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VNTR typing of *Mycobacterium leprae* in South Indian leprosy patients

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Summary

Objectives To study the suitability, stability and diversity of short tandem repeat (STR) genomic markers to elicit strain variation in the *Mycobacterium leprae* isolates within leprosy patients from Andhra Pradesh and Tamil Nadu states in South India.

Materials and Methods Slit skin smear (SSS) samples were collected from lesions and various body sites of newly diagnosed leprosy patients. The SSSs from each patient were pooled, except in the case of five patients. Total DNA was extracted from SSS samples. *M. leprae* STRs were amplified from the DNA either by multiplex PCR (MP) or single PCR methods. The number of repeats for each STR locus (the STR allele) was obtained either by fragment length analysis (FLA) or by DNA sequencing of the PCR amplicons.

Results and Conclusion Multiplex PCR minimised the use of DNA and reagents, and together with FLA, was time and cost effective for STR strain typing. After examination of the isolates of South Indian origin at 13 STR loci, it was determined that the alleles for (AC)8b, (GGT)5, 6-3a (*rpoT*), 21-3, 27-5, and 23-3 were conserved in two study populations. In a family from Andhra Pradesh, the *M. leprae* STR patterns in two patients were identical in 16 of 18 loci which indicate a common source of infection. Fourteen of 15 STR loci showed no intra-patient variation in the five patients tested in Tamil Nadu. Altogether, these studies indicate the suitability of STR strain typing for assessing short-range transmission chains.

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Introduction

For insights into the evolution of *M. leprae* and transmission of leprosy, researchers have begun to use molecular strain typing principles and techniques. Leprosy has long been endemic to the Indian subcontinent, and new cases continue to arise in large numbers in India, Nepal and Bangladesh.[1] The whole genome sequence of an Indian isolate, TN from the state of Tamil Nadu was revealed in 2001.[2] This contained short tandem repeat (STR) regions that had potential for genetic polymorphism by expansion or contraction of repeats and therefore for strain typing of *M. leprae*. A list of loci were targeted for strain typing[3,4] and multi locus variable number tandem repeat analysis (MLVA) as means for molecular differentiation of *M. leprae*, within and amongst leprosy patients thus emerged.[5,6] Studies using one or more loci have been published for isolates from Japan, Indonesia, Thailand, China, India, Mali, Malawi, and Mexico.[6–13] The TN sequence also served as a template for the identification of single nucleotide polymorphisms (SNPs) by comparison with partial genome sequences of a few other reference isolates. Three SNPs, which yielded only four patterns (SNP type 1–4) were identified in clinical isolates.[14] The origin of leprosy was ascribed to East Africa. The relative merits and information that can be garnered from polymorphism at these two types of genetic markers (STRs and SNPs) has been debated.[11]

In India, with regard to further defining the strain types of circulating *M. leprae* isolates in patients, this study utilises a larger panel of STR loci than in prior studies.[10,12] Patients originated from two states in the south, Tamil Nadu, from where the sequenced TN strain originated, and Andhra Pradesh, a neighbouring state. According to the Government National Leprosy Eradication Programme (NLEP), the prevalence rate of leprosy is 0·70/10 000 in Andhra Pradesh, India, and 10 047 new cases were detected in 2007–2008 with an annual new case detection rate (ANCDR) of 12·12/100,000 (http://nlep.nic.in/index.html), whereas in Tamil Nadu the prevalence rate is 0·55/10 000 and 5511 new cases were detected in 2007–2008 with a ANCDR of 0·82/10 000. The molecular epidemiology study in Andhra Pradesh was centred at BPRC, while the Tamil Nadu study was at The Schieffelin Institute of Health – Research and Leprosy Centre (SIH – R & LC), Karigiri in Vellore District. Vellore District was one of the first pilot districts in the country where MDT was introduced in 1982. Since then the prevalence rate has shown a steady decline, and in March 2008 it stood at 0·37 per 10 000 population, and the annual new case detection rate was 0·61 per 10 000. Between 1962 and 1997, the institution implemented the NLEP in a defined geographical area (Gudiyatham Taluk), in Vellore District, situated in the southern Indian state of Tamil Nadu which served as the field area to carry out leprosy control activities, training and research. There were a total of 42 new untreated cases detected in this area during 2008. The present prevalence rate in Gudiyatham Taluk is 0·71 per 10 000 population, and the annual case detection rate is 0·74 per 10 000 population. Household and neighborhood contact tracing of new index cases is carried out routinely as part of the epidemiological surveillance of the Taluk.

The Blue Peter Research Centre, a leprosy, TB and HIV referral clinic and research institute of LEPRA India, provides outpatient care for the patients in the city of Hyderabad and surrounding districts in Andhra Pradesh. Patients visit BPRC voluntarily, or are referred by other physicians from private, government or other LEPRA organisations. HYLEP is another LEPRA clinic unit in the city of Hyderabad.

Materials and Methods

Slit Skin Smears (SSS) were obtained after informed consent from 24 newly diagnosed LL/BL patients from Vellore District reporting to SIHRLC for diagnosis and treatment. SSS and a skin biopsy is part of the routine diagnostic protocol in the institute for all newly diagnosed leprosy patients. For the IDEAL study, SSS were collected from the four routine sites, namely ear lobe, forehead, left chin and buttock in a male and thigh in a female patient. A separate scalpel blade was used for each site. Skin scrapings from each site were stored in a separate aliquot containing 70% ethanol and then stored at $-20\,^{\circ}C$ until DNA extraction was done.

A total of 104 new or relapse cases were enrolled since April 2007 from BPRC, HYLEP and Sivananda Rehabilitation Home (SRH) for 'Molecular Epidemiology of Leprosy study (MEL)'. Another 20 leprosy patients from Hyderabad and adjacent Rangareddy districts, consulting at BPRC were recruited for the 'IDEAL' consortium study. Biopsy, SSS, and blood were collected for the MEL study, whereas only SSS samples were collected for the IDEAL study. SSS samples were collected from various sites, like ear lobes, forehead, chin, pooled and stored in 70% ethanol. Clinical details and bacteriological index (BI) was recorded. In BPRC, the Ridley–Jopling classification based on (skin biopsy) histology and clinical reports is also practiced.

The SSS samples were processed to obtain the DNA, as described elsewhere using Qiagen DNeasy Kit (in BPRC). The Vellore District DNA samples were submitted to single PCR and DNA sequencing.[15]

DNA sequencing was done at Sankara Nethralaya, Chennai. VNTR mapping of the BPRC DNA samples was carried out according to the MP-FLA protocol described in the Philippines study (Kimura *et al.*,[16] Sakamuri *et al.* in this series).[17] A new locus ML1 was discovered during the course of the studies, and included in one of the multiplex PCRs (Li *et al.* unpublished). The samples were submitted to Proteomics and Metabolomics Facility at Colorado State University for FLA.

Results

The residential locations of the 24 patients recruited from Vellore District are shown in Figure 1. Their locations are fairly widespread and there was no evidence of any contact between the families (such as household, business or social contacts). Samples from 12 of these patients were submitted for MLVA.

All the homes of the 24 patients were visited by a trained leprosy supervisor. Sixty six household contacts (HHC) were enumerated and 63 were examined for signs of leprosy. Among these HHC, eight had developed leprosy; six were classified as PB, while two were MB. All but one were smear negative, and hence not pursued for DNA analysis. The one contact who had a BI of 1 + in one selective site was submitted for DNA extraction and PCR, but unfortunately did not produce amplicons for sequencing. The VNTR data from 12 Vellore Districts are listed in Table 1.

STR locus intra-patient variation was further analysed using samples from five of the 12 patients. Except for the (GAA) 21 locus, variations were not detected in copy number at 12 loci as shown in Table 2.

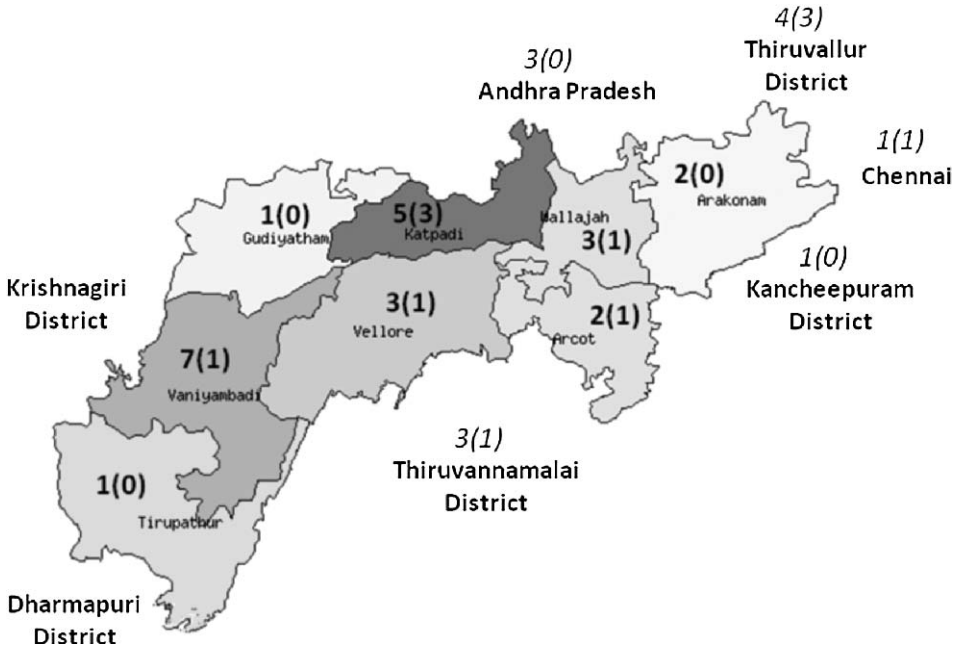

Figure 1. Map of Vellore and neighboring districts in Tamil Nadu state, India to depict the residential origins of patients enrolled in this study. The number of new cases detected during the study period, is followed in parenthesis by the number of cases in which VNTR data was collected (patients with BI > 2). Three patients were identified from the neighboring state of Andhra Pradesh. The map was adapted from http://tnmaps.tn.nic.in/district.php?dcode=04.

In the AP study, at least 39 patients were from the city of Hyderabad, 27 from adjacent Rangareddy district and remaining mostly from the northern part of Andhra Pradesh like Mehboobnagar, Nalgonda, Karimnagar, Warangal with a few patients from Eastern Andhra Pradesh districts of Vizianagaram, Vijayawada and Vishakahpatnam as shown in Figure 2, and Table 3.

The age of the patients ranged between 18–70 years, and the male to female ratio was 3:1. Majority of the patients were classified and treated as multibacillary ($n = 90$) cases. Seventy seven new patients had no previous history or treatment for leprosy. Eight patients were categorised as relapse cases. Eleven patients indicated knowledge or contact with leprosy in their families. Eighteen cases were classified as presenting with Type II reaction (Erythema Nodosum Leprosum). For the 20 cases in the IDEAL study, contact screening was conducted (total of 58 HHC); new cases were not detected.

Discussion

With regard to the VNTR analysis, alleles for all 16 loci could not be obtained for patients with BI of less than 2 ($n = 43$). SSS from cases with BI of 2 or more, yielded amplicons, although 16 samples performed poorly in PCR, and only partial allelic data was obtained. Overall, data from 68 patients are presented in the Tables 1 and 4.

Table 1. *M. leprae* VNTR genotypes detected in Tamil Nadu in S. India

ID	(AC)8b	(GTA)9	(GGT)5	(AT)17	6-3a	21-3	(AC)9	(AT)15	(AC)8a	ML1	27-5	6-7	(TA)18	(GAA)21	18-8	12-5	23-3	(TA)10	BI
M. leprae VNTR patterns in TN patients (Vellore and neighboring districts)																			
K004	7	9	4	ND	3	2	6	nd	9	nd	5	8	nd	nd	nd	4	1	8	1·75
K008	8	7	4	13	3	2	8	nd	9	nd	4	6	nd	nd	nd	4	2	8	4·75
K009	7	10	4	ND	3	2	8	nd	9	nd	6	5	nd	nd	nd	4	2	8	3·75
K011	7	8	4	11	3	2	9	nd	8	nd	5	6	nd	nd	nd	4	2	10	3·75
K014	7	9	4	9	3	2	7	nd	8	nd	5	6	nd	nd	nd	4	2	9	4·5
K015	7	9	4	13	3	2	9	nd	8	nd	5	6	nd	nd	nd	4	2	8	5·0
K016	7	7	4	11	3	2	10	nd	8	nd	5	6	nd	nd	nd	4	2	10	2·75
K017	7	10	4	13	3	2	8	nd	8	nd	6	6	nd	nd	nd	4	2	8	4·0
K002	7	12	4	9	3	2	7	nd	10	nd	5	6	nd	nd	nd	5	2	8	1·5
K006	7	11	4	11	3	2	7	nd	8	nd	5	6	nd	nd	nd	5	2	8	4·25
K007	7	10	4	12	3	2	7	nd	8	nd	5	6	nd	nd	nd	5	2	8	3·5
K013	7	9	5	10	ND	2	6	nd	9	nd	5	7	nd	nd	nd	5	1	8	5·5

nd: not done, ND: No PCR or sequence data.

Table 2. Intra-patient stability of VNTRs FLA in different Slit Skin Smears in five patients in the Vellore, TN study group

	STR loci														
DNA Sample Source	(AC)8a	(AC)8b	(AC)9	(AT)17	(GAA)21	(GGT)5	(GTA)9	(TA)10	6-7	12-5	21-3	23-3	27-5	6-3a	ML18[§]
NHDP55†	123–124	135	141	170′–171	173	159	122	190	189	286	158–159′	168′–169	243	201–202	162–163
Copy Number	10	7	8	13	12	4	10	13	7	5	2	2	4	3	2
K002‡															
Fore-Arm	123	135	139	162–163′	184	159	128	180	183	286	159	169	270	202	162–163
Leg	123′–124	135	139–140′	162	184	159	128	180	183	286	158–159′	168–169′	269–270′	202	162–163
Lt. Ear	123′–124	135	139	162	179	159	128	180	183	286	158–159′	168–169′	269–270′	201–202	163
Copy Number	10	7	7	9	U	4	12	8	6	5	2	2	5	3	2
K008‡															
Rt. EL	121–122′	137	141	171	193	159	113	180	183	274	158–159′	168′–169	242′–243	201–202	162–163
Chin	121–122′	137	141	171	193	159	113	180	183	274	158–159′	168′–169	242′–243	201–202	162–163
Lt. Thigh	121–122′	137	141	171	193	159	113	180	183	274	159	169	243	201–202	162–163
Copy Number	9	8	8	13	18	4	7	8	6	4	2	2	4	3	2
K017‡															
Rt. EL	118	135	141	171	190	159	122	180	183	274	158–159′	169	297	201–202	162–163
FH	118	135	141–142′	171	190	159	122	180	183	274	158–159′	169	297	201–202	162–163
Rt. Chin	118	135	141	171	187	159	122	180	183	274	158	169	297	202	162–163
Lt. buttock	117–118	135	141	171	187	159	122	180	183	274	158–159′	168′–169	297′–298	202	162–163
Copy Number	8	7	8	13	U	4	10	8	6	4	2	2	6	3	2
K015*															
EL	8	7	9	13	17	4	9	8	6	4	2	2	5	3	2
FH	8	7	9	13	17	4	9	8	6	4	2	2	5	3	2
Chin	8	7	9	13	16	4	9	8	6	4	2	2	5	3	2
BT/TH	8	7	9	ND	16	4	9	8	6	4	2	2	5	3	2
Copy Number	8	7	9	13	U	4	9	8	6	4	2	2	5	3	2
K013*															
EL	9	7	6	10	10	5	9	8	7	5	2	1	5	ND	ND
FH	9	7	6	10	10	5	9	8	7	5	2	1	5	ND	ND
CHIN	9	7	6	10	10	5	ND	8	7	5	2	1	5	ND	ND
BT	9	7	6	ND	10	5	ND	8	7	5	2	1	5	ND	ND
Copy Number	9	7	6	10	10	5	9	8	7	5	2	1	5	ND	ND

* STR repeat numbers determined by DNA sequencing.
† NHDP55 is DNA isolated from armadillo passaged *M. leprae* initially isolated from a US patient (see Gillis *et al.*).[15]
‡ STR allele determined by Fragment analysis followed by DNA sequencing to verify repeat number.
§ ML18 is described in Li *et al.* (Unpublished).
U = undetermined due to variation within patient; ND = not done.

Figure 2. District map of Andhra Pradesh state, India. (Map adapted from http://www.aponline.gov.in/Quick%20Links/APFactFile/info%20on%20districts/info_district.html).

Table 3. Distribution of patients from each district in Andhra Pradesh state, India (Figure 2) consulting at BPRC over period of eight years and the sampling coverage from this patient population in the current study

District	Number of Cases treated at BPRC 2000-08	Total Number of Cases included in BPRC study (4/2007-9/08)*
Adilabad	1	
Ananthapur	2	
E. Godavari	1	
Guntur	7	4
Hyderabad	83	39
Kadapa	1	
Khammam	2	
Karimnagar	6	
Krishna	1	
Kurnool	1	
Mehboobnagar	47	8
Medak	19	2
Nalgonda	37	5
Nizamabad	3	
Ongole	1	
Rajole	1	
Rangareddy	291	27
Sec'bad	24	
Vizag	6	
Warangal	17	2
W. Godavari	1	1
Andhra Pradesh	552	93
Gulburga	10	3
Other Karnataka	3	
Karnataka	13	3

* Includes patients from SRH and HYLEP.

Table 4. *M. leprae* VNTR genotypes detected in Andhra Pradesh in S. India

ID	(AC)8b	(GTA)9	(GGT)5	(AT)17	6-3a	21-3	(AC)9	(AT)15	(AC)8a	ML1	27-5	6-7	(TA)18	(GAA)21	18-8	12-5	23-3	(TA)10	BI
M. leprae VNTR patterns in AP patients mapped ≥ 16 of 18 loci																			
MEL/BPRC/077	7	13	4	14	3	2	7	18	9	1	5	6	15	15	7	4	2	8	4
MEL/BPRC/078	7	14	4	14	3	2	7	23	9	1	5	6	15	15	7	4	2	8	6
MEL/BPRC/090	8	17	4	9	3	2	7	11	8	2	5	7	19	14	8	4	3	9	5·75
MEL/BPRC/091	7	10	4	14	3	2	8	22	10	1	5	6	14	14	8	4	2	10	5·75
MEL/BPRC/079	7	8	4	12	3	2	9	13	10	2	5	6	17	12	8	4	2	11	5
MEL/BPRC/080	7	8	4	12	3	2	8	12	8	2	5	6	11	17	7	4	2	9	5
MEL/BPRC/087	7	10	4	11	3	2	9	13	8	2	5	6	9	13	7	4	2	13	5
MEL/BPRC/083	7	9	4	12	3	2	9	13	8	2	5	6	12	15	7	4	2	9	4·99
MEL/BPRC/035	8	9	4	12	3	2	8	14	6	2	5	6	10	17	7	4	2	8	4·75
MEL/BPRC/052	7	9	4	11	3	2	10	13	8	2	5	6	12	15	7	4	2	9	4·66
MEL/BPRC/067	7	10	4	14	3	2	9	18	8	2	5	6	11	15	7	4	2	21	4·5
MEL/BPRC/028	7	10	4	13	3	2	8	15	9	3	5	6	18	22	7	4	2	9	4·5
MEL/BPRC/084	7	9	4	11	3	1	9	17	9	3	5	6	12	15	7	4	2	8	3·75
MEL/BPRC/021	7	10	4	13	3	2	8	13	10	2	5	7	20	23	8	4	2	8	4
MEL/BPRC/073	7	7	4	11	3	2	9	15	8	2	5	6	10	12	7	4	2	22	4
MEL/BPRC/003	7	8	4	12	3	2	10	18	8	2	5	6	10	14	7	4	2	10	3·75
MEL/BPRC/093	7	9	4	13	3	2	9	15	10	2	5	6	12	15	7	4	2	11	3·4
MEL/BPRC/024	7	11	4	12	3	2	8	14	9	3	5	7	9	17	7	4	2	9	4·16
MEL/BPRC/057	7	13	4	11	3	2	8	16	8	3	5	6	11	16	7	5	2	20	3
MEL/BPRC/004	9	12	4	10	3	2	7	13	10	2	5	9	18	15	8	4	2	10	3·2
MEL/BPRC/050	7	9	4	10	3	2	9	13	8	2	5	7	10	19	7	4	2	9	3
MEL/BPRC/099	7	8	5	11	3	3	9	14	9		5	6	16	13	8	4	2	9	3·5
MEL/BPRC/031	7	10	4	11	3	2	9		8		5	6	16	16	7	4	2	11	3·75
MEL/BPRC/034	7	10	4	12	3	2	8		9		4	6	10	18	7	4	2	9	2·25
MEL/BPRC/076	7	9	4	9	3	2	9		8	2	5	6	15	15	7	4	2	10	5·4
MEL/BPRC/074	7	9	5	11	3	2	8	19?	10	2	5	7	16	21	9	4	2	21	4·66
MEL/BPRC/009	7	10	4	11	3	2	9	14	9	2	5	6	10	12	7	4	2	13?	4·33
MEL/BPRC/071	7	13	4	13	3		7	14	7	2	5	7	16	12	8	4	2		
MEL/BPRC/072	7	9	4	13	3	2		17	9	3	5	6	18?	15	8	4	2	8	
MEL/BPRC/026	7	9	4	13	3	2/3	8	15	8	2	5	6	13	12	8	4	2		2
MEL/BPRC/014	7	9	4	13	3	2	7	18	8	2	5	6	13	21	7	4	2	17	5·6

Table 4. *continued*

ID	(AC)8b	(GTA)9	(GGT)5	(AT)17	6-3a	21-3	(AC)9	(AT)15	(AC)8a	ML1	27-5	6-7	(TA)18	(GAA)21	18-8	12-5	23-3	(TA)10	BI
MEL/BPRC/019	8	10	4	11	3	2	7	18	10	2	5	7	15	19	8	4	2	8	6
MEL/BPRC/020	8	11	4	9	3	2	8	16	10	2	5	10	16	23	8	4	2	10	5
MEL/BPRC/101	7	10	4	13	3	2	8	25	10	2	5	8	16	20	8	4	2	8	5
MEL/BPRC/089	7	9	5	11	3	1	7	14	8	2	5	6	12	11	7	4	2	8	3·25
MEL/BPRC/065	7	13	4	13	3	2	8	15	8	2	5	6	16	16	7	4	2	19	3·33
MEL/BPRC/103	7	11	4	11	3	2	7	17	7	2	5	6	16	13	7	4	2	8	2
MEL/BPRC/102	7	9	4	12	3		9	19	8	2	5	6	11	18	6	4	2	9	4
MEL/BPRC/063	8	13	4	9	3	2	7	18/19	10	1	5	8	10	16	8	4	2	19	4·16
MEL/BPRC/082	7	9/10	4	10	3	2	10	14	8	2	5	7	12	18	7	4	2	10	4
BIDEAL17	7	10	4	11	3	2	10	17	8		5	5	20	14/15	8	5	2		4·75
MEL/BPRC/010	7	9	4	10	3	2	8	14	9	2	5	6	12	17/19	7	4	2	9	6
MEL/BPRC/025	7	9	4	14	3	1	9	15	8	2	5	6	10	22/20	7	4	2	8	4·6
MEL/BPRC/049	7	10	4	11	3	2	9	14	8	2	5	6	16	16/11	7	4	2	9	3·6
MEL/BPRC/094	7	12	4	14	3	2	9	13	8	2	5	6	10	10/16	7	4	2	8	3·5
MEL/BPRC/075	7	13	4	12	3	2	9	14	8	2	5	6	9	11/13	7	4	2	22	2·75
M. leprae VNTR patterns in AP patients with multiple alleles at more than one locus																			
MEL/BPRC/086	7	10	4	14	3	1/2	9	17/12	8	3	5	6	16	18	7/8	4	2	9	2·4
MEL/BPRC/096		10	4	12	3	2	9	14/23	8	3/2	5	7	16	17	7	4	2	8	2
MEL/BPRC/088	7	12	4	13	3	2	9	13	8	2	4/5	7/6	12?	11	7	4	2	12	5·33
MEL/BPRC/023	7	9	4	10	3	2	8	13	8/6	1	5/6	6/7	10	24/18	7	4	2	9	4·5
MEL/BPRC/062	7	9	5	14	3	2	8	13	10/8	1	5	6	11	14	7	4	2		2·5
MEL/BPRC/051	7	9	4	10	3	2	9	13	8	2		7/6	10/11	17	7	4	2	9	2
MEL/BPRC/070		9	4	12	3	2	9	16	9	2	5		14	17		4	2	11/12	2·75
MEL/BPRC/053				10	3	2	7	24/25	8	2	6	6	13/15	16/12	7	4	2		3·75
BIDEAL2	7	9	5/4	17/13	3	2	7	20	8		5	6	18	12	7	4	2	10	4
BIDEAL8	7	7	4	12	3	2	8	14	8		5	6/7	12	17/12	7	4	2		3·2
MEL/BPRC/018		11	4	10	3	2	7/8	15	8	2	5	8	16	14	8	4	2		2
BIDEAL7	8	14	4	8	3	2	10		9		5	6	12/13	15	7	4	2	12	2·6
BIDEAL6	7	10	4	11	3	2	7	15/16	13		5	7	14	11	8	5		8	2
BIDEAL18	7	9	4	11	3	2	7	16	8			6/7	15	11	8	4	3	9	0·75
BIDEAL3	8	11	4	11	3	2	8	12	8		5		19	12/16			2		4·6

Table 4. *continued*

ID	(AC)8b	(GTA)9	(GGT)5	(AT)17	6-3a	21-3	(AC)9	(AT)15	(AC)8a	ML1	27-5	6-7	(TA)18	(GAA)21	18-8	12-5	23-3	(TA)10	BI
M. leprae VNTR patterns (partial) in AP patients																			
MEL/BPRC/081	7	10	4	14	3				10	3	5	6	11	23	7	4	2		4·75
MEL/BPRC/038			4		3	2	8	17	8		6	7	13	12	8	5	2		2·6
MEL/BPRC/058		9	4	11	3		8	15	6	1			9	21	8	4	2	17	4·5
MEL/BPRC/008	7	8	4	10	3	2	7	14	8	2	5		21	14	7		2	8	2·25
BIDEAL16	7	10	4	11	3	2	8	19	8		5	6	9		7	4	2	9	4
BIDEAL11	7	9	4		3	2	8	16	7		5	6	12		7	4	2	9	1
BIDEAL12	7	9	4		3	2	8	17	7		5	6	12		7	4	2	9	0

? Weak products, Shaded cells: No PCR product or FLA signal, or multiple alleles detected.

With several high BI samples, multiple product peaks were observed at several loci. These include alleles with more than one repeat unit difference. The significance of these findings in this study setting is not clear. These may represent co-infections with different local strains, or could be variants emerging from one parent strain, or arise from experimental artifacts. Such a phenomenon has not been observed at this frequency even with low BI specimens where the product signals are difficult to detect or call above background. These data were collected from SSS, and the stored biopsy specimens could be analysed. Multiple allelic patterns, partial data or no PCR product were observed in the *M. leprae* isolates in 14 of the 34 ENL patients, two of the eight relapse patients, and 12 other patients. The significance of the samples that showed multiple alleles is not clear and needs to be studied further. In the context of the intra-patient VNTR stability as shown for the Vellore patients and in the Philippines and China studies, we propose that co-infections or repeated infections are a possibility with this patient population.

Overall, in both datasets, the alleles for (AC)8b, (GGT)5, 6-3a (*rpoT*), 21-3, 27-5, 23-3 are conserved being 7, 4, 3, 2, 5, 2 respectively. Moreover, the 12-5 allele is 4 in AP, and 4 or 5 in Vellore region. The TN like signature pattern 5-5 for the 27-5- 12-5 pair is rare in all the global datasets accumulated, but we found four in the 12 Vellore, and three of 68 in the AP set. Instead, most global isolates studied thus far, except those forming a subgroup from S. America and N. America (shown to be of SNP type 3) carry the 4–5 motif. These are discussed in the studies from Colombia and Brazil, and a possible correlation with the SNP typing system is proposed.[18,19] There are no isolates with alleles that match those of the TN sequenced strain across 12 loci, even from Vellore region. The classification of these isolates according to SNP types 1–4 has not been determined.[14]

There is one pair (MEL/BPRC 077 and MELBPRC 078) of known epidemiologically linked isolates with matching VNTRs in 16 loci but differ at two, namely (GTA)9 and (AT)15 loci (Table 3); we propose that these are genetically related. Therefore even though mainly the microsatellites which have multiple alleles, contribute to the overall strain diversity within AP, it is possible to detect transmission from a shared source of infection. Although the sampling spans urban, suburban and rural locales in districts in AP and Vellore, in TN State, there are no spatial clusters or associations. Further fine mapping using geological information and detailed patient histories are required over a longer period of study, including SNP typing.

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