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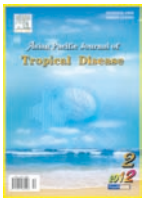

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Do diverse *Mycobacterium tuberculosis* strains circulate in Mumbai?

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ABSTRACT

Objective: To facilitate large network surveillance, we evaluated use of spoligotyping in combination with different formats (12-, 15- and 24- loci) of to Mycobacterial Interspersed Repetitive Units (MIRU)-Variable Number Tandem Repeats (VNTR) achieve maximal discriminatory power. **Methods:** Spoligotyping and 24-loci MIRU-VNTR was performed on 127 consecutive extrapulmonary *Mycobacterium tuberculosis* (*M. tuberculosis*) isolates. **Results:** Based on the analysis of 127 *M. tuberculosis* isolates, all 3 formats of MIRU-VNTR when coupled with spoligotyping (HGDI: spoligotyping +12-loci, 0.9967<spoligotyping +15-loci, 0.9996 <spoligotyping +24-loci, 1.0000) reported a similar discriminatory power in comparison to MIRU-VNTR alone (HGDI: 12-loci, 0.9951<15-loci, 0.9996<24-loci, 1.0000). **Conclusions:** Thus, we propose the use of spoligotyping as an initial epidemiological typing tool followed by the minimal subset of MIRU-VNTR typing for CAS, Beijing and EAI predominant lineages in the Mumbai population, retaining 100% discriminatory power comparable to that obtained by 24-loci MIRU-VNTR alone. For long-term surveillance, 24-loci MIRU-VNTR would an ideal typing tool for identification of predominant *M. tuberculosis* lineages in the Mumbai.

1. Introduction

Molecular fingerprinting facilitates identification of the population dynamics of the circulating *Mycobacterium tuberculosis* (*M. tuberculosis*) strains in a particular region, but the utility of a fingerprinting tool for such epidemiological based studies depends on its discriminatory power. Although IS*6110*-RFLP with high discriminatory power has been considered as the gold standard for such analysis, its use has been limited[1]. Use of PCR based tools like spoligotyping and Mycobacterial Interspersed Repetitive Units-Variable Number Tandem Repeats (MIRU-VNTR) have been encouraged due to simplicity in technique, rapid result availability, reproducibility and data exchange portability format. A recent study from this tertiary care centre in Mumbai reported 24-loci MIRU-VNTR to have a higher discriminatory power in comparison with IS*6110*-RFLP and spoligotyping, reporting the association of Beijing genotypes with multi-drug resistance[2]. Thus, we aim to evaluate the additional benefit of different formats of MIRU-VNTR (12-, 15-, 24- loci) typing when spoligotyping would be used as a first-line fingerprinting tool for appropriate lineage assignments.

The study was carried out a private tertiary care hospital in Mumbai with a referral bias towards treatment failures, and was ethically approved by the National Health and Education Society. A total of 127 culture positive *M. tuberculosis* isolates from consecutive extrapulmonary specimens were included for this study analysis. Genomic DNA was isolated from clinical *M. tuberculosis* isolates as described by van Sooligen *et al*[3]. Spoligotyping was performed using a commercial kit (Isogen, Netherlands) as per the manufacturers recommendations. MIRU-VNTR profiles were generated by performing individual PCR amplification for each of the 24 loci, followed by gel electrophoresis and analyzed as described before[1,4]. The allelic diversity (h) at each VNTR locus was calculated as $h=1-\Sigma x_{i2}\,[n/(n-1)]$, where x_i is the frequency of the ith allele at the locus, and n is the number of isolates[5,6]. The Hunter-Gaston discriminatory index (HGDI) was used to calculate the discriminatory power of each method[7]. Spoligotyping in binary format was converted to an octal code using the SITVIT2 database (http://www.pasteur-guadeloupe.fr:8081/SITVITDemo/) and compared with the

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Table 1
Detailed allelic diversity of each loci and represents a minimal loci typing for successful discrimination of all SpolDB4 classified based CAS, EAI and Beijing lineage.

MIRU-VNTR loci	Whole sample	CAS lineage	Recommended minimal loci typing for CAS lineage	EAI lineage	Recommended minimal loci typing for EAI lineage	Beijing lineage	Recommended minimal loci typing for Beijing lineage
Locus 154	0.09	−0.02		0.03		0.04	
Locus 960	0.77	0.67	X	0.64	X	0.38	X
Locus 2165	0.69	0.22		0.78	X	0.18	
Locus 2687	0.50	0.18		0.25	X	0.44	X
Locus 3690	0.66	0.18		0.78	X	0.18	
Locus 424	0.71	0.65	X	0.03		0.44	X
Locus 1644	0.56	0.32	X	0.09		0.04	
Locus 2347	0.41	−0.02		−0.03		−0.04	
Locus 2996	0.80	0.70	X	0.09		0.58	X
Locus 4052	0.91	0.91	X	0.67	X	0.87	X
Locus 577	0.54	−0.02		0.48	X	−0.04	
Locus 1955	0.83	0.55	X	0.87	X	0.19	X
Locus 2401	0.58	0.14		0.44	X	−0.04	
Locus 3007	0.10	0.02		0.21		0.04	
Locus 4156	0.80	0.68	X	0.61	X	0.47	X
Locus 580	0.44	−0.02		0.35		−0.04	
Locus 2059	0.09	0.02		0.09		0.04	
Locus 2461	0.52	0.02		0.52	X	0.44	X
Locus 3171	0.07	0.02		0.09		−0.04	
Locus 4348	0.54	0.15		0.50	X	−0.04	
Locus 802	0.56	0.52	X	0.35		0.37	X
Locus 2163 b	0.74	0.11		0.51	X	0.47	X
Locus 2531	0.47	0.02		0.15		−0.04	
Locus 3192	0.52	0.26	X	0.44	X	0.19	

X: denotes the loci recommended for fingerprinting.

SpolDB4 database for lineage identification. MIRU-VNTR profile analysis was done using the online database at http://www.MIRU-VNTRplus.org[8-10]. MIRU-VNTR clusters were defined for isolates sharing identical patterns. Dendrograms were generated using the unweighted pair group method with arithmetic averages (UPGMA) and the categorical coefficient for MIRU-VNTR analysis. For spoligotyping and MIRU-VNTR analysis, isolates with identical spoligotype international type (SIT) profiles and identical MIRU-VNTR patterns were categorized as 'clusters', whereas patterns/profiles reported for a single isolate were termed as 'unique'.

Spoligotyping revealed a total of 41 patterns (123 strains), representing 17 (13.8%) orphan strains and 12 (9.7%) unique strains. Thirteen clusters (94 strains, 76.4%) were identified in the study population: the predominant clades being Central Asian Strain (CAS) (36.5%, 45 strains), Beijing (20.3%, 25 strains) and East African Indian (EAI) (25.2%, 31 strains) genotypes followed by a small cluster of ill-defined T-lineage (8.1%, 10 strains). The HGDI was found to be 0.9182. MIRU-VNTR typing (all 3 formats: 12-, 15- and 24- loci) performed on all 123 isolates reported 24-loci genotyping to have the highest discriminatory power followed by 15-loci (3 clusters, 2 strains per cluster) and 12-loci (11 clusters, 2-5 strains per cluster) formats respectively (HGDI, 1.0000<0.9996<0.9951), a finding similar to that reported in our earlier study[2] (Supplementary material 1). Supplementary material 2 represented a UPGMA tree based on 24-loci MIRU-VNTR analysis of 123 isolates included in the study.

Congruent analysis of MIRU-VNTR with spoligotyping, did not reveal much difference in the discriminatory power when either of the MIRU-VNTR genotyping formats was combined with spoligotyping (Supplementary material 1). This coupled analysis classified 123 strains into 3 major lineages: CAS (36.5%, 45 strains), EAI (25.2%, 31 strains) and Beijing (20.3%, 25 strains); along with a minor ill-defined T (8.1%, 10 strains) clade. Of the remaining, 2 (1.6%) strains were 'unique' and 10 (8.1%) orphan strains were found belonging to LAM (3.1%, 5 strains), Haarlem (1.5%, 2 strains), Africanum (1.5%, 2 strains), and X (0.7%, 1 strain) genotypes. For the first time in Mumbai, we report the introduction of Africanum (2 patients with no epidemiological links: a tourist from South Africa and a resident of suburban Mumbai) and X (1 patient residing in Mumbai with recent history of foreign travel) genotypes. Loci 4052 (allelic diversity, 0.91) were found to be major contributors responsible for decreasing the clustering rate of *M. tuberculosis* strains (Table 1).

Based on this data, we attempted to screen out the least number of loci that if coupled with spoligotyping analysis would successfully discriminate strains of the 3 predominant clusters *i.e* CAS, Beijing and EAI in our population. Based on the allelic diversity of each loci, we recommend the use of a minimal loci system (Table 1) for successful discrimination of all isolates classified as CAS, Beijing and EAI by

Supplementary material 1

A comparative analysis of spoligotpying alone and in congruence with different formats of MIRU-VNTR typing.

Typing Method	No. of isolates	No. of clusters (size)	No. of strains clustered (%)	No. of orphans (%)	No. of Unique strains (%)	Predominant lineages (no. of strains, %)	HGDIa
Spoligotyping	127	13 (2-25)	94 (76.4)	17 (13.8)	12 (9.7)	CAS (39, 31.7) EAI (29, 23.5) Beijing (25, 20.3)	0.9182
12-loci MIRU-VNTR	127	11 (2-5)	33 (26.8)	88 (71.5)	2 (1.6)	CAS (45, 36.5) EAI (31, 25.2) Beijing (25, 20.3)	0.9951
15-loci MIRU-VNTR	127	3 (2)	6 (4.8)	115 (93.4)	2 (1.6)	CAS (45, 36.5) EAI (31, 25.2) Beijing (25, 20.3)	0.9996
24-loci MIRU-VNTR	127	0 (0)	0 (0)	121 (98.4)	2 (1.6)	CAS (45, 36.5) EAI (31, 25.2) Beijing (25, 20.3)	1.0000
Spoligotyping + 12-loci MIRU-VNTR	127	6 (2-5)	19 (15.4)	102 (82.9)	2 (1.6)	CAS (45, 36.5) EAI (31, 25.2) Beijing (25, 20.3)	0.9967
Spoligotyping + 15-loci MIRU-VNTR	127	3 (2)	6 (4.8)	115 (93.4)	2 (1.6)	CAS (45, 36.5) EAI (31, 25.2) Beijing (25, 20.3)	0.9996
Spoligotyping + 24-loci MIRU-VNTR	127	0 (0)	0 (0)	121 (98.4)	2 (1.6)	CAS (45, 36.5) EAI (31, 25.2) Beijing (25, 20.3)	1.0000
Spoligotyping + recommended minimal format loci MIRU-VNTR typing (CAS)b	45	0 (0)	0 (0)	45 (100.0)	0 (0.0)	NAc	1.0000
Spoligotyping + recommended minimal format loci MIRU-VNTR typing (EAI)d	31	0 (0)	0 (0)	31 (100.0)	0 (0.0)	NA	1.0000
Spoligotyping + recommended minimal format loci MIRU-VNTR typing (Beijing)e	25	0 (0)	0 (0)	25 (100.0)	0 (0.0)	NA	1.0000

aHGDI, Hunter-Gaston discriminatory index.
bRecommended minimal loci MIRU-VNTR typing for discrimination of CAS lineage (refer Table 1).
cNA, Not applicable.
dRecommended minimal loci MIRU-VNTR typing for discrimination of EAI lineage (refer Table 1).
eRecommended minimal loci MIRU-VNTR typing for discrimination of Beijing lineage (refer Table 1).

spoligotyping, which achieved a HGDI of 1.000, comparable to that of 24-loci typing system.

In conclusion, we recommend the use of spoligotyping as an initial typing tool followed by the MIRU-VNTR system for large scale genotyping studies in Mumbai. Also the use of minimal loci typing system would be a beneficial in a large scale genotyping studies.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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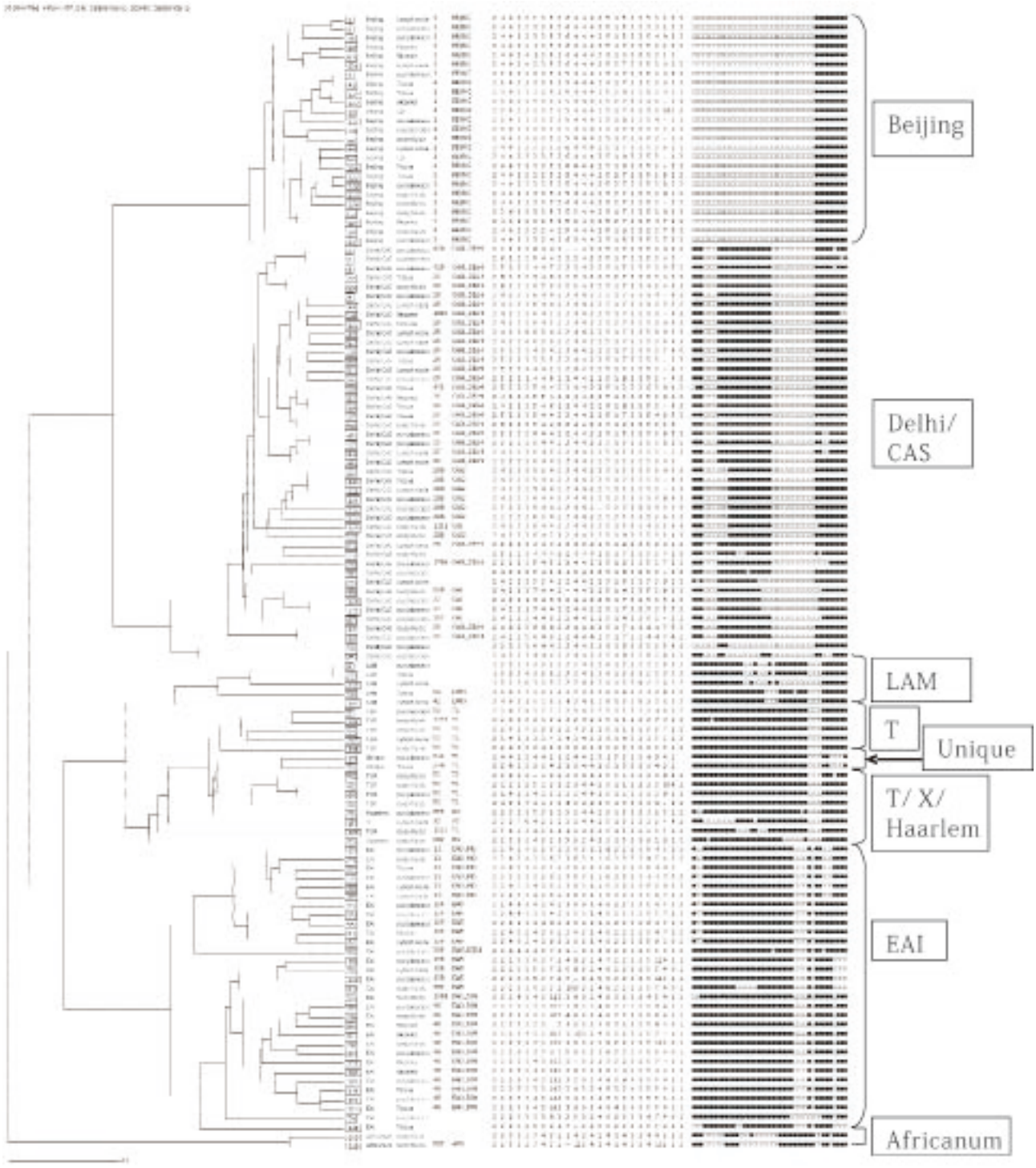

Supplementary material 2. A unweighted pair group method with arithmetic averages (UPGMA) tree based on 123 isolates included in the study. In the order, from left to right: sample ID no., MIRU-based lineage, specimen type, Spoligotype International Type, 24-loci MIRU-VNTR profile and spoligotype pattern.