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Dynamics of *Mycobacterium leprae* transmission in environmental context: Deciphering the role of environment as a potential reservoir

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ABSTRACT

Leprosy is a disease caused by *Mycobacterium leprae*. Various modes of transmission have been suggested for this disease. Transmission and risk of the infection is perhaps related to presence of the infectious cases and is controlled by environmental factors. Evidence suggests that humidity may favor survival of *M. leprae* in the environment. Several reports show that non-human sources like 'naturally' infected armadillos or monkeys could act as reservoir for *M. leprae*. Inanimate objects or fomites like articles used by infectious patients may theoretically spread infection. However, it is only through detailed knowledge of the biodiversity and ecology that the importance of this mode of transmission can be fully assessed. Our study focuses here to decipher the role of environment in the transmission of the disease. Two hundred and seven soil samples were collected from a village in endemic area where active cases also resided at the time of sample collection. Slit skin smears were collected from 13 multibacillary (MB) leprosy patients and 12 household contacts of the patients suspected to be hidden cases. DNA and RNA of *M. leprae* were extracted and amplified using *M. leprae* specific primers. Seventy-one soil samples showed presence of *M. leprae* DNA whereas 16S rRNA could be detected in twenty-eight of these samples. Samples, both from the environment and the patients, exhibited the same genotype when tested by single nucleotide polymorphism (SNP) typing. Genotype of *M. leprae* found in the soil and the patients residing in the same area could help in understanding the transmission link in leprosy.

1. Introduction

Leprosy causes severe disfigurement in many cases and has been subject to strong social stigma around the world. The estimated global prevalence of leprosy has been greatly reduced as a result of the multidrug therapy (MDT) program advocated by the World Health Organization (WHO) and its implementation with the help of governmental and non-governmental organizations. According to official reports received during 2010 from 141 countries and territories, the global registered prevalence of leprosy at the beginning of 2010 stood at 211,903 cases, while the number of new cases detected during 2009 was 244,796 (WHO, 2010). However some pockets of endemicity do remain in certain areas of Angola, Brazil, Central African Republic, Democratic Republic of Congo, India, Madagascar, Mozambique, Nepal and the United Republic of Tanzania (WHO, 2010). In India, with the widespread use of MDT the elimination target has been achieved at the national level with a recorded prevalence of 0.83/10,000 population (NLEP, 2008). A total of 134,000 cases were detected during the year 2008–09, which gives Annual Case Detection Rate (ANCDR) of 11.19 per 100,000 populations (NLEP, 2009). The remaining pockets of endemicity are localized to states of Bihar, parts of Uttar Pradesh, some parts of West Bengal, Jharkhand, and Orissa. The global use of MDT seems to have had only minimal, if any, effect on transmission of the disease (Meima et al., 2004) and an adequate explanation for this situation is lacking.

The exact mechanism of transmission of leprosy is not known. It is believed that transmission occurs due to discharge of bacilli by droplet from the nose and mouth and to a lesser extent by direct contact from an infected person to a susceptible individual (Weddell and Palmere, 1963; Barton, 1974). Limited multiplication and growth of *M. leprae* in mouse foot pad has provided a useful tool for assessing the viability of the organism and testing the drug susceptibility of clinical isolates (Shepard, 1962; Truman, 2005). The other animal models used for scientific research are the armadillo (Truman, 2005) and normal as well as athymic mice (Krahenbuhl, 1999). All these advances and tools are very important in managing the disease at patient and public health level. Detection of *M. leprae* RNA by molecular methods is considered as a potential tool for rapid detection and measurement of the viability of *M. leprae* (Kurabachew et al., 1998). The recovery of rRNA 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. 16S rRNA has been used as

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a target in several studies for assessing the viability of mycobacteria such as *Mycobacterium tuberculosis*, *M. leprae*, etc. (Hellyer et al., 1999; Kurabachew et al., 1998; Jadhav et al., 2005; Phetsuksiri et al., 2006; Martinez et al., 2009) from clinical specimens.

Various reports have suggested that *M. leprae* could be found in the environment and may have a role in continuing transmission of disease (Matsuoka et al., 1999; Lavania et al., 2008; Wahyuni et al., 2010). The important factor which may be attributed to the transmission of the disease is the viability of *M. leprae* outside human body (Desikan and Sreevatsa, 1995). Soil samples from Northern India provide the information regarding the presence of viable *M. leprae* in areas of high prevalence of the disease (Lavania et al., 2008). Presence of *M. leprae* DNA has also been reported in water samples in Indonesia (Matsuoka et al., 1999), in areas of high prevalence of the disease.

In recent years, molecular strain-typing methodologies have complemented conventional infectious disease epidemiology. With the publication in 2001 of the complete genome sequence of an isolate from Tamil Nadu, India, called the TN strain (Cole et al., 2001), selection of potential polymorphic genomic markers for strain typing was feasible. The first genetic markers that showed polymorphism were short tandem repeats (STRs) in the *M. leprae* genome. One was a six-bp intragenic sequence in the *rpoT* gene, and the second, a trinucleotide (TTC) repeat element upstream of a pseudogene (Matsuoka et al., 2000; Shin et al., 2000; Lavania et al., 2005). These sequences exhibit variable numbers of tandem repeats (VNTRs) when sequenced in different isolates. Based on these observations, 44 loci were short-listed (including the *rpoT* and TTC loci) by in silico analyses of the *M. leprae* genome. Screening of 11 STR loci was accomplished, of which nine were polymorphic when tested in a small panel of four human isolates derived from passage through armadillos (Groathouse et al., 2004). Since then, others have also shown that VNTR loci exist in *M. leprae* isolates (Truman et al., 2004; Young et al., 2004; Zhang et al., 2005). *rpo*T has been found to classify strains prevalent in North India into two groups (Lavania et al., 2007). Further studies have shown that there are at least 12 polymorphic loci which remain stable during the passages through mouse foot pad (Gillis et al., 2009) and could be useful for typing.

Molecular typing is not only used to study the global and geographical distribution of distinct strains of *M. leprae*, but also explore correlation between the *M. leprae* and the type of disease manifestation and provide some insight into historical and phylogenetic evolution of the bacillus that has affected humans and stigmatized leprosy patients for centuries (Monot et al., 2009).

Comparison of complete genome sequence of Br4923 (a Brazilian strain of *M. leprae*) and TN isolate demonstrated that there were total of only 155 SNPs of which 78 were informative. Their study also revealed that the genomes of strains from North America and Thailand had comparatively low levels of diversity. The presence of the 78 informative SNPs was subsequently surveyed in ~400 isolates, enabling classification of *M. leprae* into 16 SNP subtypes (Monot et al., 2009).

These genetic markers such as VNTRs and SNPs may hold the key to establish species and strain specific markers for identifying the sources of *M. leprae* and tracing transmission patterns.

Our study basically focuses on the detection of viable *M. leprae* and preliminary assessment of its genotype so that it could be used to correlate the link between the patient, household contact and the environment.

2. Materials and methods

2.1. Collection of sample

Soil samples were collected from different places of endemic areas of Purulia district, West Bengal. Soil was dug (3–4 inch deep) and collected in clean plastic containers (10 g each) with a help of "trowel" and labeled with site code and the village name. The collected samples were transported to the laboratory at room temperature (within 2 days) for the study and thereafter stored at 4–8 °C till further processing. Two hundred and seven soil samples were collected from different places like bathing place, common sitting place, near the entrance of the house, areas around house used as washing place by the inhabitants, common water ponds and near bore well. 82 samples were from near the houses of the leprosy patients whereas 125 samples were from the areas near washing and bathing places.

Slit skin smears were collected from 13 multibacillary (MB) leprosy patients and 12 household contacts of the patients suspected to be hidden cases (hypopigmented anesthetic patches later on confirmed as paucibacillary (PB) leprosy. Smears were taken from both the earlobes and lesion site in 70% ethanol in a tube and transported to the laboratory at room temperature. The tubes were kept at 4 °C until use.

2.2. DNA extraction from soil samples

DNA extraction was carried out using Proteinase K and Sodium Dodecyl Sulfate lysis followed by extraction with TENP buffer (50 mM Tris, 20 mM EDTA, 100 mM NaCl and 1% Polyvinylpolypyrrolidone). DNA preparation was passed through Sepharose 4B column for complete removal of humic acid and other PCR inhibitory substances. To ensure complete removal of PCR inhibitory substances and successful DNA extraction, we spiked some of the samples during processing with *Mycobacterium smegmatis* and checked for the DNA using PCR. In brief 100 mg of soil was weighed and dried in 1.5 ml microfuge tube. Five hundred microliters of ethanol was added to the tube along with 0.1 mm glass or zirconium beads and homogenized using bead beater to ensure and facilitate lysis of the cells. Our standardization experiments in the laboratory have shown that homogenization with bead beater improves yield of the DNA extracted from soil samples (data not shown). It was then centrifuged at 10,000 rpm 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. 30 µl 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 vortexing regularly at 5 min intervals. This was followed by centrifugation at 3000 rpm for 10 min. Supernatant was then passed through Sepharose 4B column (small column: 4–5 cm prepared in syringe) for the removal of humic acid components and the flow through was collected. DNA was precipitated by adding 70% ethanol and incubation at −20 °C for 2–3 h (preferably overnight). Subsequently the tubes were centrifuged at 10,000 rpm for 15 min and supernatant discarded. The pellet was air dried for about 15–20 min to ensure complete removal of ethanol. Pellet was resuspended in 50 µl 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 solution. The DNA solution was then passed through the inhibitor removal resin columns (Epicentre, USA SR04350) prior to storage at −20 °C until further use.

2.3. DNA extraction from samples collected from leprosy patients and their contacts

M. leprae DNA was isolated from slit skin smear of 13 multibacillary (MB) leprosy cases and their household contacts. Smears collected in 1 ml 70% ethanol were centrifuged at 10,000 rpm (8000*g*) for 10 min. Supernatant was discarded and pellet was air dried for