extra human source or reservoirs of *M. leprae*, if any.

**Key words:** Environment, rpoT genotyping, transmission, viable *Mycobacterium leprae*

**Introduction**

In 1874, Armauer Hansen identified leprosy as an infectious disease caused by *Mycobacterium leprae*. India is home to 60% of the leprosy patients worldwide, a situation that has remained unchanged despite massive decline in global prevalence of the disease since the inception of effective multi-drug therapy (MDT) in 1985. According to official reports received during 2012 from 105 countries and territories, the globally registered prevalence of leprosy at the beginning of 2012 stood at 181,941 cases whereas the number of new cases detected during 2011 was 219,075. However, some pockets of endemicity do remain in certain areas of Angola, Brazil, Bangladesh, China, Democratic Republic of Congo, India, Madagascar, Mozambique, and Nepal. In India, the year 2011–12 started with a total of 0.83 lakh leprosy cases on hand as on 1st April 2011, with prevalence rate 0.68/10,000 showing 33 States/Union Territories attaining the level of leprosy elimination. A total of 528 districts (81.4%) out of total 649 districts also achieved elimination by March 2014. The remaining pockets of endemicity where elimination figure has not been yet attained are localized to states of Bihar, Odisha, Chhattisgarh, Uttar Pradesh, West Bengal, and Jharkhand.
The global use of MDT seems to have had only minimal, if any, effect on transmission of the disease, and an adequate explanation for this situation is lacking. Some of the possible reasons could be that MDT does not kill all *M. leprae* in all patients, as evidenced by significant relapse rates associated with both 24 months and 12 months multibacillary MDT regimens, especially when patients have been followed up for extended periods. There is cessation of active case finding by most countries which will mean that untreated and unreported cases in the community can continue transmitting disease. Also, a single LL patient can transmit infection to many people before symptoms become sufficiently severe to induce healthcare seeking. Long incubation period of the disease is another problem in evaluating transmission of the disease. Emergence of drug resistance in *M. leprae* has been reported but its implications in transmission is not yet well documented. The exact mechanism of transmission of leprosy is still not known, it is believed that transmission occurs due to droplet infection carried through the discharge of bacilli as droplet from the nose and mouth and by direct contact from an infected person to a susceptible individual. Limited multiplication and growth of *M. leprae* in the mouse foot pad has proved it to be a useful tool for assessing the viability of the organism and testing the drug susceptibility of clinical isolates. However, recently developed advanced molecular biological techniques proved the existence of *M. leprae* in the environment and further pointed out to their role in continuing transmission of disease. The important factor which may be attributed to the transmission of the disease is the viability of *M. leprae* outside human body. Soil samples obtained from some endemic regions of Northern India provided the proof of existence of viable *M. leprae* in areas of high prevalence of the disease. Presence of *M. leprae* DNA has also been reported in water samples in Indonesia. There is coincidental observation, as is seen in developed countries that improved hygiene, better sanitation, covered/underground drainage system, improved mechanized farming practices, treated, piped water supply, etc., could have contributed to disappearance of leprosy. Hence, organism shed by the patient in the environment, if remains viable then it could possibly have implications in transmission of the disease. Current study has been focused on the detection of viable *M. leprae* from the environmental samples and on genotyping of *M. leprae* specific rpoT gene target to identify similarities if any, in the number of copies of this gene between *M. leprae* obtained from patient and the environment. This study in turn might throw light on the possible transmission link of *M. leprae* from the patient to the environment and vice versa.

**Materials and Methods**

**Ethical approval**

Informed consent was obtained from all the patients and the study was approved by the Organization Ethical Committee of The Leprosy Mission Trust, India.

**Collection of sample**

Samples were collected from patients and from different places of environment in endemic region of Purulia District, West Bengal. Slit skin smears (SSSs) were collected from the leprosy cases in 70% ethanol in 1.5 ml tube. Ground soil was dug 4 inch deep and soil was collected in clean plastic containers (10 g each) with the help of “hand trowel” and labeled with site code and the village name. The collected samples were transported to the laboratory at room temperature (within 2 days) and stored at 4–8°C till further processing. One hundred and sixty soil samples were collected from different places such as hospital campus and resettlement village of the leprosy patients. Specific locations for the collection of samples were the bathing place (back side of the house), common sitting place, near the entrance of the house; areas around house used for washing and place near the bore well.

**DNA extraction from slit skin smears samples**

SSS from 50 bacteriological index positive cases were collected in 1 ml of 70% ethanol and were centrifuged at 10,000 rpm (8000 ×g) for 10 min. Supernatants were discarded and pellets were air dried for the removal of ethanol. After the removal of ethanol, samples were lysed overnight in lysis buffer (100 mM Tris buffer pH 8.5 with 1 mg/ml proteinase K and 0.05% Tween 20) at 60°C followed by deactivation of proteinase K at 95°C for 15 min. This lysate preparation was further used for polymerase chain reaction (PCR).

**DNA extraction from soil samples**

The soil samples were processed for DNA extraction using the standard protocol described earlier. Briefly, in ethanol, 100 mg of soil and zirconium beads (0.1 mm) were added in 1.5 ml microcentrifuge tube and homogenized using bead beater at 4800 rpm to ensure and facilitate lysis of the cells. The lysate was then centrifuged at 10000 rpm for 10 min at 4°C and ethanol was discarded. To the pellet, 250 μl of lysis buffer was added and incubated at 60°C overnight in a water bath and followed by inactivation of protease K at 97°C for 15 min. Thirty micro liters of 10% sodium dodecyl sulfate (SDS) was added to the tube and incubated at 60°C for 1 h in a water bath. Subsequently, 500 μl of TENP buffer (pH 9.0) was added and the tube was incubated for 1 h at room temperature with vortexing regularly at 5 min intervals. This was followed by centrifugation at 3000 rpm for 10 min. DNA was precipitated by adding 70% ethanol followed by overnight incubation at −20°C. Subsequently the tube was centrifuged at 10,000 rpm for 15 min and supernatant was discarded. The pellet was air dried for about 15–20 min to ensure complete removal of ethanol. Pellet was resuspended in 50 μl of 10 mM TE buffer (pH 8.0) and incubated at 37°C in a water bath for 1 h to
ensure that DNA goes in to solution. The DNA solution was then passed through the inhibitor removal resin columns (Epicenter, USA SR04350) and stored at −20°C until further use.

**RNA extraction**

Standard method was used for RNA extraction from soil samples which was used earlier.[9] Briefly, the precautions were taken to prevent degradation of RNA by RNase during the purification steps which included pretreatment of all glasswares with diethyl pyrocarbonate (DEPC) and baking them overnight at room temperature. All buffers were prepared in DEPC-treated water prior to autoclaving. 0.1 g of soil was weighed in 1.5 ml micro-centrifuge tube. Five hundred microliters ethanol was added to the tube along with 0.1 mm glass or zirconium beads and homogenized using bead beater for six pulses of 1 min at 4800 rpm with 30 s break time on ice to ensure and facilitate lysis of the cells. The lysate was then centrifuged at 10,000 rpm for 10 min at 4°C and ethanol was discarded. To the pellet, 250 μl of lysis buffer (100 mM Tris buffer pH 8.5 with 1 mg/ml proteinase K and 0.05% Tween 20) was added and incubated at 60°C overnight in a water bath. Reaction was terminated by inactivating proteinase K at 97°C for 15 min. Thirty microliters of 10% SDS was added to the tube and incubated at 60°C for 1 h in a water bath. Subsequently, 500 μl of TENP buffer (pH 9.0) was added, and the tube was incubated for 1 h at room temperature with overtaxing regularly at 5 min intervals. Supernatant was used for the acid phenol extraction to eliminate DNA in the organic phase. The acid phenol was equilibrated with 2 M acetate buffer prior to use and NaCl was added to the samples to make up the final concentration of 0.5 M. The aqueous phase after the extraction was re-extracted with chloroform to remove traces of phenol and then passed through the sepharose 4B columns. It was then treated with RNase free DNase I (Ambion, USA) to ensure complete removal of impurities of DNA followed by isopropanol precipitation by overnight incubation and RNA was pelleted by centrifugation at 12,000 rpm for 10 min at 4°C. Supernatant was carefully decanted, and the pellet was washed with 70% ethanol and then the pellet was air dried. The pellet was finally suspended in 50 μl of TE buffer.

**Polymerase chain reaction of Mycobacterium leprae by using RLEP region**

PCR amplification of RLEP region was carried out in a total 25 μl of reaction volume that contained 2 μl of template DNA, primers at final concentration of 0.5 μM (forward and reverse) and 1X Genei Mix (Merck India). We used *M. leprae* specific RLEP primers sequences PS1 5’-TGC ATG TCA TGG CCT TGA GG -3’ and PS 2 5’-CAC CGA TAC CAG CGG CAG AA-3’. Amplification consisted of first stage of single cycle of denaturation at 95°C for 5 min followed by second stage of 35–45 cycles of 30 s at 94°C, 30 s at 58°C, and 1 min at 72°C and final stage of single cycle of 10 min at 72°C. PCR reaction without DNA was used as negative control (reagent control). Purified *M. leprae* DNA was used as positive control. PCR product containing amplified fragment of the target region was electrophoresed in a 2% agarose (Sigma) gel using tris-borate-ethylenediaminetetraacetic acid (EDTA) buffer at 100 volts constant voltage.

**Reverse transcriptase-polymerase chain reaction of Mycobacterium leprae using 16S rRNA gene target**

As 16S rRNA RNA reverse transcriptase-PCR (RT-PCR) has already provided evidence for viability of *M. leprae*,[12] RT-PCR was carried out by using One Step RT-PCR Kit (Qiagen - 210212). Control reactions to test DNA contamination were also performed simultaneously with each experiment by carrying out PCR without prior reverse transcription. 16S RNA gene region was amplified using *M. leprae* specific primers P2 and P3 as described earlier.[13] The total volume (50 μl) of PCR amplification mixture contained 10 μl of 5X RT-PCR buffer, 2 μl of dNTPs, 10 μl - 5X Q Solution, 50 ng of each primer, 2 μl of RT-PCR enzyme, 0.25 μl of RNase inhibitor, and remaining RNAse free water and 10 μl of sample (template). The cycling profile for the amplification reaction was in two stages. In the first stage, reverse transcription was carried out at 50°C for 30 min followed by inactivation step at 95°C for 15 min. In the second stage, amplification was carried out using 95°C for 5 min for initial denaturation followed by 37 cycles, each cycle consisting of denaturation at 94°C for 2 min, annealing at 60°C for 2 min and extension at 72°C for 3 min followed by a final extension at 72°C for 10 min. The amplification products were electrophoresed in a 2% agarose (Sigma) gel at 100 volts constant voltage using Tris-Borate-EDTA buffer containing ethidium bromide dye at final concentration of 0.5 μg/ml and the gels were observed on a Gel Documentation System (Alphalmager).

**rpoT genotyping by polymerase chain reaction**

PCR amplification was carried out as per the protocol described earlier.[14] A total 25 μl volume of reaction mix containing 4 μl of template DNA, primers at final concentration of 0.5 μM (forward and reverse) and Genei™ PCR Master Mix (2x) was used. Primer A (VIC-5’-ATG CCGAACC CGACCT CGGAGT GA-3’) and B (5’-TCGCT TCTCGGAGG TCGAGA-3’) (GenBank Accession No. AB019194) which were used for amplification span the 91 bp (containing three repeats) or 97 bp (containing four repeats) fragments of the rpoT gene target. PCR reaction without DNA was used as negative control. Purified *M. leprae* DNA (Zen-4 1mg) was used as a standard reference strain procured from NIH Japan (Dr. Masanori Matsuoka). The
amplification was carried out in a thermal cycler (Corbett JH BIO) under the conditions of 95°C for 5 min for initial denaturation followed by 40 cycles, each cycle consisting of denaturation at 95°C for 30 sec, annealing at 60°C for 2 min and extension at 72°C for 3 min followed by a final extension at 72°C for 10 min. For identifying the differences in the repetitive region of the rpoT gene, 15 μl of each PCR product containing amplified fragment of the target region was electrophoresed along with DNA ladder of 100 base pair in a 4% low melting point agarose gel (Sigma) using Tris borate EDTA buffer at 100 volts constant voltage.

Fragment length analysis

For confirmation of the number of 6 bp tandem repeats in rpoT, the PCR products were sent for fragment length analysis (FLA) to a commercial lab (Xplorigen, New Delhi, India).

Results

Polymerase chain reaction amplification and reverse transcriptase-polymerase chain reaction

PCR based amplification and FLA of 50 SSS samples to determine the number of repeats of the rpoT gene was accomplished. Samples from the leprosy resettlement village area as well as from the hospital area showed the presence of three copies of rpoT gene. Out of the 160 soil samples collected from different areas, 52 samples (32.5%) were PCR positive for RLEP region [Figure 1]. The positive samples were primarily from the inhabitant areas of the active leprosy patients such as the bathing place, areas close to the house of the leprosy patient, place where the patient sits in the leisure time, lanes between the houses courtyard and hospital area. From the 52 positive soil samples that showed presence of M. leprae DNA, we could detect M. leprae 16S rRNA from 16 samples (10%) [Table 1]. Twelve samples (54.5%) out of 22 M. leprae positive samples collected from the areas near the bathing places, one sample (7%) out of 14 M. leprae positive samples collected at the entrance of the houses of the patients, one sample (8%) out of 12 M. leprae positive samples collected from sitting places of the patient area and two samples (50%) out of 4 M. leprae positive samples collected from hospital area around the wards of the leprosy patients showed presence of viable M. leprae by 16S rRNA based amplification. The % PCR positivity for 16S rRNA was significantly higher (P < 0.005) from around the bathing places of the leprosy patients than that of the areas where the patients sit and from the entrance of the houses.

Genotyping of Mycobacterium leprae from slit skin smear and soil samples

M. leprae DNA positive PCR product run on agarose gel showed same mobility and were of the size 91 bp compared with 50 base pair DNA ladder [Figure 2]. Further confirmation of PCR product was carried out with labeled primers and the amplicons were sent for FLA for the size determination. The data obtained from 50 SSS and fifteen soil samples showed that isolates contain 91 bp, i.e., three copies of 6 bp tandem repeats of the rpoT gene [Figure 3]. Out of these 15 soil samples, 11 samples were from bathing place, two from sitting place, one from entrance of the house, and one from the hospital campus. Out of these 15 samples, 14 were also positive for 16S rRNA.

Discussion

In spite of leprosy being an ancient disease of mankind, the transmission of leprosy is very poorly understood. The success of MDT is confined only to treatment and cure of the disease. To realize the dream of leprosy eradication, a strategy effective in prevention of the disease is also required along with MDT. This can be achieved only by better understanding the modes of transmission and potential sources or reservoir of the pathogen in environment, so that proper intervention strategies can be used to break the transmission chain. In order to understand

![Figure 1: Detection of Mycobacterium leprae from environmental samples targeting RLEP region. Polymerase chain reaction amplification of RLEP region of Mycobacterium leprae obtained from environmental samples from Purulia, polymerase chain reaction products were electrophoresed on a 2% agarose gel. Samples were lane 1: Negative control; lane 2: Positive control; lane: 3 100 bp ladder; lane: 4–11 environmental samples](www.ijmm.org)

<table>
<thead>
<tr>
<th>Location</th>
<th>Number M. leprae PCR positive (RLEP region target)</th>
<th>Number reverse transcription-PCR (16S rRNA gene target) positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leprosy colony</td>
<td>22</td>
<td>12 (54)</td>
</tr>
<tr>
<td>Bathing place</td>
<td>12</td>
<td>1 (8)</td>
</tr>
<tr>
<td>Sitting place</td>
<td>14</td>
<td>1 (7)</td>
</tr>
<tr>
<td>Entrance of the house</td>
<td>14</td>
<td>1 (5.7)</td>
</tr>
<tr>
<td>Hospital area</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Sitting place</td>
<td>14</td>
<td></td>
</tr>
</tbody>
</table>

PCR: Polymerase chain reaction, RLEP: Repetitive element, rRNA: Ribosomal ribonucleic acid
the prevalence of high endemicity of leprosy in certain areas, it is necessary to understand the environment around the leprosy patients, the route of infection and the mode of transmission. Although the exact route of infection is not fully known, the evidence of transmission through the skin and the respiratory route has gained support of experimental evidences. Laboratory experiments of development of leprosy infection in immunocompromised mice after exposure to M. leprae aerosol have strongly suggested that droplet infection through respiratory route is possible. Epidemiological evidences have also suggested that M. leprae can be carried nasally in an endemic region. With the realization of the importance of the nose as a portal of exit, there has been increased emphasis on the respiratory tract as the portal of entry. In addition, numerous studies indicate that leprosy is transmitted from person-to-person by close contact between an infectious patient and a susceptible host. This contact may be direct (e.g., skin to skin) or indirect (e.g., contact with soil, and fomites such as contaminated clothes and linen).

A study reported from Southern USA in wild armadillos and leprosy patients found that both armadillos and patients were infected with the same strain of M. leprae. Armadillos are a large natural reservoir for M. leprae, and leprosy may be a zoonotic in that region. It might be possible if one genotype M. leprae strain from soil where these armadillos are residing we will get the presence of same strain. To understand this, in our study, we genotyped both soil and patients’ samples from the same area. It may also be transmitted via breast milk from lepromatous mothers to the offspring, by insects, arthropods (fleas, ticks, mosquitoes, and flies) or by tattooing needles. Transmission of M. leprae through abraded mucus membrane has been experimentally demonstrated to infect nude mice. Research studies have shown that number of tandem repeats in rpoT gene used as marker for understanding of the origin among the strain of M. leprae in patient from Northern India.

In the present study, we have detected M. leprae DNA from SSS of leprosy patients and soil samples around the patient’s inhabitant area. SSS samples from patient and their surrounding soil samples were analyzed for genotyping on the basis of rpoT which showed three copies of rpoT gene. We have detected both dead and viable M. leprae from soil samples from these areas by demonstration of DNA and RNA of M. leprae using specific M. leprae primers. The possibility of transmission depends upon the viability of the organism outside the human body. It has shown that M. leprae can survive outside human body for 46 days in wet soil. Various reports have suggested that M. leprae is found in the environment and may have role in continued transmission of disease. In earlier studies M. leprae DNA has been detected from environmental samples by using RLEP sequences. In our study, we used repetitive (RLEP) sequences for and 16S rRNA as markers for detection and viability, respectively. It has been reported that RLEP gene region is repeated 37 times in the M. leprae chromosome. Though there is a single set of rRNA gene in M. leprae there could be 1000–10,000 copies of rRNA in a cell. It has been shown that 16S rRNA/RLEP-RT-PCR acts as a better target compared to superoxide dismutase mRNA/RLEP RT-PCR for viability testing of M. leprae. The soil samples that showed presence of M. leprae were from the specific areas such as the common bathing points, places used for washing by the patients, areas close to the house of the leprosy patient, places used by patients as sitting areas near the ward in the hospital. Our results showed that viable M. leprae is present in the areas where active leprosy patients reside. This suggests that there could be a possibility of contamination of the soil as well as indirect exposure of M. leprae to inhabitants around the area. We also observed that the proportion of samples which were positive for M. leprae specific 16S RNA was much higher in those collected from areas near the bathing places compared to that of the samples collected from areas near the houses of the leprosy patients. This indicates that the dampness and wet conditions probably help M. leprae to survive for longer time in the environment.

The sequence of the rpoT gene region from the SSSs as well as the soil samples reveal the presence of three copies
of 6 bp tandem repeats which indicates that the patients and environment has the same source of \textit{M. leprae}. Hence, the environment could play a role in maintaining the transmission of infection to man. Further studies may be pursued using the molecular markers such as rpoT gene to identify various genotypes within leprosy patient families from the endemic region which might give deeper insights into understanding the transmission chain in leprosy.

Though significance of viable environmental \textit{M. leprae} in transmission is not yet clear, the results fall in line with established concept that hygiene and sanitation play important role in the disease transmission. This study provides information on the presence of viable \textit{M. leprae} in soil samples with genetic similarity to the one seen in the patients. This might further help to have better insights into investigating the transmission dynamics of leprosy.

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Conflicts of interest

There are no conflicts of interest.

References


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