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Genotyping of *Mycobacterium leprae* strains from a region of high endemic leprosy prevalence in India

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ABSTRACT

Leprosy is still a major health problem in India which has the highest number of cases. Multiple locus variable number of tandem repeat analysis (MLVA) and single nucleotide polymorphism (SNP) have been proposed as tools of strain typing for tracking the transmission of leprosy. However, empirical data for a defined population from scale and duration were lacking for studying the transmission chain of leprosy. Seventy slit skin scrapings were collected from Purulia (West Bengal), Miraj (Maharashtra), Shahdara (Delhi), and Naini (UP) hospitals of The Leprosy Mission (TLM). SNP subtyping and MLVA on 10 VNTR loci were applied for the strain typing of *Mycobacterium leprae*. Along with the strain typing conventional epidemiological investigation was also performed to trace the transmission chain. In addition, phylogenetic analysis was done on variable number of tandem repeat (VNTR) data sets using sequence type analysis and recombinational tests (START) software. START software performs analyses to aid in the investigation of bacterial population structure using multilocus sequence data. These analyses include data summary, lineage assignment, and tests for recombination and selection. Diversity was observed in the cross-sectional survey of isolates obtained from 70 patients. Similarity in fingerprinting profiles observed in specimens of cases from the same family or neighborhood locations indicated a possible common source of infection. The data suggest that these VNTRs including subtyping of SNPs can be used to study the sources and transmission chain in leprosy, which could be very important in monitoring of the disease dynamics in high endemic foci. The present study strongly indicates that multi-case families might constitute epidemic foci and the main source of *M. leprae* in villages, causing the predominant strain or cluster infection leading to the spread of leprosy in the community.

1. Introduction

Leprosy (Hansen's disease) is a disease of great antiquity, having been recognized from Vedic times in India and from Biblical times in the Middle East. Armauer Hansen discovered the leprosy bacillus in 1873, in Bergen, Norway. With this discovery *M. leprae* became the first bacterial pathogen to be associated with any human disease. Leprosy may be defined as a chronic, infectious disease of low contagiousness caused by an acid-fast bacillus, *Mycobacterium leprae* that affects primarily the superficial parts of the body especially nerves and appendages of the skin like sweat and sebaceous glands in susceptible hosts after a varying incubation period.

Implementation of WHO multidrug treatment (MDT) regimen in the treatment of leprosy globally for last two and half decades has resulted in a dramatic decline in the prevalence of leprosy although there is a much slower decline in the detection of new cases. In 1981, when the presence of the disease was considered to be at its peak in India, the prevalence rate (PR) was 57 per ten thousand population. MDT was introduced in India 1982 onwards. In April 2014 it had fallen to 0.68/10,000 (NLEP, 2014).

According to WHO during 2014, globally 180,618 leprosy patients were on record for treatment. The prevalence rate was estimated as 0.32 per 10,000 population and the new case detection rate globally was 3.81 per 100,000 population (WHO, 2014). Three decades after the introduction of multi-drug therapy (MDT) the prevalence of leprosy has come down from 4.2 to 0.68/10,000 from 2002 to 2014 in India (NLEP, 2014). The remaining pockets of endemicity are localized to the states of Bihar and Maharashtra, parts of Uttar Pradesh, some parts of West Bengal, Jharkhand and Orissa. This does indicate the continued transmission of the disease in these areas.

The exact mode or mechanism of transmission is not known. It is believed that the main reservoir of *M. leprae* are the highly bacillated lepromatous patients, but occasionally, also the sparsely bacillated tuberculoid cases may be a source of infection. Inanimate objects or fomites like the articles used by infectious patients can theoretically spread infection, but sputum or nasal mucus and sneezed droplets

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(Weddell and Palmere, 1963; Barton, 1974), particularly the infected dust particles or air-borne droplets discharged from a highly bacillated patient are more likely to propagate and spread the infection. The relationship between environment and transmission is also reported to be important in continued incidence of the disease (Lavania et al., 2006; Turankar et al., 2012).

Molecular typing using genetic markers are expected to be important in establishing strain specific polymorphisms in *M. leprae* which should be helpful for improving our understanding of the epidemiology of leprosy. The current approach of molecular typing largely involves locus-to-locus comparisons, such as microsatellite analysis (Groathouse et al., 2004). Unlike locus-directed methods of genotyping, current advances in technology do allow insertion, deletion, and single-nucleotide polymorphism detection on a genome-wide basis. For bacterial organisms that are not cultivable, it is difficult to obtain sufficient amounts of genomic DNA, particularly from clinical material. Since *M. leprae* cannot be cultured amplification-based techniques are potentially more applicable and preferred for *M. leprae* as compared to other organisms. Molecular typing is useful to study the global and geographical distribution of different strains of *M. leprae* and the potential application in studying the transmission dynamics. This would also provide some insight into the historical and phylogenetic evolution of the bacillus (Monot et al., 2005, 2009).

While the potential of different genotyping methods has been speculated, actual usefulness of these markers to identify genotypic differences has not been investigated adequately. Very little information about the genetic diversity among *M. leprae* is known from India which still has a large pool of infection. Using these targets, new molecular system/schemes need to be developed. Our study focuses on the identification of *M. leprae* strains from different parts of India by using 10 polymorphic loci short tandem repeats (STRs) and SNP subtyping.

2. Material and methods

2.1. Ethical approval

Informed consents were obtained from all the patients and the study was approved by the Organisation Ethical Committee of The Leprosy Mission, India.

2.2. Collection of specimens

Overall, 70 slit scrapings were obtained from BI positive leprosy patients who attended the Out Patient Department of TLM hospitals at Shahdara (Delhi), Naini (UP), Purulia (West Bengal) and Miraj (Maharashtra) during 2007–2010 (Fig. 1). Among these 70 samples 41, 14, 12 and 3 were from Shahdara, Miraj, Purulia and Naini respectively. All cases were diagnosed and classified as multibacillary (MB) type following standard clinical criteria (NLEP guidelines — http://nlep.nic.in/).

2.3. Isolation of DNA

DNA was isolated from slit scrapings by following a procedure described earlier (Jadhav et al., 2001). Smears collected in 1 ml of 70% ethanol 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 ethanol removal samples were kept 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. The reaction was terminated at 97 °C for 15 min. This lysate preparation was further used for PCR. PCR targeting the RLEP gene region was performed to confirm the diagnosis of leprosy. Reference *M. leprae* NHDP63 and *Thai 53* DNA (obtained from Colorado State University, USA) were used as positive controls and distilled water used as negative controls included in each set of experiment (data not shown).

2.4. Detection of VNTRs using PCR and fragment length analysis

The sequences of primer pairs as described by Gillis et al. (2009) for the 10 loci (AC8b, GTA9, GGT5, 21-3, AC9, AC8a, 27-5, 6-7, 12-5 and 23-3) were listed in Table 1a. PCR was performed using the Hotstart-PCR kit (Qiagen). Each reaction mixture (20-μl final volume) was comprised of 10 μl of 2× Qiagen PCR master mix, 2 μl Q solution, 2 μl (each) of the forward- and reverse-primer (described by the IDEAL group[2]) working stocks, and 2 μl of template DNA; the volume was adjusted with distilled water. The final concentration of each primer was thus 0.2 μM. Following an initial denaturation step at 95 °C for 15 min, 40 cycles of PCR were run as follows: denaturation at 94 °C for 30 s, primer annealing at 60 °C for 90 s, and primer extension at 72 °C for 90 s. The PCR was terminated with a final extension at 72 °C for 10 min. 5 μl of PCR products were electrophoresed in a 2% agarose gel (Sigma, India) using Trisborate EDTA buffer (1×) at 50 V constant current for approximately 2 h. After electrophoresis DNAs were further sent for commercial fragment length analysis (FLA) to Xplorigen Technologies Ltd., Delhi, India.

2.5. SNP typing

SNP loci 1, 2, and 3 of *M. leprae* (nucleotide positions 14676, 1642875, and 2935685, respectively, on the sequenced TN strain) were amplified using previously reported primer sequences (Monot et al., 2005). Details of primers used for genotyping are given in Table 1b. 20 μl of reactions contained *M. leprae* DNA from different samples, 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 1.5 U Taq DNA polymerase (Qiagen, India) and 200 nM of forward and reverse primers. PCR conditions were similar as per Monot et al. (2005).

After PCR amplification restriction digestion assays were done according to the protocol described by Sakamuri et al (2009) using SmlI, CviKI-1, and BstUI (New England Biolabs, MA) for SNP loci 1, 2, and 3, respectively. The PCR products (5 μl) were digested with 1 unit of the enzymes. The SmlI, BstUI, and CviKI-1 reactions were performed at 55 °C, 60 °C, and 37 °C, respectively, for 1 h. The SmlI- and BstUI-uncut and -cut DNAs were subjected to electrophoresis on 3% agarose gels. The gels were stained with ethidium bromide and visualized by UV transillumination using a gel documentation system.

2.6. PCR for SNP sub typing

Subtyping of SNP loci 1 (nucleotide positions 8453, 313361, 61425 and 1642879 respectively) were amplified using previously reported primer sequences as mentioned in Table 1b (Monot et al., 2009).

The reaction mix (25 μl) consisted of 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 1.5 U Taq DNA polymerase (Qiagen, India), 200 nM of each primers and 2 μl DNA sample. PCR was carried out using initial denaturation at 95 °C for 5 min followed by 40 cycles consisting of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min, with a final extension at 72 °C for 10 min in a thermal cycler. PCR products were submitted for commercial sequencing (The Centre of Genomic Application, Delhi, India). Sequence data were analyzed by using CodonCode Aligner.

[2] IDEAL (Initiative for Diagnostic and Epidemiological Assays for Leprosy) group — we are one of the member of this consortium. The IDEAL consortium consists of thirty leprosy research groups, half of which is established in countries where leprosy still occurs.