Original Article

Viability of *Mycobacterium leprae* in the environment and its role in leprosy dissemination

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ABSTRACT

Background: Leprosy, a chronic disease caused by *Mycobacterium leprae,* is a public health concern in certain countries, including India. Although the prevalence of the disease has fallen drastically over time, new cases continue to occur at nearly the same rate in many regions. Several endemic pockets have been observed in India and elsewhere. The precise dynamics of leprosy transmission are still not clearly understood. Both live bacilli as well as *M. leprae* DNA have been detected in the soil and water of endemic areas; they possibly play an important role in disease transmission. **Aims:** To study the occurrence of viable *M. leprae* in environmental samples collected from areas of residence of patients with active leprosy. **Methods:** The study was conducted on 169 newly diagnosed leprosy patients in Ghatampur, Uttar Pradesh, India. Soil and water samples were collected from their areas of residence using a standardized protocol. An equal number of soil and water samples were also collected from non-patient areas of the same or adjoining villages. The environmental samples collected from the patients surroundings were subjected to 16S ribosomal RNA gene analysis after obtaining informed consent. **Results:** About a quarter of the environmental samples collected from patient areas, (25.4% of soil samples and 24.2% of water samples) were found to be positive for specific 16S ribosomal RNA genes of *M. leprae*. Environmental samples collected from non-patient areas were all found negative for *M. leprae* 16S ribosomal RNA genes. **Limitations:** The major limitation of the study was that the sample size was small. **Conclusion:** The study demonstrated the presence of viable strains of *M. leprae* in skin smear samples of paucibacillary patients and multibacillary patients, as well as in the environmental samples obtained from around their houses. This could play an important role in the continued transmission of leprosy.

Key words: 16S ribosomal RNA, leprosy, multibacillary, *Mycobacterium leprae*, paucibacillary

INTRODUCTION

Leprosy is a slow and chronic infection caused by *Mycobacterium leprae*, affecting both sexes and all age groups, in many parts of the world. According to the WHO report, leprosy is a public health problem in 105 countries (including 28 in Africa, 28 in the Americas, 11 in South East Asia, 22 in the Eastern Mediterranean region and 16 in the Western Pacific region).[1] India achieved 'elimination' (prevalence < 1/10000) in December 2005 but new cases of leprosy continue to be detected in some endemic pockets. A total of 542 (84.7%) districts out of total 640 districts also achieved 'elimination' of leprosy by March 2012.[2] However, the new case detection rate continues to pose

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a challenge for program managers as it has either remained stationary or showed only a very slow decline since 2007.

Numerous studies suggest that leprosy is transmitted from person to person by close contact of a healthy individual with an infectious patient. Till date, the exact mechanisms of leprosy transmission are not clearly understood. Even the widely advocated methods of spread including person to person contact or contact with respiratory secretions from infected individuals have not been conclusively established so far. Different authors have suggested that *M. leprae* may be present in the soil, in water, on plants or in various animal species including amoeba, insects, fish, primates and armadillos.[3-15] The role of soil and water in the transmission of leprosy has only been speculated upon; it is yet to be recognized and supported by experimental proof. Indirect evidence for this possibility comes from the fact that in the recent past, contaminated water supply systems have been responsible for several hospital and community outbreaks of mycobacterial infections.[16]

Studying transmission dynamics is complicated by the inability to grow *M. leprae* in culture and the lack of animal models. Infection from the animal source, that is, armadillos (*Dasypus novemcinctus*) has been ruled out in India since armadillos are not found in nature in this country. The nearly constant detection of new cases in some pockets, with no clear cut history of close and prolonged contact with leprosy patients, indicates that other reservoirs of infection, possibly environmental, could be responsible for the continued transmission of leprosy.

METHODS

Collection of environmental samples

Soil and water samples were collected from different places of Ghatampur, Kanpur Nagar, Uttar Pradesh, an endemic pocket for leprosy. The environmental samples (both soil and water) were collected from 169 patient areas and 169 non-patient areas. Patient areas included the vicinity of houses where patients lived and dwelled. Different portions of the same or adjoining villages, where leprosy cases had not been detected during the last 5–6 years, were designated as non-patient areas. To collect soil samples, soil was dug (4”deep) and collected in clean plastic containers (10 g each) with the help of a “khurpi” and labelled with the patient ID and the village name. Likewise, water samples were collected from the drainage outlet of the patient’s (or non-patient’s) house, bathing place and drinking water source (bore well). The collected samples were transported to the research centre (National JALMA Institute for Leprosy and Other Mycobacterial Diseases, Tajganj, Agra, UP, India) and stored at 4–8°C till they were processed further. The geomapping of patient samples was done using Geoplaner online software (www. geoplaner. com).

Isolation of *Mycobacterium leprae* total RNA

Extraction of *M. leprae* total RNA from soil and water samples was done by the method of Miskin *et al*.[17] Briefly, 5 ml (1 mg/ml) of soil suspension was taken in a sterile 25 ml screw-capped tube with sterile glass beads and centrifuged at 8000 g for 30 minutes. The pellets were treated with 2 ml of extraction buffer (0.12M sodium phosphate buffer, 10 mg lysozyme, 1% β-mercaptoethanol) and shaken for 20 seconds. The homogenates were transferred to new vials containing 500 µl of 10% (w/v) sodium dodecyl sulfate, incubated at 80°C for 30 minutes with vigorous shaking at 10 minute intervals in a water bath and centrifuged at 2800 g for 15 minutes at 4°C. The supernatants were transferred to fresh tubes and held on ice while the pellets were re-extracted with 2.5 ml of extraction buffer and re-centrifuged at 2800 g for 15 minutes at 4°C. The supernatants were pooled, mixed with twice their volume of 30% polyethylene glycol 6000 and kept for 2 hours at room temperature for nucleic acid precipitation and centrifuged at 5000 g for 30 minutes at 4°C. The pellets were re-suspended in 1 ml diethylpyrocarbonate treated water (to inactivate RNase enzymes). One hundred microliters of 7.5 M potassium acetate were added to make a final concentration of 0.5 M and it was centrifuged at 8000 g for 5 minutes to remove the precipitated humic acid (which interferes with polymerase chain reactions). Nucleic acid was precipitated with the addition of twice the volume of chilled ethanol and keeping overnight at −20°C. RNA was pelleted by centrifuging at 8000 g for 15 minutes and dissolved in 20 µl of diethylpyrocarbonate-treated/HPLC-grade water. RNA preparations were treated with DNaseI for removing any traces of contaminating DNA. DNaseI was inactivated by incubation at 80°C for 10 min. Then isolated RNA was stored at −70°C till further use.

Preparation of complementary DNA and polymerase chain reaction amplification of 16S ribosomal RNA gene

Complementary DNA synthesis was done using Revert Aid First strand complementary DNA synthesis kit (Fermentas) from the RNA of environmental samples. Prepared complementary DNA was stored at −20°C for further use. The 16S ribosomal RNA gene of *M. leprae* was amplified using the primers F-5' TCGAACGGAAAGGTCTCTAAAAAATC 3' and R-5' CCTGCACCGCAAAAAGCTTTCC 3' to determine the presence of viable *M. leprae* in environmental samples (soil and drainage water samples).[18]

Sequencing of 16S ribosomal RNA gene

Polymerase chain reaction products of the 16S ribosomal RNA gene were purified using polymerase chain reaction clean-up kit. All the polymerase chain reaction products were sequenced directly in the ABI 3031XL big dye terminator sequencer according to the manufacturer's instructions.

Basic local alignment search tool (BLAST) search and sequence alignment

Initially, the sequences were subjected to the basic local alignment search tool search (BLAST) at NCBI to determine their molecular taxonomic identity. For basic local alignment search tool search, sequences were converted to FASTA format and entered into the NCBI web page (http://blast.ncbi.nlm.nih.gov/blast), selecting the reference data domain as nucleotide collection (nt/nr) for highly similar megablast search. The taxonomic identities of the strains were determined after comparing the search results. Five sequences from basic local alignment search tool search results were aligned with our sequences. The sequence alignment was done using MEGA 4.0 (www.megasoftware.net.mega4).

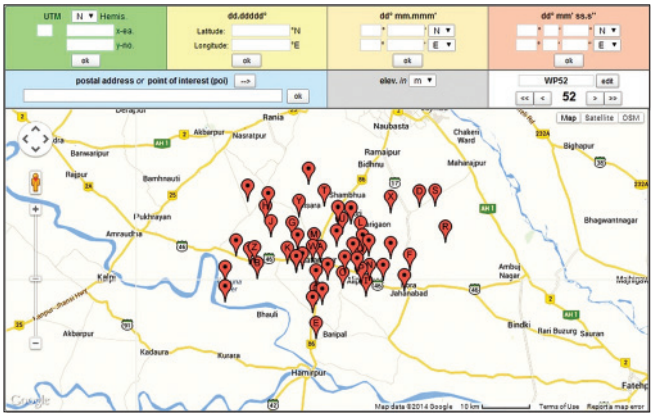

Figure 1: Geographic location and the prevalence of leprosy in environmental samples collected from Ghatampur tehsil. The spots showed the samples collected from the patient area

RESULTS

Of the patients from whom 169 slit skin smear samples were collected, 4 were indeterminate, 93 were borderline tuberculoid (BT) type, 66 were (BB) type, 5 were borderline lepromatous (BL) type and 1 was found to be lepromatous (LL) type diagnosed clinically. These 169 patients belonged to 132 nuclear and joint families hailing from 96 villages. The mapping of patient samples is depicted in Figure 1. Of the environmental samples collected from the areas where patients were residing, 43 (25.4%) soil samples and 41 (24.2%) water samples were found to be positive for 16S ribosomal RNA on amplifying the product of complementary DNA. In 37 (21.8%) areas, both soil and water samples were positive for 16S ribosomal RNA. The environmental samples showing positivity were obtained from the areas where 34 multibacillary and 17 paucibacillary patients resided. The amplified product of the 16S ribosomal RNA gene was about 172 bp [Figure 2]. On the other hand, no environmental samples taken from non-patient areas were found positive for *M.leprae* 16S ribosomal RNA. Basic local alignment search tool (BLAST) search results confirmed that the sequenced product was the 16S ribosomal RNA gene of *M. leprae*. The cDNA sequences from soil and water samples clearly matched with *M. leprae* Tamil Nadu strain (AL583920), *M. leprae* cosmid B1549 (U00014), *M. leprae* strain (X55022), *Mycobacterium lepromatosis* Br1 (GQ900374) and Uncultured bacterium clone (JQ374245), with identities scores of 100%, 100%, 100%, 97% and 96%, respectively. The nucleotide differences between *M. leprae* and uncultured bacteria were found in the nucleotide positions 1276 (G to A), 1315 (T to C), 1316 (G to A), 1342 (G to A), 1355 (A to T) and differences between

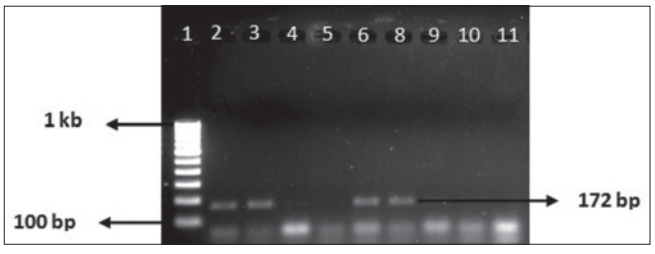

Figure 2: Polymerase Chain Reaction product of 16S ribosomal RNA gene amplified from complementary DNA. Lane 1: 100 bp ladder, Lane 2: Positive control, Lane 3–10: Environmental samples, Lane 11: Negative control

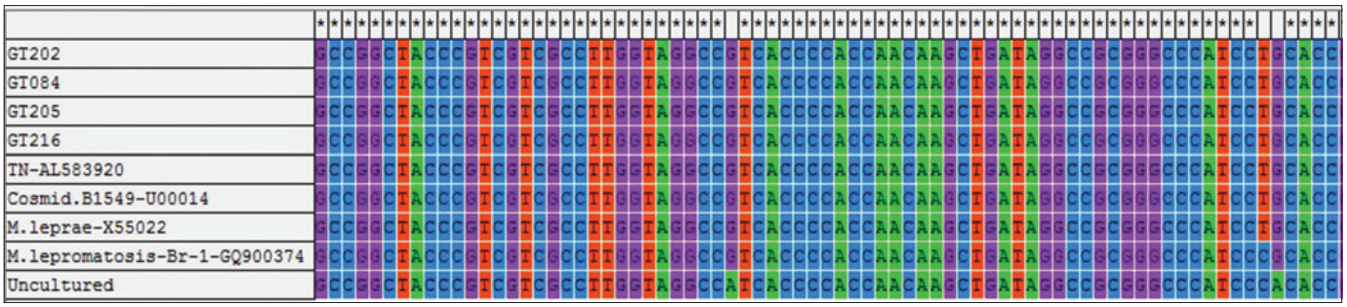

Figure 3: Multiple sequence alignment of partial sequence 16S ribosomal RNA gene of *Mycobacterium leprae, Mycobacterium leprae* cosmid, *Mycobacterium lepromatosis* and uncultured bacterium

M. leprae and *M. lepromatosis* were in the nucleotide positions 1315 (T to C), 1378 (A to G), 1387 (A to G) and 1409 (C to T) [Figure 3].

DISCUSSION

Leprosy is still a public health problem in several countries including India. The disease is said to be transmitted from person to person by respiratory secretions from infected individuals but this theory has not been conclusively proven so far. Several studies have provided insight into the non-human sources of *M. leprae*.[5,7,19-21] There is growing recognition in recent years that soil and water may be important vehicles of transmission of *M. leprae* and could be responsible for the continued presence of leprosy. In the soil, several microorganisms live in the same niche in a symbiotic or commensal relationship. *M. leprae* is known to survive in the soil for upto 40 days.[22] Our study investigated the soil and water obtained from patient residential areas as well as non-patient areas; we had a larger sample size compared to that of earlier studies from other parts of India.[3,20,21] Using the specific primers designed for *M. leprae* by Jadhav *et al*. we specifically amplified the 172 bp 16S ribosomal RNA gene of *M. leprae*.[18] In this study, RNA was used instead of DNA to detect *M. leprae* not only for its presence but also to find out the viability of *M. leprae* in the soil. We found that 25.6% soil and 24.2% water samples from patient areas were positive for viable leprosy bacilli. This might have ome about due to the movement of an infected person in the surrounding environment and resultant shedding of leprosy bacilli, which then continue to live in the environment by symbiotic or commensal mechanisms Our results corroborate with the work of different researchers who have provided evidence of the existence of viable *M. leprae* in environmental samples taken from areas where patients reside.[3,20,21] Those environmental samples that we collected from the non-patient areas did not show the presence of *M. leprae*. Thus, the environmental *M. leprae* is a connective link in the continuation of leprosy in several endemic pockets where multiple cases are detected in the same family or in nearby families. The GenBank accession number JQ374245 represents an uncultured bacterium found in 6 terrestrial ecosystems of USA, having 96% similarity with the 16S ribosomal RNA gene of *M. leprae*.[23] As the bacterium had 96% similarity with *M. leprae* and was unculturable, we conclude that the bacterium found in the terrestrial ecosystem might be *M. leprae*. Matsuoaka *et al*.[5] strongly suggested that non-human sources such as soil and water may be responsible for continued leprosy transmission. Jadin reported the presence of *M. leprae* in amoeba, thus confirming our results in favor of the existence of *M. leprae* in the environment and its role in further transmission.[24]

There are two limitations of this study (1) the sample size of the study was very small (2) 16S rRNA gene analysis for viability study was limited to environmental samples only.

CONCLUSION

Our study showed the presence of viable *M. leprae* in the soil and water of the patient areas. To confirm the role of environmental *M. leprae* in leprosy transmission, more extensive studies are required.

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Conflicts of interest

The authors declare no conflicts of interest.

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