Distribution of Mycobacterium leprae Strains among Cases in a Rural and Urban Population of Maharashtra, India

Sanjana Kuruwa, Varalakshmi Vissa, and Nerges Mistry

The elimination of leprosy continues to be a challenge, with the disease remaining endemic in several countries. India accounts for the highest number of cases, and the identification of child cases indicates recent transmission. Genetic markers, like variable-number tandem repeats (VNTRs) and single-nucleotide polymorphisms (SNPs), have been identified to track transmission of the pathogen Mycobacterium leprae. They were used to describe M. leprae strains detected in 48 skin biopsy specimens from leprosy patients in the state of Maharashtra in western India in rural and urban areas near Mumbai. Ninety-three percent of strains across both settings belonged to the SNP type 1D, with three of SNP type 1B being identified in patients living within 3 km of each other. The VNTR profiles of the Maharashtrian strains clustered with those from Southern India reported previously and a few other Asian strains, indicating that the Indian strains are genotypically conserved at the level of many VNTR loci. Taken together, SNP and VNTR markers are sufficiently reliable and suitable for both localized and broad geographical genotype associations. VNTR profiles of additional cases may aid in distinguishing the SNP type 1B and 1D strains.

Global efforts to eradicate leprosy have been largely successful in controlling its spread. Despite these efforts, the disease remains endemic in some countries, with 244,849 cases reported globally in 2009 (24). India accounted for 54.6% of the cases reported in 2009, emphasizing the need for greater scrutiny of its epidemiology. India has achieved an 80% reduction in the prevalence of leprosy; however, its rate of incidence has remained constant over the past 3 years. In India, 184,000 cases were reported in 2009, about 9% of which were children below the age of 14 years (12). The state trends in leprosy show Maharashtra as one of the states which continues to report a high number of newly detected cases in some districts (12, 13).

In addition to this, a survey conducted by the Foundation for Medical Research (FMR) in a rural (Panvel) and an urban (M east ward) area of western Maharashtra identified significant numbers of previously undetected cases, 30% of which were among children, indicating the possibility of a recently acquired infection (18). Thus, the difficulty of tracing the origins of leprosy and its complicated modes of transmission is compounded by the lack of active case detection systems and the ambiguity of the incubation period.

Identifying strains of Mycobacterium leprae in these areas may reveal such chains of transmission. Currently available knowledge of the M. leprae genome has revealed a very high degree of conservation with a limited number of identified genetic markers, which may aid in genotyping M. leprae strains. Among the markers identified, variable-number tandem repeats (VNTRs) with a broad range of allelic diversity have been shown to be effective in tracking strain transmission within and among strains in different regions of the world (1, 3, 9, 16, 17, 19, 21, 22). In addition to these, single-nucleotide polymorphisms (SNPs), which have revealed a global pattern of distinct types of M. leprae strains (SNP types 1 to 4), have been identified (10). These 4 SNPs have been further categorized into 16 subtypes designated SNP 1A to -D, SNP 2E to -H, SNP 3I to -M, and SNP 4N to -P (11).

In the Philippines, a relationship between the VNTR alleles at two loci, 21-3 (1 or 2 copies) and (GGT)5 (4 copies), and SNP type 3 was observed (16), while the association between the 27-5 and 12-5 alleles was highly correlated with SNP type 3 or 4 in South America (3). Several such signature allele combinations between different markers in different regions exist, which allows their separation into clusters (20). Other loci then add to the strain resolution.

There have been rapid advances in molecular epidemiology techniques, including strain typing (4, 5, 25). In this context, M. leprae strain types from patients detected in two locales of active transmission in India were surveyed. An urban and a rural population near the city of Mumbai, in western Maharashtra, were studied using VNTR and SNP. This was done to assess their discriminatory potential, which may aid in determining markers effective for tracking the transmission of leprosy and any associations between these markers. The genetic profiles of M. leprae from these patients were compared between areas and between the rural and urban groups, as well as to a global database of strain types.

MATERIALS AND METHODS

Patient samples. A survey conducted by the FMR in a rural and an urban area, both located in western Maharashtra, between June and December 2007 identified 199 undetected leprosy cases (18). This survey covered 5 rural Primary Health Centres (PHCs), viz., Apta, Ajivali, Nere, Vavanja (these areas are referred to as Rural 1, Rural 2, Rural 3, and Rural 4, respectively), and Gawat (no samples were obtained from this area), and 9 urban health posts (all urban cases were analyzed as a group). Each PHC covers a total population of 20,000 to 30,000, among which these cases were identified. Of 199 cases detected, 109 were subjected to a skin biopsy.
which was used for strain typing and to ascertain the diagnosis of leprosy, and were classified as multibacillary (MB) or paucibacillary (PB) on the basis of clinical examination. These 109 cases included 64 males and 45 females whose details are summarized in Table 1.

These 109 cases were identified across an area covered by 4 rural PHCs in Panvel and 6 urban health posts in the M east ward. The 4 rural PHCs included 65 cases (25 PB and 40 MB). These included 7 PB and 8 MB child cases from Panvel.

Forty-four cases (28 PB and 16 MB) were detected in the urban M east ward, which included 11 PB and 4 MB child cases.

In addition, archived *M. leprae* isolates from 2 rural patient biopsy specimens, which were amplified in mouse footpads (MFP), were also typed.

**Ethics review.** While written informed consent was obtained from the patients prior to collection of the biopsy specimens, consent of a parent or guardian was taken in child cases (less than 14 years old). This study was approved by the Institutional Ethics Committee (FMR-IEC-LEP01-2007).

**DNA extraction.** A piece (40 to 60 mg) of each biopsy specimen collected in RNAlater (Ambion, USA) was used as a source for *M. leprae* DNA for strain typing.

Total DNA was extracted using the DNeasy blood and tissue kit (Qiagen, Germany) according to the manufacturer’s instructions. A negative “buffer-only” control was included for every five biopsy samples at each step of the extraction procedure. Aliquots (20 µl) of these extracted DNAs were prepared and stored until use. Reference *M. leprae* NHD63 DNA prepared from bacteria from infected armadillo liver or spleen was obtained from Colorado State University (CSU) (5).

**Amplification and detection of VNTRs using multiplex PCR and fragment length analysis (FLA).** A panel of 17 VNTRs were assessed as described previously (9), except the ML 1 locus. VNTRs that did not amplify in the multiplex PCR were retested with single primer sets using the Hot Start *Taq* PCR kit (Qiagen) for amplification under the same PCR conditions as for multiplex PCR. Following PCR, the samples were diluted 1:40 and subjected to capillary electrophoresis on the Applied Biosystems 3130 Avant Genetic Analyzer at Sankara Nethra, Chennai, India. After separation, the electropherograms were visualized and analyzed by two individuals using PeakScanner version 1.0 software (Applied Biosystems, USA) to determine the allele for each VNTR locus. PCR-amplified products of both MFP and 5% of the samples were also tested at CSU.

**SNP typing.** Previously identified *M. leprae* SNPs at positions 14676, 1642875, and 2935685 (designated positions 1, 2, and 3, respectively) on the sequenced TN strain (2) were amplified and classified using a previously reported restriction fragment length polymorphism (RFLP) protocol (15). Five percent of the samples were also sequenced at Sankara Nethralaya, Chennai, India. After separation, the electropherograms were visualized and analyzed by two individuals using PeakScanner version 1.0 software (Applied Biosystems, USA) to determine the allele for each VNTR locus. PCR-amplified products of both MFP and 5% of the samples were also tested at CSU.

**RESULTS**

**VNTR strain typing of *M. leprae* in rural and urban leprosy patients from Maharashtra.** DNA was extracted from 109 (65 rural and 44 urban) samples, as well as 2 archived MFP amplified samples (Table 2). Within this set of 109 patient biopsy specimens, gender was found to be significantly associated with the clinical classification of cases, with males being associated with an MB profile and females with PB (*P* = 0.006) (Table 1).

No deviation was observed between the results obtained at the FMR and those obtained at CSU for quality control except at 1 locus, (TA)18, which has been reported to be prone to stuttering (9).

Among those typed, 10 samples were fully typed, 12 had data missing at 1 locus, 14 samples failed to amplify at 2 to 5 loci, and 13 yielded data at fewer than 10 out of 16 loci. Among the 13 cases that were typed at fewer than 10 loci, 5 could be included in N-N analysis. Cases that could not be typed did not amplify in at least 9 VNTR loci of the 14 required for N-N analysis despite repeated attempts. Thirty-one rural and 10 urban samples were typed at a minimum of 9 of 14 loci. Four samples were typed by SNP markers but not by VNTR due to missing data at some loci.

**VNTR loci** (GGT)5, 6-3a (rpoT), 21-3, and 23-3 were non-morphic in the studied population with alleles of 4, 3, 2, and 2, respectively. The observed allele numbers for each of the 12 polymorphic VNTRs [except the (TA)18 locus] are presented in Table 3. Table 4 summarizes the strain types across 16 loci.

**SNP typing of *M. leprae* strains in rural and urban leprosy patients from Maharashtra.** All 45 samples assessed belonged to SNP type 1, which is predominant in India and Southeast Asian countries (10, 11). Subtyping revealed that 37 of these belonged to SNP subtype 1D, with 3 isolates from the rural area belonging to subtype 1B (Table 4).

Among the 4 samples for which an SNP type was available but that could not be typed by VNTR, 3 were SNP 1D and 1 rural sample was SNP 1B. Two of the subtype 1B samples (case no. 35

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**Table 1 Age and gender distribution of cases included in this study**

<table>
<thead>
<tr>
<th>Age group (yr)</th>
<th>Urban</th>
<th>Rural</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Under 14</td>
<td>8</td>
<td>12</td>
<td>20</td>
</tr>
<tr>
<td>15–25</td>
<td>5</td>
<td>9</td>
<td>14</td>
</tr>
<tr>
<td>26–60</td>
<td>11</td>
<td>16</td>
<td>27</td>
</tr>
<tr>
<td>Above 60</td>
<td>0</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
<td>40</td>
<td>64</td>
</tr>
</tbody>
</table>

**Table 2 Numbers of successfully typed samples across the clinical spectrum**

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Case classification</th>
<th>Rural</th>
<th>Urban</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MB</td>
<td>38</td>
<td>22</td>
<td>60</td>
</tr>
<tr>
<td>2</td>
<td>PB</td>
<td>25</td>
<td>6</td>
<td>31</td>
</tr>
<tr>
<td>3</td>
<td>Relapse (MB)</td>
<td>5</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>MFP amplified</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>67</td>
<td>31</td>
<td>98</td>
</tr>
</tbody>
</table>
and 37) belonged to the same village and 1 (case no. 41) to a different village about 3 km away.

Comparative analysis of M. leprae genotypes based on VNTR. Nei’s index of diversity (14) was calculated and compared for each of the 16 loci among different PHC areas and rural and urban samples. Western Maharashtra VNTR profiles were also compared to global VNTR profiles as recorded in the M. leprae database (1, 3, 4, 7, 9, 16, 19, 21, 22). The 18-8 locus was not assessed in the urban samples, as it failed to amplify in all samples. The (TA)18 locus was not included due to inconsistencies in analysis. Commonly occurring alleles for each locus are shaded. Note that VNTR loci (GGT)5, 6-3a (rpoT), 21-3, and 23-3 were nonpolymorphic in the studied population with alleles 4, 3, 2, and 2, respectively.

This may indicate consistency of allele frequencies within populations across most loci (Table 5). The variability in diversity between all samples and fully typed samples may be attributed to missing data and, in the case of urban areas, to nonamplification at the 18-8 locus. The diversity was calculated separately for each rural area, but most urban profiles were collectively assessed as a group, since the total number of urban samples was low. Analyzing diversities between the rural and urban groups revealed similar indices across all loci except the 12-5 locus (a 5-copy allele that was detected only in Rural 1 and Rural 2). On a global scale, the diversity indices of these samples were comparable to those in India and Southeast Asia.

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The VNTR profiles obtained in this study were compared to VNTR profiles from a published M. leprae VNTR database (1, 3, 4, 7, 9, 16, 19, 21, 22) using the modified structure–nearest-neighbor method as described previously (6). Nine strain types clustered within the respective groups rather than with a strain type in the reference database, while 3 from the rural and urban groups were related to strains from the urban and rural groups, respectively. On the other hand, 16 strains preferentially clustered with strains from patients enrolled within India but outside Maharashtra and 5, 3, and 1 isolates with strains from Thailand, Colombia, and the Philippines, respectively. One isolate was similar to a strain from the rural group and one from another location in India, while another urban sample had similarity to a Thai strain and a Colombian strain (Table 6).

Five out of 7 samples from Rural 2 were noted to be distinct from others in Panvel (rural) and M east ward (urban), as they clustered with Thai strains on the basis of VNTR. This may be partly associated with the 12-5 VNTR, which showed 5 copies in most of these samples (5 samples showed 5 copies, 2 samples showed 4 copies, and 1 sample had data missing for this locus), whereas most other samples showed 4 copies (P = 0.01).

All samples that were segregated by N-N analysis were MB cases (n = 32), with the exception of 9 PB cases. The strain types from the 9 PB cases clustered with Indian (5 cases) and Thai (4 cases) M. leprae strains. An MST was constructed using VNTR profiles across 16 loci for samples used for N-N analysis in order to represent clusters. The MST also separated Rural 2 samples that showed 5 copies of the 12-5 locus (Fig. 1).

In this study, typing of samples across the disease spectrum revealed no association between the strain type and a particular clinical form of leprosy or smear positivity, as the distribution of all sample types was mixed and spread across the groups.

### DISCUSSION

The markers used for identification of M. leprae strains have shown extremes in their discriminatory potentials. A few SNPs are capable of revealing a global pattern of deeper lineages (11). On the other hand, multilocus VNTRs prove useful in tracking transmission within communities across short distances and in separating strains within and between countries (1, 3, 16, 17, 19, 21, 22, 23).

The objectives of this study were to compare our strains to a global strain type database, to identify the closest strain types using globally conserved SNP markers, and eventually to compare strain types internally within the studied population with VNTRs. The isolates studied in a western region of Maharashtra demonstrated a relationship between the strain type and the geographical region (Table 6).

Three SNP 1B isolates were isolated from cases residing within a distance of 3 km, indicating the possibility of a common source of transmission. Their VNTR profiles showed similarity to a common SNP 1D sample obtained from another area (case no. 37 and 41 showed case no. 43 as the closest match). This indicates similarity in VNTR profiles but a lack of relationship between the VNTR profile and the SNP type, which may be attributed to missing data, as sample 43 had missing data at 5 loci (Table 4).
On the basis of diversity indices, VNTR profiles from western Maharashtra were similar to those from India and Southeast Asia. This may have varied with a greater sample size, including missing loci, particularly at the 18-8 locus. The (TTC)21, (AC)9, (GTA)9, and (AT)17 loci had high discriminatory indices in the present study area that was considered for the N-N analysis, indicating that, although suitable for VNTR classification on a global scale, they may not be suitable for characterizing samples in the present study area.

When these isolates were compared to the groups deduced using N-N analysis on the global database (7), most samples were similar to Indian profiles (Table 6). Additionally, most samples from the Rural 1, Rural 3, and Rural 4 PHC areas showed a sample from India as their nearest neighbor, whereas those from Rural 2 had VNTR profiles similar to those of isolates from Thailand. This indicates a distinction between Rural 2 strains and those from the other PHCs.

The distribution of M. leprae strains in Maharashtra, India is presented in Table 4. The table includes VNTR and SNP profiles of M. leprae isolates from Maharashtra.

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Clinical class</th>
<th>BI</th>
<th>Area</th>
<th>No. of samples for locus:</th>
<th>SNP Subtype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### TABLE 4

**VNTR and SNP profiles of M. leprae isolates from Maharashtra**

- **Typed by VNTR**
- **Typed by SNP**
- **Partially typed samples**

*Blank cells failed to amplify. SNP 1B isolates are highlighted. BI, bacterial index.*

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panvel and eastward India 3
Rural area/south India 1
Urban areas 7
Thailand 3
Colombia 2
Total 31
East ward (urban) India 3
Rural areas 3
Urban areas 2
Thailand/Colombia 1
Philippines 1
Total 10

acknowledgments
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We gratefully acknowledge Vanaja Shetty, Anju Wakade, and Fatema Kambati (FMR) for the supply of M. leprae-infected human and mouse tissues and Pushpendra Singh (École Polytechnique Federal de Lausanne, Lausanne, France) for helpful discussions on SNP typing. Anirvan Chattterjee (FMR) and Rama Murthy Sakamuri (Colorado State University)

Table 5: Diversity indices of individual VNTR loci and M. leprae haplotypes in Maharashtra

<table>
<thead>
<tr>
<th>Locus</th>
<th>Geographical origin of case</th>
<th>Geographical origin of nearest neighbor</th>
<th>Total no. of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panvel (rural)</td>
<td>South India</td>
<td></td>
<td>13</td>
</tr>
<tr>
<td>Rural area/south India</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Rural areas</td>
<td></td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>Urban areas</td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Thailand</td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Colombia</td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>31</td>
</tr>
<tr>
<td>M east ward (urban)</td>
<td>India</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Rural areas</td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Urban areas</td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Thailand/Colombia</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Philippines</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>10</td>
</tr>
</tbody>
</table>

Panvel and eastward India appear to be partly associated with the 12-5 VNTR locus, although significance has been estimated for only 8 samples and needs to be verified with testing of more samples. Additionally, a higher number of copies of (GTA)9, ranging from 9 to 17 copies, were observed in this area, which showed that the 9-copy allele occurred most frequently in the other areas. Such differences in allele copy numbers may be useful in classifying samples from areas where diversity is variable. This was corroborated by the construction of an MST (Fig. 1).

We were able to verify the similarity of strains across both rural and urban samples in western Maharashtra, as the samples belonged mostly to SNP type 1D. Nearest-neighbor analysis showed that most of the samples were similar to other isolates from India, with about 46% of rural samples and 50% of urban samples being genetically similar to other samples from Maharashtra. Genetic similarity among samples from both rural and urban areas on the basis of both VNTR and SNP has been observed. However, we were unable to track transmission among familial contacts due to smear negativity in many cases that could not be successfully typed.

The samples had varying degrees of identity to those from the same region when assessed only on the basis of VNTR, using select VNTRs to infer the result (Table 5). Identifying isolates on the basis of SNP type and subtype added a level of discrimination that would aid in giving a broader classification. A subsequent VNTR analysis would provide further discrimination. In addition to this, no distinctive VNTR pattern was observed to segregate SNP types 1B and 1D (Fig. 1). More SNP 1B samples and such polymorphic VNTR loci need to be studied in combination in order to identify such associations.

Some loci showed allele profiles similar to those observed across India, such as 4 copies of (GGT)5 and 27-5, 3 copies of rpoT, and 2 copies of 21-3 and 23-3. Based on 3 samples detected, no particular VNTR allele was identified with the SNP 1B type. A combination of both VNTR and SNP is better suited to provide a more accurate classification of strains, even across short ranges of transmission.

Genotypes showed similarity to Southeast Asian strains by N-N analysis, which included some loci that were conserved in western Maharashtra. The alleles observed at these conserved loci may result in these strains being classified within the Indian sample set of the global database. The polymorphic loci, in addition to missing data, classified each strain as unique when all 16 VNTRs were used.

Overall, the genotypes that were recorded in this study came predominantly from males (71.5%). This may be explained by the fact that most females were diagnosed with a PB clinical profile, which would yield a smaller amount of M. leprae DNA.

Finally in accordance with our study, SNP 1A/B/C/D have been reported in India and other Southeast Asian countries (11). These results are also in agreement with studies carried out in China, where genetic profiles of samples differ between the north and south but are conserved with respect to their SNP profiles (type 3) (22, 23, 25).
This study captures M. leprae strain patterns from 2 areas in Maharashtra showing a high prevalence of leprosy. However, complementation of conventional and modern epidemiological tools with molecular techniques is likely to be useful in the further clarification of regional transmission patterns of M. leprae.
FIG 1 A minimum spanning tree was constructed using VNTR profiles to represent clusters. Rural 1, Rural 2, Rural 3, and Rural 4 are the rural areas. Samples from urban areas were collectively analyzed as the urban group. SNP type 1B and isolates from Rural 2 with a 5-copy allele of the 12-5 locus are specified. Each sample is represented as having a distinct profile, which may be attributed to polymorphic VNTR loci, as well as missing data at some loci.

helped significantly in the standardization and quality control aspects of genotyping. We also acknowledge the involvement of N. Soumittra and Sankara Nethralaya (Chennai, India) for performing FLA and sequencing of samples.

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