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A continuation: study and characterisation of *Mycobacterium leprae* short tandem repeat genotypes and transmission of leprosy in Cebu, Philippines

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Summary

Objective To study the stability and allelic diversity of tandem repeat loci in *M. leprae* in leprosy patients of Cebu, Philippines, and the suitability of multilocus variable number of tandem repeat (VNTR) analysis (MLVA) typing for detecting transmission.

Methods Seventy newly diagnosed leprosy patients consulting at the Leonard Wood Memorial, Cebu Skin Clinic Total DNA was extracted from slit skin smear (SSS) scrapings of each patient and used for amplification of 13 *M. leprae* VNTR loci by single locus or multiplex PCR. Number of repeats for each VNTR locus was obtained by DNA sequencing or fragment length analysis methods. Medical, social and geographic details were included in the molecular epidemiology database.

Results and conclusions Multiplex PCR (MP) and fragment length analysis (FLA) methods were found to be more efficient and accurate compared to single short tandem repeat (STR) amplification and DNA sequencing. Intra-patient MLVA patterns from four different samples were conserved in the minisatellites, while differences in one or more of the polymorphic and stutter prone microsatellites was observed, in four of five patients. The 13 loci could differentiate *M. leprae* strains in Cebu, however, MLVA patterns were stable enough during incubation and transmission between individuals within multi-case families. Thus *M. leprae* MLVA has potential for strain typing and transmission studies in Cebu.

Introduction

A micro-epidemiology project under the auspices of the IDEAL consortium has been active in several countries, including the Philippines, which has a national case detection rate (CDR)

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of 2·6 per 100,000. The Philippines consists of around 7000 islands and Cebu, one of the larger islands, historically had a higher incidence of leprosy than most others. The leprosy CDR for Cebu is now 5·4 per 100,000 although there is considerable variation between districts; in Lapu Lapu (an urban area adjacent Cebu City) the CDR was 16·6 per 100,000 in 1997 and 10·3 per 100,000 in 2007. In 1930, the Leonard Wood Memorial research facility was established in Cebu and the epidemiology of leprosy was intensively studied in the district of Cordova near Cebu City.[1]

Because it is an island with a well-functioning health infrastructure, it was felt that Cebu would be an ideal setting to study the micro-epidemiology of leprosy. The movements of patients and their inter-relationships should be easier to map.

Materials and Methods

CLINICAL SAMPLES

Seventy slit skin smears (SSS) and biopsy samples were collected in 70% ethanol from the patients attending Leonard Wood Memorial (LWM), Skin Clinic in Cebu. These samples were shipped to Colorado State University (CSU) in two separate batches.

Besides obtaining basic details of the medical history including the leprosy condition and treatment regimen, information about the patients' geographical area of residence (village, city and province), types/place(s) of employment and education, nature of dwelling, sources of drinking, cooking, and bathing water spanning a prior 20 year period, was captured with a standardised questionnaire at the time of recruitment.

The average bacteriological index was 3·5. Nineteen patients reported knowledge of at least one known leprosy patient in their families. Two multi-case families (MCFs) were identified in this IDEAL study patient population. In some patients, several specimens were obtained to identify any intra-patient variations which may be present.

The SSS samples were processed to obtain the DNA. Briefly, the SSS containing tubes were centrifuged to pellet the particulate material. The pellet was re-hydrated in 0·5 ml PBS buffer (Invitrogen) at room temperature for 1 hour, followed by a centrifugation step to recover the SSS material. Buffer (0·1 M Tris, 0·05% Tween 20) was prepared, filter sterilised and distributed into 3–5 ml aliquots. Proteinase K powder was added to the buffer (10 mg/ml) just before its use and filter sterilised. This lysis buffer (200 μl) was added into the re-hydrated SSS sample and allowed to incubate overnight at 60 °C. Then the lysate was centrifuged at 13, 000 rpm for 10 minutes, the supernatant was divided into two aliquots. One aliquot was stored at − 20 °C and the other tube was stored at 4 °C for further analysis. DNA from the biopsy samples were obtained by using DNeasy Tissue kit (Qiagen).

VNTR MAPPING

Single short tandem repeat (STR) amplification was done according to the IDEAL protocol[2] and the Multiplex PCR (MP) amplification was done according to a protocol developed at CSU.[3] MP was performed to amplify four or five VNTR loci simultaneously from the clinical isolates, by utilising the primer sequences described by us or others, in four combinations.[3–5] Primer sequences used for M-PCR combinations are from Kimura *et al.*[3] Ten microsatellites [(AC)8a, (AC)8b, (AC)9, (AT)15, (AT)17, (TA)10, (TA)18, (GGT)5, (GTA)9, (GAA)21] and seven minisatellites [6-7, 12-5, 18-8, 21-3, 23-3, 27-5, and rpoT] were included in this

study. Minisatellite copy number was inferred by 2·5–3% agarose gel electrophoresis using the EZ load™ 20 bp Molecular Ruler (Bio-Rad) for product length determination. The copy number (allele) for microsatellite loci was determined by direct sequencing of PCR products. Sequence was obtained for representative products at the Macromolecular Resource Facility (MRF) (now known as Proteomics and Metabolomics Facility) at CSU.

MP products were subjected to fragment length analysis (FLA) for determining the allele number for each VNTR locus, henceforth this strain typing method will be called as MP-FLA.[3] After the M-PCR, the diluted PCR product was combined with deionised formamide (Applied Biosystems), and LIZ-500 DNA standard (Applied Biosystems). The sample was denatured and subjected to capillary electrophoresis on the Applied Biosystems Genetic Analyzer 3130 at MRF/PMF at CSU. Following the separation, the electropherograms were visualised and analysed using GeneMapper Version 3.7 software (Applied Biosystems) to determine the major allele for each VNTR locus, in each Multiplex-PCR combination.[3]

Results

DNA from 60 SSS and some of their paired biopsy samples were extracted. Single PCR for 13 VNTR loci were done for some of the samples. Allele numbers for the minisatellite loci like 12-5, 21-3, 23-3 and 27-5 were obtained from 3% agarose gel. However, the sequence data obtained from the PCR products of the microsatellite loci were hard to analyse to determine the exact allele number. Therefore, MP-FLA method was used for obtaining the VNTR profiles for all samples (Table 1).

In order to test the reproducibility of the VNTR data, nine SSS DNA samples were also sent to another laboratory. Thirteen VNTR loci data for the nine SSS samples were obtained by using single PCR method and conventional sequencing at a partner laboratory, whereas at CSU MP-FLA method was used. When the VNTR data from both the laboratories were compared 14 mismatches were observed. When the sequence files were re-read for 12 of these mismatches, it was found that seven of the sequence data was hard to analyse or five of them were misinterpreted due to mixed signals, stutter or unidentifiable end points of repeats in the sequence. The FLA results were therefore accepted. In general, MP followed by FLA was found to be easier to interpret for allele numbers for the VNTR loci and the data obtained from this approach may be more accurate and also useful to detect mixed alleles.

In order to study the intra-patient *M. leprae* VNTR profiles, SSS from different anatomical sites were collected. A biopsy specimen was also obtained from one of the lesions. These SSS and biopsy samples were subjected to M-PCR and FLA. It was found that the patterns were mostly similar in the minisatellites, while variations were observed in the more variable and stutter prone microsatellites like (AT)17 and (GAA)21 (Table 2).

The VNTR profiles of the isolates from the SSS samples within the two MCFs were compared. IDEAL50 and IDEAL53 of MCF-1 were found to have identical VNTR profiles, indicating that the infections were from the same isolate. In another MCF (IDEAL68, IDEAL69 and IDEAL70) the isolates in the patients IDEAL69 and IDEAL70 were found to be closely matching, but different from that of IDEAL68 (Table 3) at 4/13 VNTR loci.

When the epidemiological information for the second MCF was considered, it was found that this family has a history of five other leprosy patients (two uncles, two sisters and a brother). Therefore, multiple sources of infection may be possible in this family context.

Table 1. *M. leprae* VNTR profiles in leprosy patients in the Cebu study population

	(AC)8b	(GTA)9	(GGT)5	(AT)17	21-3	(AC)9	(AT)15	(AC)8a	27-5	6-7	(TA)18	(GAA)21	18-8	12-5	23-3	(TA)10	rpoT	BI
IDEAL6	8	9	5	14	3	9	15	9	5	7	20?	24	8	5	2	12	3	2·0 +
IDEAL7	9	9	5	15	2	8	17	9	5	6	14	36	8	4	2	11	3	3·8 +
IDEAL8	7	9	5	17	3	9	16	10	5	7	18	21	8	5	2	11	3	2·33 +
IDEAL9	6	9	5	10	2	8	17	9	5	7	15	16	7	5	2	7	3	3·66 +
IDEAL10	7	9	6	14	3	8	20	10	5	7	24	23	8	4	2	11	3	3·0 +
IDEAL11	8	9	5	13	3	9	15	9	5	8	21	19	8	4	2	11	3	3·17 +
IDEAL12	7	10 + 12	4	14	3 + 1	9	14	9	5	7	17	20	7/8	4	2	11	3	2·8 +
IDEAL13	7	10	4	14	1	9	14	9	5	7	17	20	7	4	2	11	3	4·5 +
IDEAL14	8	9	5	15	3	9	14	9	5	7	19	25	8	5	2	11	3	5·0 +
IDEAL15	8	10	5	15	3	9		8	5	7	21	26	8	4	2	11		4·7 +
IDEAL16	8	9	5	14	3	8	17	9	5	7	21	25	8	4	2		3	1·2 +
IDEAL17	8	8	5	13	3	8	14	10	5	6	22	27	8	4	2	11	3	3·33 +
IDEAL18	8	11	5	13	3	10	18	8	5	7	14	31	8	4	2	10		3·5 +
IDEAL19	8	11	4	15	2	8	19	8	5	6	13	9	8	4	2	8	3	2·2 +
IDEAL20	7	12	5	13	3	8	22	9	5	7	21	31	8	4	2	11	3	4·0 +
IDEAL21	8	9	5	11	3	9	13	9	5	8	20	18	8	4	2	11		4·66 +
IDEAL22	8	11	5	15	3	10	16	9	5	7	15		8	4	2	10	3	4·5 +
IDEAL23	8	9	5	20	3	9	15	8	4	8	25	19	8	4	2	10	3	5·33 +
IDEAL24	8	9	5	13	3	9	11	8	5	7	14	16	8	4	2	9	3	5·0 +
IDEAL25	8	9	4/5	15	3	9	15	9	5	7	16/19	24	8	5	2	13	3	5·5 +
IDEAL26	8	11	5	12	3	9	16	9	5	7	19	21	8	4	2	9	3	3·8 +
IDEAL27	8	11	5	15	3	8	14	9	5	6	14	22	8	4	2	13	3	5·0 +
IDEAL28	8	12	5	13	3	10		9	5	7	23	28	8	4	2	11		1·7 +
IDEAL29	8	13	5	13	3	9		9	5	7	15	31	8	4	2	10		4·8 +
IDEAL30	8		5	13	3	8		9	5	7	15	19			2	10	3	1·0 +
IDEAL31	8	9	5	15	3	9	13	10	5	7	23	19		4	2	12	3	2·5 +
IDEAL32		12	4	13	3	8	17	9		7	15	12		4	2		3	0·33 +
IDEAL33	8		6	18	2/3	8		9	4	7	15		8	4	2		3	0·17 +
IDEAL34	7	9	6	18	3	9	23	10	5	7	11	22	8	4	2	16		2·5 +
IDEAL35	8	10	5	14	3	9	15	9	5	7	18	25	8	5	2	12	3	4·33 +
IDEAL36	8	12	5	13	3	8/10	17	8	5	5/7	16	23	8	4	2	10	3	5·0 +
IDEAL37	8	11	5	14	3	10	16	8	5	7	14	30/33	8	4	2	10	3	4·17 +
IDEAL38	8	9	5	21	3	9	15	8		7	15	27	8	6	2	11	3	4·7 +
IDEAL39	8	13	5	13	3	9	15	9	5	7	16	31	8	4	2	10	3	4·5 +
IDEAL40	8	11	5	14	3	9	15	8	5	7	17	26	8	4	2	10	3	4·2 +
IDEAL41	7	9	6	17	3	9	22	10/9	5	6	17	23	8	4	2	13	3	4·2 +

Table 1. *continued*

	(AC)8b	(GTA)9	(GGT)5	(AT)17	21-3	(AC)9	(AT)15	(AC)8a	27-5	6-7	(TA)18	(GAA)21	18-8	12-5	23-3	(TA)10	rpoT	BI
IDEAL42					2	8	15	9	6			32	8	5	2		3	0·33 +
IDEAL43	8	11	5	14/10	3	8		10	5	7	17	26	8	4	2	10		0·17 +
IDEAL44	8	12	5	14	3	9	16	11	5	7	17	23	8	4	2	14		4·66 +
IDEAL45	8	13	4	15	3	9	16	9	5	7	21	27/25	8	4	2	11	3	4·5 +
IDEAL46	6	12	4	12	2	8	16	9	5	6	14	23	8	4	2	7	3	4·0 +
IDEAL47	7	9	6	17	3	9	15	9	5	7		26	8	4/5	2		3	1·0 +
IDEAL48	8	9	4/5	15/16	3	9	15	9	5	7	17	25	8	5	2	11	3	5·0 +
IDEAL49	8	9	5	12	2	10	14	9	5	7	15		8/7	4	2	10	3	4·2 +
IDEAL50	8	10	5	14	3	8	15	8	5	5	18	24	8	4	2	10		5·0 +
IDEAL51	7	8	4	17	1	9	14	9	5	8	14	18	7	4	2	10	3	5·0 +
IDEAL52	6	9	5	10	2	8	18	9	5	6	13	14	8	5	2	7	3	5·0 +
IDEAL53	8	10	5	14	3	8	15	8	5	5	16	24/25	8	4	2	10	3	3·5 +
IDEAL54	8	12	5	14	3	8	18	9	5	7	23	18	7	4	2	11	3	3·17 +
IDEAL55	8	11	5	17	3	9	14	10	5	8	18	17	8	4	2	11	3	3·2 +
IDEAL56	7		6	12	2/3	9	14	11	5	7		18	8	4	2		3	1·2 +
IDEAL57	8	12	5	13	3	9	15	9	5	7	19	27	6	4	2	10	3	4·0 +
IDEAL58	8	9	5	15	3	9	11	8	5	8	16	17		4	2	9	3	4·2 +
IDEAL59	8	9	5	18	3	9	15	8	5	7	21	24	4	5	2	12	3	5·0 +
IDEAL60																		0·17 +
IDEAL61	8	9	5	15	3	9		9	5	7	17	18	8	5	3	11	3	3·7 +
IDEAL62	8	9	5	15	3	9	15	9	5	7	18	25	8	5	2	12	3	4·0 +
IDEAL63	8	10	5	24	3	9	16	9	5	7	16	22	8	5	2	14	3	2·7 +
IDEAL64	8	10	5	17	3/2	10	13	9	5	7	18	25	8	4	2	12	3	4·2 +
IDEAL65	7	9/10	6		3	9	16	10	5	7	15	23	8	4	2	12	3	1·5 +
IDEAL66	8		5	14	3	9	13	9	5	8	15	19	8	4	2	11	3	2·7 +
IDEAL67	8	11	5	15	3	9	16	8	5	6		27	8	4	2	11	3	5·0 +
IDEAL68	7	9	6	15	3	9	14	9	5	7	17	26	8	4	2	13	3	5·0 +
IDEAL69	9	9	5	15	3	9	14	9	5	7	17	23	8	5	2	13	3	4·8 +
IDEAL70	9	9	5	15	3	9	14	9	5	7	17	25	8	5	2	13	3	4·2 +
					Weal PCR product				NO PCR product									

+ indicates two alleles for the VNTR locus; /indicates either of the alleles, small font number indicates a minor allele of the locus.

Table 2. Comparison of VNTRs from different body sites; Biopsy (B) and Slit Skin Smears (A, B, C)

		STR locus												
Sample ID	Sample type	(AC)8a	(AC)8b	(AC)9	(TA)10	(AT)17	(GGT)5	(GTA)9	(GAA)21	6-7	12-5	21-3	23-3	27-5	Sample collection sites
IDEAL1A	SSS	9	8	9	10	13	5	12	28	7	4	3	2		Ear lobe right
IDEAL1B	SSS	9	8	9	10	13	5	12	29	7	4	3	2	5	Elbow right
IDEAL1C*	SSS	9	8	9	10	13	5	12	29	7	4	3	2	5	Elbow left
IDEALB01	Biopsy	9	8	9	10	13	5	12	29	7	4	3	2	5	Elbow left
IDEAL2A	SSS	10	8	8	11	13	5	11	30 + 34	7	4	3	2	5	Ear lobe right
IDEAL2B*	SSS	10	8	8	11	13	5	11	31	7	4	3	2	5	Back right A
IDEAL2C*	SSS	10	8	8	11	13	5	11	30	7	4	3	2	5	Back right C
IDEALB02	Biopsy	10	8	8	11	13	5	11	31	7	4	3	2	5	Back right
IDEAL3A	SSS	9	NA	8	NA	10	NA	9	12	NA	4	3	NA	5	Ear lobe left
IDEAL3B	SSS	10	6	8	7	10	5	9	15	7	5	2	2	5	Arm left posterior
IDEAL3C*	SSS	10	6	8	NA	10	5	10	14	7	5	2	2	5	Back left
IDEALB03	Biopsy	10	6	8	7	10	5	10	14	7	5	2	2	5	Back left
IDEAL4A	SSS	10	7	9	NA	18	6	9	24	7	4	3	2	5	Ear lobe right
IDEAL4B*	SSS	10	7	9	NA	19	6	9	23	7	4	3	2	5	Back right
IDEAL4C	SSS	10	7	9	12	19	6	9	23	7	4	3	2	5	Back left
IDEALB04	Biopsy	10	7	9	12	19	6	9	23	7	4	3	2	5	Back right
IDEAL5A	SSS	10	6	8	7	9	5	9	14	7	5	2	2	5	Ear lobe left
IDEAL5B	SSS	10	6	8	7	9	5	9	14	7	5	2	2	5	Arm left posterior A
IDEAL5C*	SSS	10	6	8	7	9	5	9	14	7	5	2	2	5	Arm left posterior B
IDEALB05	Biopsy Weak product	10	6	8	7	9	5	9	14	7	5	2	2	5	Arm left posterior B

* SSS is from the biopsy site, NA: No PCR product.

Table 3. MLVA profiles of two multi-case families

		STR locus														
Family Number	Patient ID	(AC)8a	(AC)8b	(AC)9	(TA)10	(AT)17	(GGT)5	(GTA)9	(GAA)21	6-7	12-5	21-3	23-3	27-5	Barangay	City
MCF-1	IDEAL50	8	8	8	10	14	5	10	24	5	4	3	2	5	Kinalumsan	Lapu-lapu
	IDEAL53	8	8	8	10	14	5	10	24/25	5	4	3	2	5	Kinalumsan	Lapu-lapu
MCF-2	IDEAL68	9	7	9	13	15	6	9	26	7	4	3	2	5	Upper Gahab	Sibonga
	IDEAL69	9	9	9	13	15	5	9	23	7	5	3	2	5	Upper Gahab	Sibonga
	IDEAL70	9	9	9	13	15	5	9	25	7	5	3	2	5	Upper Gahab	Sibonga

Alleles Differences when compared with *M. leprae* found in siblings in the multi-case family.

Discussion

MLVA differentiated *M. leprae* in this study of 70 leprosy patients consulting at the Cebu Skin Clinic; adding to an existing strain type database of more than 200 Cebu patients. Although a formal phylogenetic or cluster analysis of the strain types in the 70 patients is not shown here, the alleles, their frequencies and diversity, and the VNTR patterns are consistent with earlier findings. The strain types can be assigned to five major groups.[6] The two multi-case families in this study (as in others identified in the earlier study)[6] are of interest in that two individuals in each family had identical strains of *M. leprae*, indicating that the VNTR profile can remain stable for at least as long as the incubation period of these cases. This is also suggested by the finding that the VNTR profile is stable within one individual. This suggests that if the right specimens can be collected, evidence of direct transmission between individuals could be obtained.

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References

[1] Guinto RS, Doull JA, Bancroft H *et al.* A field study of leprosy in Cordova, Philippines; resurvey in 1941 after eight years. *Int J Lepr*, 1951; **19**: 117–135.
[2] Gillis T, Vissa V, Matsuoaka M, *et al.* Characterization of Short tandem repeats for genotyping *Mycobacterium leprae*. *Lepr Rev*, 2009; **80**, 250–260.
[3] Kimura M, Li W, Sakamuri RM *et al.* Rapid single nucleotide polymorphism typing of *Mycobacterium leprae* and association with short tandem repeat alleles in the Philippines. *J Clin Microbiol*, 2009; **47**: 1757–1766.
[4] Groathouse NA, Rivoire B, Kim H *et al.* Multiple polymorphic loci for molecular typing of strains of *Mycobacterium leprae*. *J Clin Microbiol*, 2004; **42**: 1666–1672.
[5] Zhang L, Budiawan T, Matsuoka M. Diversity of potential short tandem repeats in *Mycobacterium leprae* and application for molecular typing. *J Clin Microbiol*, 2005; **43**: 5221–5229.
[6] Sakamuri RM, Kimura M, Li W *et al.* Population based molecular epidemiology of leprosy in Cebu, Philippines. *J Clin Microbiol*, 2009; **47**: 2844–2854.