Intrapatient comparison of *Mycobacterium leprae* by VNTR analysis in nasal secretions and skin biopsy in a Brazilian leprosy endemic region

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Summary
Background: This study compares the strains of genotypes of *M. leprae* from nasal secretions (NS) and skin biopsy (SB) in the same patient, supplementing conventional epidemiology to gain insight into the infection of leprosy in Fortaleza, Brazil.

Methods: The sample consisted of 38 newly diagnosed leprosy patients attending the National Reference Center of Dermatology Dona Libania (CDERM), in Fortaleza, who tested positive for *M. leprae* by PCR in DNA extracts of nasal secretions. DNA was also extracted from skin biopsy (SB) scrapings of each patient and used for multiplex PCR amplification of *M. leprae* VNTR loci. The number of repeats at 15 loci were determined by the fragment length analysis method.

Results: Locus VNTR genotypes were achieved in 38 NS, and in 38 SB specimens. *M. leprae* strains differed in their genotypes in paired specimens in all but two of 38 patients. The genotype similarity in the remainder ranged from 53% to 87%.

Conclusion: *M. leprae* 15 VNTR loci genotypes of paired nasal and biopsy skin samples from five patients were identical, while as many as seven loci differed in the 33 other patients. When the NS and biopsy genotypes were pooled and compared, it was found that there was a great variability among different VNTR markers. It is important to investigate other molecular markers suitable for typing genetic variations of the bacilli.

Keywords: leprosy, VNTR loci, nasal cavity, biopsy, genotype

Introduction

Leprosy is a public health problem in Brazil. In 2013, a new case detection rate of 3.78 cases/100,000 inhabitants was reported globally.\(^1\) In the same year, the State of Ceará reported the 2,069 new cases, with an overall new-case detection rate/100,000 population of 24/100,000. In 2015, only 36 out of the 184 municipalities in Ceará did not report new cases, and 18 were considered hyperendemic (over 40 new cases per 100,000 population).\(^2\) In 2014, Fortaleza, the capital of Ceará State, had a coefficient of detection of new cases of 24.4/100,000.\(^3\) The factors contributing to these high rates of incidence are poorly understood, requiring additional tools and knowledge to address questions of source, routes and mode of infection and transmission. In the last few years, the conventional epidemiology of leprosy has been supplemented by *M. leprae* genotyping, to aid in addressing such gaps. A more accurate description of leprosy transmission can then be provided by a synthesis of both molecular and epidemiological data, leading to more effective control of the disease. The first reported as a genetic marker in *M. leprae* was an intragenic single tandem repeat (STR) of six base pairs in the *rpoT* gene that showed two alleles.\(^3\) The repetitive trinucleotide, TTC, in a pseudogene was the second marker, which was detected as allelic variations from 10 to 37 repeats.\(^4,5\) Other researchers initiated a more comprehensive analysis of other potential STRs in *M. leprae*. Following, unique patterns have emerged and several independent loci of STRs from clinical samples have been evaluated. Thus, approximately 50 VNTR loci have been
identified in *M. leprae*.\textsuperscript{3,5–10} Kimura et al.\textsuperscript{11} reported the development of a test and reproducibility of the multilocus variable number of tandem repeat analysis (MLVA) of *M. leprae* in armadillos and strains derived from clinical materials. These authors have standardised amplification of 17 loci in four combinations of multiplex PCR. All combinations were optimised with *M. leprae* DNA from the strain NHDP63 obtained from infected armadillo tissue, and since then, this DNA has been used as a reference for mapping VNTR.

The study of genetic diversity of *M. leprae* is of extreme importance. The genotyping methods can provide insights into the leprosy transmission network within populations and in addition, genomic markers can be experimentally evaluated in the population of interest. Studying the *M. leprae* genotypes in nasal secretions provides additional knowledge towards assessing the strains harboured by patients in the state of Ceará. NS specimens are collected in a less invasive manner than through skin biopsy (SB). In this study, our aim has been to apply MIRU-VNTR loci typing to compare *M. leprae* isolates from the same patient, from NS and SB.

**Materials and Methods**

**SUBJECTS**

A cross-sectional study was conducted from June 2009 to December 2010 in Fortaleza, north-east Brazil. During the period, a total of 1,103 new leprosy cases were diagnosed by specially trained dermatologists from the National Reference Center of Dermatology Dona Libania (CDERM). Cases were confirmed by clinical skin examination, skin smear and biopsy according to Ridley-Jopling\textsuperscript{12} criteria based on histological study, and bacilloscopic index (BI). Recruitment was conducted 2 days per week. December, January, July and holidays had reduced recruitment. A total of 185 consenting new leprosy cases were enrolled in the study, and were tested using *M. leprae* RLEP PCR\textsuperscript{13} from nasal samples. Out of the 185 cases, 128 were RLEP PCR positive; of these 38 cases were taken NS and SB biological specimens within the same patient and processed for genotyping. At the same time, nasal and biopsy specimens were taken from each patient.

**ETHICAL CONSIDERATIONS**

All participants signed an informed consent form and authorised the collection of samples. This project was approved by the CDERM Ethics Committee and guidelines of the National Ethical Committee were followed to conduct the research.

**EPIDEMIOLOGY SURVEY, SPECIMEN COLLECTION, AND DNA EXTRACTION**

A standardised questionnaire was completed for all leprosy cases to collect demographic, socioeconomic, environmental and behavioural data.

NS were obtained from all participants by gently rubbing a perinasal swab previously wetted with Tris-EDTA buffer (pH 8.0), in one side of each nostril over the lateral conchae. After collection, each swab was immersed in a sterile and labeled tube and stored at $-$20°C until processing. DNA was extracted as described in Lima et al.\textsuperscript{14}
The SB samples were collected through a 5 mm punch. The removed tissue was placed in a sterile 1.5 mL tube and stored at $-20^\circ$C. DNA was extracted by using the DNeasy Blood & Tissue kit (Qiagen) following the manufacturer’s guidelines.

**M. leprae-specific repetitive element (RLEP) PCR**

DNA from nasal biological specimens of all participants were searched for a 238-pb fragment of RLEP2 in a nested PCR reaction following the protocol described in Lima et al.\textsuperscript{14} The PCR products were purified using the QIAquick PCR Purification Kit prior to sequencing in an ABI Prism 3730 automated DNA sequencer using the ABI PRISM BigDye Terminator v 3.0 sequencing kit (Applied Biosystems). The sequences were identified using SecScape software v.2.7 (Applied Biosystems). A reference RLEP2 sequence (GenBank accession NC002677) was used to align the sequences.

**Single nucleotide polymorphisms (SNP) typing of M. leprae**

To amplify three SNP loci, 1, 2 and 3 at nucleotide positions 14676, 1642875 and 2935685 on the sequenced TN strain, *M. leprae* genomic DNA (RLEP-positive samples) was amplified and classified using a previously reported restriction fragment length polymorphism (RFLP) protocol.\textsuperscript{15,16} The genotypes were classified into SNP types 1/2, 3 and 4.

**MLVA (multi locus VNTR analysis)**

SB and NS were used to study the genotypic variability of *M. leprae* in the same individual by analyses of 15 VNTR loci [(AT)\textsuperscript{17}, (GGT)\textsuperscript{5}, (GTA)\textsuperscript{9}, (AC)\textsuperscript{8b}, (AC)\textsuperscript{8a}, (AT)\textsuperscript{15}, (AC)\textsuperscript{9}, 21-3, (GAA)\textsuperscript{21}, (TA)\textsuperscript{18}, 6-7, 27-5, (TA)\textsuperscript{10}, 23-3 and 12-5]. VNTR was performed by amplifying the 15 MIRU-VNTR loci as described elsewhere with some modifications.\textsuperscript{11,17} The VNTRs were separated into four combinations for multiplex PCR. For each locus one primer was 5’ fluorescently labeled.

The PCR amplifications were performed using the Qiagen Multiplex PCR kit (Qiagen) in a final volume of 20 $\mu$L containing 10 $\mu$L of 2 $\times$ Qiagen Multiplex PCR Master Mix, 2 $\mu$L of 5 $\times$ Q solution, 2 $\mu$L (each) of a 0.2 $\mu$M mix of forward and reverse primers and 2 $\mu$L of template DNA. Final volume was adjusted with sterile distilled water. Identical thermocycling conditions were used for all VNTRs: an initial denaturation at 95$^\circ$C for 15 minutes followed by 40 cycles of 94$^\circ$C for 30 seconds, 60$^\circ$C for 90 seconds, 72$^\circ$C for 90 seconds followed by a final elongation step of 72$^\circ$C for 10 minutes.

**Analysis of fragments**

PCR products were diluted 30–60 times in water and then 1 $\mu$L was sized on a ABI 3130 Genetic Analyzer (Applied Biosystems) using 0.3 $\mu$L of GeneScan™ 500 LIZ® size standard (Applied Biosystems) and 8.7 $\mu$L deionized formamide.

The material was denatured at 94°C for 5 minutes and subjected to capillary electrophoresis (length 50 cm, polymer POP-7) by applying a voltage of 1 kV for 22 seconds. Capillary electrophoresis was carried out at 15 kV over 60°C with a running time of 45 minutes.\textsuperscript{11,17}
The electropherograms were visualised and analysed and the number of repetitions of the 15 loci VNTRs were determined using Peak Scanner™ software version 1.0 (Applied Biosystems).

The allelic diversity \( h \) at a given MIRU-VNTR locus was calculated as

\[
h = \frac{n}{(n - 1)} \times 1 - \sum x_i^2,
\]

where \( x_i \) is the frequency of the \( i \)th allele at the locus and \( n \) is the number of isolates.\(^{18}\)

**Results**

**GENOTYPING RESULTS USING BIOPSY AND NASAL SAMPLES**

Since \( M. leprae \) is not cultivable, the identification of intrapatient variability was based on MIRU-VNTR screening in order to identify clonal complexity directly from the bacilli containing specimens. Paired nasal and biopsy biological specimens taken from 38 leprosy cases were analysed by 15 MIRU-VNTR loci. Genotyping of the isolates from our study revealed noticeable intrapatient variability in seven cases (Table 1). No associations between genotype and demographic, socioeconomic, environmental and behavioural data were observed.

**FREQUENCY OF GENOTYPES DEFINED BY SNP ANALYSIS AND BI**

We obtained conclusive SNP genotypes for all biological specimens of the 38 patients. SNP genotype 4 was predominant (\( n = 35, \) 92.1%), genotype 3 was identified in two patients (5.3%) and genotype 1/2 was detected less frequently in only one case (2.6%). The distribution of each SNP genotype is demonstrated in Table 1. The same genotype was obtained for NS and SB of the same patient. The BI ranged from 1.4 to 6.0, median of 4.56 ± 0.94 (Table 1). No correlation was found between BI, SNP typing and VNTR genotypic diversity.

**ANALYSIS OF THE VNTRS MARKERS**

In intrapatient genotypic comparison of NS and SB samples from the same individual, some loci of dinucleotide repeats [(TA)10, (AT)17, (AT)15, (AT)18, and (AC)8b] of samples of NS did not amplify. Also allelic variations at one or more loci were observed. Differences were seen due a lost or gain of one or more repeat units.

**INTRAPATIENT ALLELIC DIVERSITY ACCORDING TO MLVA**

Table 2 shows the allelic diversities of the 15 MIRU-VNTR loci based on the total of samples.

For the SB the loci (AT)18, (AT)15, (AT)17, (GAA)21, and (GTA)9 were highly discriminative (≥0.6), loci (AC)8a, (AC)9, (AC)8b, 6-7, and (TA)10 were moderately discriminative (≥0.3), and loci 27-5, 12-5, (GGT)5, 21-3, and 23-3 were poorly discriminative (<0.3) when ranked according to the method of Sola et al.\(^{19}\) The allelic diversities for NS were similar to the BS, excepting to loci 27-5 that was moderately discriminative (≥0.3). When observing the discriminatory power of the different STRs, highest allelic diversity was presented at locus (AT)18, presenting 11 different
Table 1. Comparison of VNTRs from different body sites: Nasal Secretions (NS) and Skin Biopsy (SB) from the same patient

| Sample ID | Sample type | BI SNP | (AT)17 | (GGT)5 | (GTA)9 | (AC)8b | (AC)8a | (AT)15 | (AC)9 | 21-3 | (GAA)21 | (TA)18 | 6-7 | 27-5 | (TA)10 | 23-5 | 12-5 | Number of allele differences |
|-----------|-------------|--------|--------|--------|--------|--------|--------|--------|--------|------|--------|--------|------|------|--------|------|------|-----------------------------|
| 91845     | NS          | 4.0    | 4      | 11     | 4      | 10     | 7      | 10     | 16     | 8    | 2      | 9      | 20   | 7    | 5      | 8    | 2    | 4 | 7 |
|           | SB          | 4      | 17     | 4      | 9      | 8      | 8      | 9      | 16     | 8    | 2      | 15     | 23   | 6    | 5      | 8    | 2    | 4 |
| 87728     | NS          | 3.8    | 4      | 12     | 4      | 10     | NP     | 9      | 18     | NP   | 2      | 10     | NP   | 5    | NP    | 11   | 2    | NP | 6 |
|           | SB          | 4      | 18     | 4      | 10     | 8      | 8      | 25     | 7      | 2    | 19     | 23    | 6    | 5    | 8     | 2    | 4 |
| 88053     | NS          | 5.8    | 4      | 14     | 4      | 10     | NP     | 9      | 19     | 8    | 2      | 11     | 16   | 5    | 5      | 8    | 2    | 4 |
|           | SB          | 4      | 12     | 4      | 9      | 7      | 9      | 18     | 6      | 2    | 10     | 16    | 6    | 5    | 10    | 2    | 4 |
| 91979     | NS          | 4.0    | 15     | 4      | 10     | 8      | 9      | NP     | 7      | 2    | 14     | 16    | 7    | 5    | 8     | 2    | 4 |
|           | SB          | 4      | 13     | 4      | 9      | 7      | 9      | 16     | 7      | 2    | 11     | 14    | 6    | 5    | 8     | 2    | 4 |
| 86340     | NS          | 3.8    | 4      | 14     | 4      | 9      | 7      | 8      | 22     | 7    | 2      | 10     | NP   | 6    | 5      | 9    | 2    | 4 |
|           | SB          | 4      | 13     | 4      | 9      | 7      | 9      | 14     | 8      | 2    | 12     | 30    | 6    | 5    | 9     | 2    | 4 |
| 88119     | NS          | 4.0    | 1/2    | 12     | 4      | 13     | NP     | 10     | 15     | 7    | 2      | 10     | NP   | 6    | NP    | 5    | 2    | 4 |
|           | SB          | 1/2    | 16     | 4      | 7      | 7      | 10     | 20     | 7      | 2    | 14     | 18    | 7    | 5    | 8     | 2    | 4 |
| 88742     | NS          | 1.4    | 4      | 13     | 4      | 10     | 7      | 9      | 16     | 8    | 2      | 11     | 26   | 6    | 5      | 8    | 2    | 4 |
|           | SB          | 4      | 13     | 4      | 9      | 7      | 9      | 15     | 8      | 2    | 13     | 25    | 5    | 5    | 8     | 2    | 4 |
| 84267     | NS          | 4.6    | 4      | 13     | 4      | 10     | 8      | 8      | 16     | 8    | 2      | 12     | 18   | 6    | 5      | NP   | 2    | 4 |
|           | SB          | 4      | 12     | 4      | 9      | 7      | 9      | 16     | 8      | 2    | 12     | 18    | 6    | 5    | 8     | 2    | 4 |
| 86776     | NS          | 6.0    | 4      | 13     | 4      | 9      | 7      | 8      | 21     | 8    | 2      | 12     | 16   | NP   | 5      | NP   | 2    | 4 |
|           | SB          | 4      | 13     | 4      | 9      | 8      | 9      | 22     | 8      | 2    | 12     | 17    | 6    | 5    | 8     | 2    | 4 |
| 86962     | NS          | 3.8    | 4      | 17     | 4      | 10     | 8      | 8      | NP     | 7    | 2      | 17     | 19   | 6    | 5      | NP   | 2    | 4 |
|           | SB          | 4      | 25     | 4      | 10     | 8      | 8      | 34     | 7      | 2    | 16     | 14    | 6    | 5    | 8     | 2    | 4 |
| 87197     | NS          | 3.8    | 4      | 18     | 4      | 9      | NP     | 9      | NP     | 7    | NP    | 14     | NP   | 7    | NP    | NP   | 3    | 4 |
|           | SB          | 4      | 18     | 4      | 11     | 8      | 9      | 21     | 7      | 2    | 15     | 17    | 6    | 5    | 8     | 2    | 4 |
| 87669     | NS          | 5.2    | 4      | 11     | 4      | 12     | NP     | 9      | 19     | 7    | 2      | 12     | NP   | 6    | 4      | 8    | 2    | 4 |
|           | SB          | 4      | 15     | 4      | 12     | 7      | 9      | 17     | 7      | 2    | 15     | 17    | 6    | 2    | 8     | 2    | 4 |
| Sample ID | Sample type | BI SNP (AT)17 (GGT)5 (GT\(A\)9) (AC)8b (AC)8a (AT)15 (AC)9 21-3 (GAA)21 (TA)18 6-7 27-5 (TA)10 23-3 12-5 | Number of allele differences |
|-----------|-------------|--------------------------------------------------------------------------------------------------|-----------------------------|
| 90148     | NS 4.0 4   | 13 4 10 8 9 10 7 2 13 15 7 5 8 2 4 4                                                         | 4                           |
|           | SB 4 20 4  | 10 8 8 29 7 2 14 15 7 5 8 2 4                                                               | 4                           |
| 86855     | NS 5.5 4   | 18 4 10 8 8 NP 7 2 14 19 7 5 NP 2 4 3                                                        | 3                           |
|           | SB 4 14 4  | 10 8 9 25 7 2 14 20 7 5 8 2 4                                                               | 4                           |
| 87188     | NS 5.0 3   | 18 4 10 NP 9 NP 7 2 14 NP NP NP NP 2 3                                                       | 3                           |
|           | SB 3 20 4  | 10 8 8 28 7 2 14 17 6 5 8 2 4                                                               | 4                           |
| 87370     | NS 3.5 3   | 18 4 9 NP 9 13 8 NP 10 19 7 5 NP 2 5 3                                                        | 3                           |
|           | SB 3 19 4  | 9 7 9 12 8 2 10 19 7 4 10 2 5                                                               | 5                           |
| 87851     | NS 5.0 4   | 15 4 NP NP 8 16 7 2 12 15 6 4 8 2 4 3                                                        | 3                           |
|           | SB 4 16 4  | 15 7 8 16 7 2 12 14 6 5 8 2 4                                                               | 4                           |
| 88079     | NS 4.4 4   | 16 NP NP 8 NP NP NP 14 13 6 4 8 2 4 3                                                        | 3                           |
|           | SB 4 16 4  | 10 8 8 30 6 2 13 5 6 5 8 2 4                                                               | 4                           |
| 88689     | NS 5.0 4   | 16 4 10 8 8 NP 7 2 15 NP 7 5 8 2 4 3                                                        | 3                           |
|           | SB 4 17 4  | 10 8 8 22 7 2 16 25 6 5 8 2 4                                                               | 4                           |
| 88899     | NS 4.0 4   | 11 4 11 NP NP 20 NP NP NP 14 NP NP NP 9 2 NP 3                                                | 3                           |
|           | SB 4 13 4  | 9 8 8 22 7 2 14 19 6 5 9 2 4                                                               | 4                           |
| 88935     | NS 4.0 4   | 13 4 12 7 9 18 8 2 11 18 7 4 10 2 4 3                                                        | 3                           |
|           | SB 4 13 4  | 12 7 9 19 8 2 11 19 6 4 10 2 4                                                               | 4                           |
| 89160     | NS 4.0 4   | 11 4 10 7 8 16 8 2 11 NP 7 5 8 2 4 3                                                        | 3                           |
|           | SB 4 11 4  | 10 7 8 15 8 2 12 21 6 5 8 2 4                                                               | 4                           |
| 89167     | NS 4.4 4   | 12 4 9 7 9 15 8 2 12 17 5 5 9 2 4 3                                                        | 3                           |
|           | SB 4 12 4  | 9 7 10 14 8 2 12 17 6 5 9 2 4                                                               | 4                           |
| 85886     | NS 5.0 4   | 13 4 9 7 9 17 8 2 12 15 7 5 NP 2 4 2                                                        | 2                           |
|           | SB 4 13 4  | 9 7 9 18 8 2 12 17 7 5 8 2 4                                                               | 4                           |
| 87126     | NS 3.7 4   | 22 4 10 8 8 NP 7 2 13 17 7 5 NP 2 4 2                                                        | 2                           |
|           | SB 4 12 4  | 10 8 8 29 7 2 14 17 7 5 8 2 4                                                               | 4                           |
| Sample ID | Sample type | BI | SNP (AT)17 (GGT)5 (GTA)9 (AC)8b (AC)8a (AT)15 (AC)9 21-3 (GAA)21 (TA)18 6-7 27-5 (TA)10 23-3 12-5 Number of allele differences |
|-----------|-------------|----|--------------------------------------------------|
| 87214     | NS 5.3      | 4  | 18 4 10 8 8 NP 7 2 14 22 NP NP NP 2 4 2          |
|           | SB 4        | 18 4 10 8 8 33 7 2 13 25 6 5 8 2 4             |
| 87250     | NS 5.8      | 17 4 10 NP 8 NP 7 2 14 17 7 NP NP 2 4 2         |
|           | SB 4        | 17 4 10 8 8 26 7 2 15 16 7 5 8 2 4             |
| 87549     | NS 2.8      | NP 4 10 8 9 NP 7 2 NP 22 NP 4 NP NP NP 2        |
|           | SB 4        | 11 4 10 8 9 16 7 2 13 14 6 5 8 2 4             |
| 87914     | NS 6.0      | 16 4 10 8 8 16 7 2 14 18 6 5 10 2 4 2          |
|           | SB 4        | 16 4 10 8 8 15 7 2 15 18 6 5 10 2 4             |
| 89230     | NS 4.6      | NP 4 7 NP 9 NP 8 2 11 20 NP NP 8 2 NP 2         |
|           | SB 4        | 12 4 9 7 9 16 8 2 12 20 6 5 8 2 4             |
| 91796     | NS 5.5      | 13 4 9 7 9 16 8 2 11 25 7 5 8 2 4 2            |
|           | SB 4        | 13 4 9 7 9 16 8 2 11 21 6 5 8 2 4             |
| 92070     | NS 4.8      | 14 4 10 7 11 16 9 2 9 NP 7 5 10 2 4 2          |
|           | SB 4        | 14 4 10 7 11 16 9 2 9 26 6 5 8 2 4             |
| 86187     | NS 5.4      | NP 4 10 8 8 22 7 2 15 15 6 5 NP 2 4 1          |
|           | SB 4        | 21 4 10 8 8 23 7 2 15 15 6 5 8 2 4             |
| 86190     | NS 4.6      | 11 4 9 7 9 NP 8 2 11 21 6 5 NP 2 4 0          |
|           | SB 4        | 11 4 9 7 9 28 8 2 11 21 6 5 8 2 4             |
| 86555     | NS 5.0      | 11 4 10 7 9 16 8 2 12 21 6 5 NP 2 4 0        |
|           | SB 4        | 11 4 10 7 9 16 8 2 12 21 6 5 8 2 4             |
| 87156     | NS 5.8      | 10 4 9 NP 9 16 7 2 14 19 6 5 NP 2 4 0        |
|           | SB 4        | 10 4 9 7 9 16 7 2 14 19 6 5 8 2 4             |
| 87212     | NS 4.4      | 12 4 8 7 9 14 8 2 13 23 6 5 NP 2 4 0            |
|           | SB 4        | 12 4 8 7 9 14 8 2 13 23 6 5 8 2 4             |
| 87401     | NS 5.0      | 19 4 10 8 8 NP 7 2 14 16 6 5 NP 2 4 0         |
|           | SB 4        | 19 4 10 8 8 24 7 2 14 16 6 5 8 2 4             |

NS: Nasal sample; SB: Skin Biopsy; BI: Bacilloscopic Index from slit skin smear; SNP: Single Nucleotide Polymorphisms genotype; NP: No PCR product.

Grey shaded boxes show mismatch allele (copy number); Boxes in the last column highlights the paired samples with the highest number of loci with mismatched alleles ($\geq$ 5).
Table 2. Allele types, frequency and allelic diversity of *M. leprae* VNTR loci in nasal and biopsy samples from Brazilian leprosy patients

| Allele type and frequency | (AT)17 | (GGT)5 | (GTA)9 | (AC)8b | (AC)8a | (AT)15 | (AC)9 | 21-3 | (GAA)21 | (TA)18 | 6-7 | 27-5 | (TA)10 | 23-3 | 12-5 |
|---------------------------|--------|--------|--------|--------|--------|--------|--------|------|----------|--------|-----|-------|--------|------|------|
| Allelic diversity*        | 0.870  | 0.026  | 0.601  | 0.478  | 0.556  | 0.779  | 0.507  | 0.810| 0.892    | 0.569  | 0.300| 0.484 | 0.484  | 0.143|
| Locus name                | (AT)17 | (GGT)5 | (GTA)9 | (AC)8b | (AC)8a | (AT)15 | (AC)9 | 21-3 | (GAA)21 | (TA)18 | 6-7 | 27-5 | (TA)10 | 23-3 | 12-5 |
| Nasal samples             |        |        |        |        |        |        |        |      |          |        |     |       |        |      |      |

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| Locus name | (AT)17 | (GGT)5 | (GTA)9 | (AC)8b | (AC)8a | (AT)15 | (AC)9 | 21-3 | (GAA)21 | (TA)18 | 6-7 | 27-5 | (TA)10 | 23-3 | 12-5 |
|------------|--------|--------|--------|--------|--------|--------|--------|------|--------|--------|------|------|--------|------|------|
| Allelic diversity* | 0.882 | 0.629 | 0.485 | 0.552 | 0.885 | 0.532 | 0.835 | 0.902 | 0.324 | 0.125 | 0.299 | 0.026 |
| Allele type and frequency | 25 | 1 | 4 | 38 | 15 | 1 | 8 | 18 | 11 | 1 | 34 | 1 | 9 | 1 | 2 | 38 | 19 | 1 | 30 | 1 | 7 | 7 | 5 | 35 | 10 | 4 | 2 | 38 | 5 | 1 |
| Brazilian population | 0.86 | 0.09 | 0.76 | 0.05 | 0.57 | 0.92 | 0.27 | 0 | 0.81 | 0.873 | 0.44 | 0.43 | 0.86 | 0 | 0.41 |

*Allelic diversity ($h$) was defined as $h = 1 - \sum x_i$, where $x_i$ is the frequency of the $i$th allele at the locus.\textsuperscript{18} NP, No PCR product.
alleles that ranged in copy number between 5 and 30 copies in SB and between 13 and 26 copies in NS.

Samples from five patients (IDs 86555, 87212, 87156, 86190, and 87401) did not diverge in any of the amplified loci; the NS from two out of these five patients did not amplify one locus (TA)10 and NS strains from the other three patients did not amplify two loci [(AT)15 and (AC)8b].

With regard to the seven patients with high genotypic diversity, one patient (ID 91845, Table 1) had strains that diverged at seven loci. This patient demonstrated NS and SB with the most different strains, based on the fact that all loci could be. In other three patients (IDs 87728, 88053, and 91979, Table 1) the NS and SB differed in six loci, while other three patients (IDs 88742, 86340, and 88119) the NS and SB differed in five loci, but with several non-amplified loci, it is not possible to make any inferences on their relatedness. Most M. leprae strains that were found in these patients with different VNTRs demonstrated an increased number of copies in the SB compared to NS samples.

Discussion

This study was one of the first to use NS for M. leprae genotyping, in which a larger number of loci and samples were evaluated, than in previous studies. It also includes comparison of the intrapatient genotypes in NS and SB specimens.

Genotyping M. leprae is more complex than that of many other bacteria due to its long incubation period, low pathogenicity and the difficulty of obtaining DNA, due to restriction to biological specimens. Thus, one point of this study was to demonstrate the ease of collecting nasal samples and their utility for research. However, M. leprae from nasal samples demonstrates higher contamination with microorganisms derived from the community and from the environment, as demonstrated by other studies. In addition, it is suggested that nasal microbiota composition is susceptible to environmental modification. Also, the M. leprae DNA extracted from this material is of poor quality (degradation demonstrated by no amplification of some loci) and often in quantities insufficient (low detection of some fragments compared to SB) for study purposes. While all SB samples gave PCR positive VNTR alleles, 31 (82%) NS DNA samples were PCR negative at least one locus. All 15 loci mapped were PCR negative in at least one NS sample. The loci which were most affected in NS were (TA)10, (AT)15, (AC)8b and (TA)18 with 20 (53%), 14 (37%), 13 (34%) and 10 (26%) specimens not yielding a PCR product. There was no relationship with the BI and PCR efficiency for NS (Table 1).

In this context, of known substantial missing alleles in the overall NS dataset, it is difficult to assess the differences between VNTR profiles of SB and NS. However, a few paired cases for which the 15 locus VNTR profile was nearly complete are interesting. For example, notable differences in NS and SB samples were observed in one patient (patient ID 91845, 7 loci), in other three patients (IDs 87728, 88053 and 91979, 6 loci) and in three patients (IDs 86340, 88119, and 88742, 5 loci).

While SNP typing subtyping provides a higher level of phylogenetic classification, it lacks the strain discrimination for communities infected by the same SNP type. In MLVA, certain loci are prone to slipped strand replication more than others such as the AT/TA type dinucleotide loci. It is of note, that these are also the loci prone to degradation as seen in the NS samples. Despite this, it should be clarified that higher rates of mutation due to slipped
strand synthesis is the basis for their use as typing markers, as they generate strain diversity. It is the balance between stability and change that determines the value of typing systems for various applications. With regard to *M. leprae* VNTRs, it was clear, in the study demonstrating zoonotic transmission of leprosy in patients and armadillos from different distant states in the US, that even potentially ‘unstable’ AT/TA type loci had matching alleles. Identical to highly similar VNTR profiles have been identified in multiple studies using skin biopsies of independent patients linked by household and even broader geographic scales such as neighbourhood and townships. Such studies have adequately demonstrated that MLVA is suitable for identifying transmission links over a background of highly discriminatory VNTR profiles. Thus, *M. leprae* VNTRs have sufficient stability for tracing transmission, although the time scale and in vivo conditions for evolution of VNTR microsatellites are not yet completely understood. Skin biopsies from lesions, which represent ongoing clinical infections, have been shown to be suitable sampling site for *M. leprae* for transmission studies, where VNTR relatedness has been retained in linked cases. Yan et al. have studied VNTR stability in skin biopsy from multiple time points. On the other hand, in human leprosy, where patients have coexisted over hundreds of years in endemic communities, and multiple types of strains are prevalent, the relevance of NS in the infection process and in transmission are not known. This study attempted to address this gap. The results indirectly suggests that though it is easier to obtain a nasal swab from a patient than SB, even in patients with high BI, the NS do not harbour sufficient bacilli compared to the SB and that the bacilli may not be sufficiently viable (DNA is highly degraded).

With these considerations, we suggest that the NS samples from which full VNTR profiles were obtained but which diverged considerably from the corresponding SB VNTR may represent dual infections in the same patient in two different anatomical locations. Since there are VNTR differences ranging from two to even six units among several markers, Monot et al. and Gillis et al. also had raised the possibility of infection with two different strains in the same individual. Entry into the host provides an opportunity for a limited number of bacteria to undergo clonal expansion in a new fairly isolated niche. This situation could allow the emergence of an initially subdominant population either through a purely random process, or as a result of some selective advantage. The bacilli can undergo different adjustments to pressure from host immune response and physiological conditions at the different anatomical sites. For other bacteria, including *Staphylococcus aureus*, the nasal microbiota is strongly influenced by the environmental bacteria. We may suggest that for *M. leprae* bacilli from NS in patients may represent the strains circulating in the community while the bacilli in the SB are those that are already established in the patient and may have undergone clonal expansion. The intergenic or interpseudogenic location of VNTRs loci makes it unlikely that the differences in repeat copy numbers itself confer any biological advantage, but they may act as a marker for some biologically distinct subpopulations of bacteria.

This study demonstrated the predominance of SNP genotype 4, similar to what previously described by Fontes et al. Since this marker shows a high stability, no differences were found within biological specimens from the same patient. This finding was similar to what described by Monot et al. This SNP genotype is characteristic of African continent. During almost three centuries it was estimated 4.9 million slaves from Africa to Brazil, most of them were working sugar plantations in the North-east of Brazil. In the South and South-east of Brazil the SNP genotype 3 is predominant. Comparing the VNTR profile of SNP genotype 4 with genotype 3 described in Yunnan Province of China, the median repeat
length at (GAA)\textsubscript{21} was similar with 13 versus 14 copies, while a different median in the (AT)\textsubscript{15} was of 16 versus 13 to type 4 and 3, respectively and similar to described in Monot \textit{et al.}\textsuperscript{22} Since type 4 is descendant from type 3, the same authors suggested that reduction observed in SNP type 4 strains are due to allelic diversity.

Young \textit{et al.}\textsuperscript{9} had noticed an increase in the copy number in SB compared to nerve within the same patient; we did not observe this pattern in all markers. In fact, the majority of the differences were upshifts in NS compared to SB, contrary to their observation. They had compared a profile obtained with only three microsatellite markers (TTC, AGT and AT) in contrary to our study with 15 markers. The same authors also suggested that the higher number of allele copies in nerve samples may be related to some characteristic of the replication of \textit{M. leprae} in the nerve, such as a rapid doubling time, promoting a change in the number of copies of the VNTR locus in a way that results in a change in the globally dominant genotype. Increasing in pathogenicity of SB strains cannot be ascribed due to allelic diversity by MLVA markers. The main differences can be attributed to clonal instability within VNTR.\textsuperscript{25} Overall, the allelic diversity of our samples were similar compared to other Brazilian samples of \textit{M. leprae}.\textsuperscript{28} Some differences in our samples were seen in the loci (AC)\textsubscript{9} and (AC)\textsubscript{8b} that had a moderate discriminatory index (\(>0.3\)) while in Fontes \textit{et al.} had an allelic diversity of 0.27 and 0.05 respectively.\textsuperscript{28} Opposite to this was seen at locus (TA)\textsubscript{10} that had shown a higher degree of diversity in the same study. These results might be attributed to regional differences in the allelic range (number of alleles) and frequencies of each of the STR loci.

Whatever the underlying mechanism, the finding of differences in \textit{M. leprae} subpopulations among NS and SB samples makes clear the importance of a better understanding of the factors involved in tissue invasion by \textit{M. leprae}. For example, the high genetic variability of \textit{M. leprae} could be proportional to the number of disseminated cases, suggesting the existence of different \textit{M. leprae} strains in Fortaleza.\textsuperscript{30,31}

Several limitations should be addressed. One could argue that the use of MLVA to determine genetic relatedness may have identified isolates as belonging to different strains that could have been identified as belonging to the same strain. However, several studies have shown that MIRU-VNTR is a typing method with adequate discriminatory power and stability to differentiate among strains.\textsuperscript{32,33} Another limitation is the lack of data on other potential factors that may influence the rate of acquisition or the source of clonal diversity, such as overcrowded family conditions or family members living in colonies.

When observing the VNTR profile, we also observed a greater variation in repeat lengths in the AT rich dinucleotide repeats [(AT)\textsubscript{17}, (AT)\textsubscript{15}, (TA)\textsubscript{18}, and (TA)\textsubscript{10}], followed by the trinucleotides [(GAA)\textsubscript{21} and (GTA)\textsubscript{9}].\textsuperscript{6,8} Our study demonstrates also some regional differences in the allelic range and frequencies pointed by previous authors.\textsuperscript{26,28} In the overall, the VNTR profile of particular alleles of the 38 patients were similar to what described in previous studies conducted in Brazil. It is worth notice the predominance of four copies of STR 12-5, against five copies in the South of Brazil. Also is observed a higher copy number of the markers (GAA)\textsubscript{21}, (TA)\textsubscript{18} and (AT)\textsubscript{17} compared to the south region and other countries.\textsuperscript{11,16,25}

In the study in North India by Lavania \textit{et al.}\textsuperscript{34} the loci 12-5, 21-3, 18-8 and 27-5 were polymorphic. In Yunnan Province of China, they did not vary.\textsuperscript{29} This demonstrates that loci that are polymorphic may present in different ways, which makes it necessary to conduct studies with VNTRs to complement the database on the \textit{M. leprae} genotypes prevalent in many areas of the world and to understand whether the observations are associated with the
study region or sample size, leprosy history, clinical manifestations, and conditions of its persistence and spread.

Further studies of genetic diversity in samples with known epidemiological links will be important to assess how microsatellites can be used as reliable markers of transmission events. This study demonstrated that it is possible to apply NS that are less invasive and easier to collect, but inadequate for a molecular epidemiological approach to the study of leprosy. Recommendations that follow are that for better discrimination of the strains more VNTR loci and other molecular markers suitable for typing genetic variations of the bacilli should be discovered. Since whole genomic sequencing is limited in developing countries, we need to improve classification systems capable of providing information on the \textit{M. leprae} dynamics of transmission and use these to help in the search for interventions that reduce the number of new cases of leprosy.

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