Single nucleotide polymorphism-based molecular typing of *M. leprae* from multicase families of leprosy patients and their surroundings to understand the transmission of leprosy

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Abstract

The exact mode of transmission of leprosy is not clearly understood; however, many studies have demonstrated active transmission of leprosy around a source case. Families of five active leprosy cases and their household contacts were chosen from a high endemic area in Purulia. Fifty-two soil samples were also collected from different areas of their houses. DNA was extracted from slit-skin smears (SSS) and soil samples and the *Mycobacterium leprae*-specific RLEP (129 bp) region was amplified using PCR. Molecular typing of *M. leprae* was performed for all RLEP PCR-positive samples by single nucleotide polymorphism (SNP) typing and confirmation by DNA sequencing. SSS of these five patients and six out of the total 28 contacts were PCR positive for RLEP whereas 17 soil samples out of 52 showed the presence of *M. leprae* DNA. SNP typing of *M. leprae* from all RLEP PCR-positive subjects (patients and smear-positive contacts) and 10 soil samples showed the SNP type 1 genotype. *M. leprae* DNA from the five leprosy patients and the six contacts was further subtyped and the D subtype was noted in all patients and contacts, except for one contact where the C subtype was identified. Typing followed by subtyping of *M. leprae* clearly revealed that either the contacts were infected by the patients or both patients and contacts had the same source of infection. It also revealed that the type of *M. leprae* in the soil in the inhabited areas where patients resided was also of the same type as that found in patients.

Keywords: Environmental samples, leprosy multicase families, *M. leprae*, SNP typing, transmission
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Introduction

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae*. It is one of the oldest recorded diseases of mankind. The changes in the registry pattern after the implementation of Multi drug therapy (MDT) in the vertical programme have drastically brought down the prevalence (PR) of leprosy, whereas the incidence has not come down in many places in the world [1], including India [2], where a prevalence of <1/10 000 has been attained. A total of 11 districts with an incidence rate of >50/100 000 population still exist in Chhattisgarh, Gujarat, Maharashtra, West Bengal, Dadra & Nagar Haveli, Orissa and Delhi [2]. The global use of MDT seems to have had only minimal, if any, effect on transmission of the disease [3] and an adequate explanation for this situation is lacking. Multibacillary (MB) leprosy patients harbour enormous numbers of leprosy bacilli and discharge them freely from their skin, nasal ulcers and saliva [4,5]. Coughing and sneezing can give rise to formation of droplets and droplet nuclei, which in turn enter the respiratory system of close contacts. In a house or in a community such leprosy patients are in contact with

households and neighbours and have other social relationships. The contacts of leprosy patients are known to have an increased risk of contracting leprosy themselves. The estimated risk of leprosy was about nine times higher in households of patients and four times higher in direct neighbouring houses of patients compared with households that had no such contact with patients [6, 7]. The highest risk of leprosy was associated with households of multibacillary patients [6]. It has been reported that a single mouthwash of an MB patient may yield 1.6 million *M. leprae* [8].

Various studies have suggested that *M. leprae* can be found in the environment and may have a role in continuing transmission of the disease [9–11]. The presence of *M. leprae* DNA has also been reported in water samples in Indonesia [9] and soil samples from high prevalence areas of northern India [10]. In such endemic areas molecular tools can be of help in devising techniques for understanding the epidemiology of leprosy and identifying sources, as well as finding out the source of persisting foci of infection. Application of molecular methods to elicit strain differences within the leprosy bacillus would be of utmost importance for this purpose. Molecular typing will make it feasible not only to study the global and geographical distribution of distinct clones of *M. leprae* in the population, but also to explore correlation between the *M. leprae* and the type of disease manifested and provide insight into historical and phylogenetic evolution of the bacillus [12]. Ultimately these genetic markers may hold the key to establishing species and strain-specific markers for identifying the sources of *M. leprae* and tracing transmission patterns.

Recently, a complete genome sequence of an isolate from Tamil Nadu, India (TN strain), helped to develop a polymorphic genomic marker for *M. leprae*. After screening a large number of *M. leprae* isolates from different parts of the world, *M. leprae* has been classified into four single nucleotide polymorphism (SNP) types and 16 subtypes [12,13]. Therefore, SNPs may hold the promise of establishing species and strain-specific markers for identifying the sources of *M. leprae* and tracing transmission patterns. The aim of present study was to detect *M. leprae* DNA by using PCR and perform typing based on SNP PCR, followed by restriction enzyme digestion from slit-skin smears of leprosy patients and their household contacts and environmental samples.

Materials and Methods

Ethical approval

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

Slit-skin smear samples

Slit-skin smear collection is an invasive procedure where 5 mm long and 2 mm deep incisions are made on the left and right earlobe, patches and forearm, after blanching the area between the thumb and forefinger, and the superficial tissue material is scraped four times with horizontal strokes of the blade without any contamination with blood along the skin-slit, and mixed with 700 μL of 70% ethanol in a micro-centrifuge tube. Later the samples were transferred to the laboratory and centrifuged to sediment a pellet of tissue material. After receiving consent, slit-skin smear samples were collected from both earlobes of five active MB leprosy patients and their household contacts. The contacts selected for the study had clinical assessment data available from the records of The Leprosy Mission Hospital, Purulia.

We observed that the mean bacterial index (BI) of all five patients was 3+ and six contacts on follow-up were found to be MB cases with an average BI of 2+. BI is an expression of the extent of bacillary load. It is calculated by counting six to eight stained smears under the 100× oil immersion lens. The BI 2+ means at least one bacillus observed in every 10 fields of slides under the microscope and BI 3+ indicates one bacillus in every field. Intense clinical assessment of the contacts in each family revealed a total of six contacts manifesting cardinal signs of leprosy and 22 contacts without any signs and symptoms of leprosy. Slit-skin smear samples were collected and AFB staining was performed for all. (Table 2**)**. The slit-skin smear samples (SSS) were collected during field visits in different blocks of Purulia District, West Bengal, and were transported in 70% ethanol in micro-centrifuge tubes to the laboratory at room temperature (25°C). The tubes were kept at 4°C until further use.

Environmental samples

Fifty-two soil samples were collected from different places around the houses of leprosy patients. Soil samples were collected from areas used for bathing, drinking, sleeping and sitting and the entrances of the houses. Initially soil was dug (3–4 inches deep) and 10 gm from each site was collected in clean plastic containers with the help of a 'trowel' and labelled with a unique specific code. The collected samples were transported to the laboratory at room temperature (within 2 days) and thereafter stored at 4–8°C till further processing.

DNA extraction from slit-skin smears

The Proteinase K Lysis method was used for *M. leprae* DNA extraction from slit-skin smear samples [14]. In brief, smears collected in 1 mL 70% ethanol were centrifuged at 8000 *g* for 10 min. The supernatant was discarded and the pellet was air-dried for the removal of ethanol. After ethanol removal, samples were kept for overnight lysis in lysis buffer (100 mM