ABSTRACT

Despite near elimination of leprosy as a public health problem, several problems in leprosy still remain. These include early detection, determining efficacy of the treatment and differentiating relapses from re-infection. These aspects have important impact on the patients undergoing treatment and also have a bearing on understanding transmission dynamics in the community. While early diagnosis and management do not need major technological inputs, various reports have suggested that \textit{M. leprae} is found in the environment and may have a role in continued transmission of disease. In earlier studies from other parts of world the presence of \textit{M. leprae} DNA in the environment has been investigated both by microbiological and molecular studies. In the present study, an attempt was made to extract \textit{M. leprae} DNA from soil samples, which were collected from eighteen different locations including 3 from our Institute area and 15 from different villages of Ghatampur area. We optimized a protocol for the extraction of DNA and amplified a fragment of \textit{M. leprae} using specific primers targeting RLEP sequences. It was found that 33.3% of these soil samples collected from areas inhabited by leprosy cases gave positive result for \textit{M. leprae} specific DNA. The utility of this method needs to be explored on a larger scale to establish the presence of \textit{M. leprae} in the environment, and its role in the spread of the disease.

Detection of \textit{Mycobacterium leprae} DNA from soil samples by PCR targeting RLEP sequences


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INTRODUCTION
Leprosy is one of the oldest infectious diseases afflicting man\textsuperscript{1}. But even today, largely due to the inability to cultivate the ethiologic agent \textit{Mycobacterium leprae} in conventional media or in tissue culture cell lines\textsuperscript{2}, our knowledge about \textit{M. leprae} basic physiology as well as of the mechanism(s) of pathogenicity is limited. Prevalence rate of India at the end of November 2005 was 1.06 per ten thousand population 128,336 patients were under treatment at that point of time and 107,726 new patients had been detected in the country since first of April 2005\textsuperscript{3}. Pockets of endemicity exist in certain areas as experience in the surveys from Ghatampur, Kanpur \textsuperscript{4}. Higher than the estimated prevalence has also been observed in other areas like Agra\textsuperscript{5}. Despite this concept of an exclusive human reservoir of leprosy, there was an old German belief that the disease might have had some links with the soil. Direct spread of leprosy is certainly important, but infection may also be indirect. \textit{M. leprae} has been said to be capable of survival outside the human body for as long as several months under favourable conditions\textsuperscript{6}. It has also been found in the soil\textsuperscript{7}. To find out reasons for persistence of high endemic pockets, the potential of soil, water or environment to act as a reservoir for \textit{M. leprae} needs to be explored. Though these extra human sources may or may not facilitate the growth of \textit{M. leprae} they could act as a potential reservoir. Various reports have suggested that \textit{M. leprae} can survive in the environment and may have a role in continuing transmission of disease. PCR analysis provides a sensitive and specific means to detect and monitor microorganisms in complex environmental samples. Successful detection and characterization of microbial DNA in the environment requires efficient extraction of the DNA from environmental samples and adequate purification from the co-extracted contaminants that inhibit PCR. Soils and sediments vary greatly in chemical and organic composition. They also contain abundant humic and fulvic acids that are inhibitory to \textit{Taq} DNA polymerase and other enzymes\textsuperscript{8,9}. Soils are therefore one of the most challenging environmental matrices from which to obtain microbial DNA that will be useful for PCR detection. PCR is now being used to detect \textit{M. leprae} in various clinical samples and can also be used to detect live \textit{M. leprae} in various environmental from the leprosy endemic pockets \textsuperscript{10}. The objective of the present study was to investigate the feasibility of detecting \textit{M. leprae} DNA in soil samples using PCR so as to determine possible sources of transmission that may be contributing towards the spread of leprosy.

MATERIAL AND METHODS

Sample Collection
Eighteen soil samples were collected from different locations around JALMA and the 15 villages of Ghatampur, an endemic area of leprosy. We collected two samples from each village. These samples were stored at 4°C.

Extraction of \textit{M. leprae} genomic DNA
These soil samples were processed for DNA extraction using method described. 100 mg of dried soil was weighed in a 1.5 ml microcentrifuge tube. 500 μl of 0.125 M EDTA pH 8.0 was added. Samples were lysed in 100 μl of lysis buffer (Proteinase K and Tween 20 concentration) and incubated at 60°C overnight in a water bath. The reaction was terminated by inactivating Proteinase K at 95°C for 10 mins. 30 μl of 10% SDS and incubated at 60°C for 1 hour in a water bath. After centrifugation at 825x g (3000 rpm) for 10 mins, supernatant was collected and DNA was precipitated by adding 70% ethanol overnight. Next day centrifuge at 8000x g (10,000 rpm) for 15 mins. The pellet was air-dried and resuspended in 25 μl of Tris-EDTA buffer before being used for PCR.

**PCR-RFLP analysis targeting RLEP gene**

PCR reactions were performed in 50 μl reaction mixtures consisting of 5 μl of DNA template, 0.2 mol l⁻¹ deoxynucleoside triphosphates, 0.5 mol l⁻¹ primers for RLEP gene and 1.5U Taq polymerase (Bangalore Genei, Bangalore, India). 129 bp fragment was amplified by using primers and procedure described by Donoghue et al. The forward primer for RLEP was, 5' TGATGTCATGGCCTTGAGG 3' and the reverse primer was, 5'CACCGATACCACCGGCGAGA 3'.

The PCR conditions for RLEP gene were 95°C for 5 min, 58°C for 2 min and 72°C for 2 min for single cycle. Then 94°C for 30 sec, 58°C for 30 sec and 72°C for 1 min followed by 35 cycles and then a final extension at 72°C for 10 min.

**Fig-1** Agarose Electrophoresis of DNA extracted from soil samples and PCR performed using primers targeted for *M. leprae* specific region in RLEP region.
phenolic glycolipid-I antigen, which is unique to the *M. leprae* cell wall, is present in soil. *M. leprae* specific DNA has been reported to be present in 21 out of 44 water sources used daily by villagers for bathing and washing.

Our findings confirm the presence of *M. leprae* DNA in soil. While these initial results of our study suggest that *M. leprae* is present in the environment in this Ghatampur field area, in-depth long term follow-up studies are required to confirm the role of soil in the epidemiology of leprosy. Data need to be expanded and correlated with other epidemiological parameters.

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An aliquot (25 µl) of each amplification reaction was analysed on 2% w/v agarose gels cast and run in TBE buffer (pH 8.0). Gels were stained with ethidium bromide and photographed using transmitted U.V. light. A 100 base pair marker (Bangalore, Genei) was included on every gel.

**RESULTS**

*Mycobacterium leprae* DNA targeting RLEP were detected by PCR in 6 out of 18 (33.3%) soil samples collected from the villages of Ghatampur. One of the three samples around JALMA had demonstrable *M. leprae* DNA (Fig-1).

**DISCUSSION**

Leprosy has been considered to occur only after exposure to a human case. However, evidence has been accumulating that this conventional view may not be fully correct and that environmental nonhuman source(s) may be important. The observations, some of which date back to the nineteenth century, support soil, vegetation, water, arthropods, and armadillos (*Dasypus novemcinctus*) as environmental sources of leprosy. Disparate clinical, epidemiologic, and microbiologic evidence has been critically reviewed in light of the fact that 50%-70% of sporadic cases of leprosy in well-studied populations occur in persons who have had no known contact with human leprosy. Historical data and current information also substantiate the concept of possible nonhuman environmental sources of the disease. Recent observations have shown that...


