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Short Communication

Efficient discrimination by MIRU-VNTRs of *Mycobacterium tuberculosis* clinical isolates belonging to the predominant SIT11/EAI3-IND ancestral genotypic lineage in Kerala, India

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ABSTRACT

The present study evaluated the ability of MIRU–VNTRs to discriminate *Mycobacterium tuberculosis* (MTB) clinical isolates belonging to the SIT11/EAI3-IND ancestral genotypic lineage, which is highly prevalent in Kerala, India. Starting from 168 MTB clinical isolates, spoligotyping (discriminatory index of 0.9113) differentiated the strains into 68 distinct patterns, the biggest cluster being SIT11/48 SIT11 ($n = 48$). The present study shows that 12-loci MIRUs and 3 ETRs allowed an efficient discrimination of these isolates (discriminatory indexes of 0.7819 and 0.5523, respectively).

India ranks first in the burden of tuberculosis (TB), and along with the other Asian countries, like China, Pakistan, Bangladesh and Indonesia, accounts for over 50% of the TB burden of the world [1]. Different methods of genotyping such as IS6110–RFLP, spoligotyping and MIRU-VNTR (Mycobacterial Interspersed Repetitive Unit-Variable Number Tandem Repeats) have helped in studying the evolution and dissemination dynamics of *Mycobacterium tuberculosis* (MTB) [2]. IS6110–RFLP, long considered the gold standard for genotyping of MTB, has disadvantages such as the requirement of good quality DNA and the inability to type strains with low/no copy number of *IS6110* insertions. In addition, the difficulties in inter-laboratory comparison of the RFLP patterns hinder its generalized use [3].

PCR-based spoligotyping has helped to distinguish different MTB lineages; it also allows easy inter-laboratory comparison of data thanks to huge international databases [4]. Another PCR-based typing method using MIRU-VNTR mini-satellites has advantages similar to spoligotyping for inter-laboratory comparison; but in addition, it shows a significantly higher discriminatory power – equal to that of the IS6110–RFLP gold standard [5]. However, both the MTB population under study as well as the discriminatory power of the method(s) in question is important to choose a typing

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technique. Though IS6110–RFLP has been used worldwide for successful genotyping, MTB populations from Kerala, South India contain low or no copy of IS6110 in their genomes, and therefore this method fails to discriminate the isolates [6]. In such a case, spoligotyping has an advantage over IS6110–RFLP.

The study population consisted of 168 clinical isolates of MTB obtained from patients attending different TB clinics in Kerala, India [7]. This included 9 multidrug resistant (MDR) and three extremely drug resistant (XDR) isolates. The spoligotyping of the isolates was done as described by Kamerbeek et al. [8]; 12 loci MIRU-VNTRs and Exact Tandem Repeats-A, B & C (ETR A, B & C) were performed as reported previously by Supply et al. [9] and Frothingham and Meeker-O'Connell [10]. The amplicons were separated manually on 2% agarose gels, and the copy number of each locus was scored by comparing the corresponding bands with appropriate DNA size markers. Both spoligotypes in octal format and MIRU patterns were compared with the SITVIT2 proprietary database of the Institut Pasteur de la Guadeloupe, which is an updated "in-house" version of the recently released SITVITWEB database [4]. In this database, Spoligotype International Type (SIT) and MIRU International Type (MIT) designate identical patterns shared by two or more patient isolates, whereas "orphan" designates patterns reported only for a single isolate.

In this study, spoligotyping differentiated the 168 strains into 68 distinct patterns, of which 51 ($n = 151$ or 59.88%) corresponded to different SITs whereas 17 patterns ($n = 17$ or 10.12%) corresponded to orphans. The majority of the isolates ($n = 108$ or 64.28%) belonged to the ancestral East-African Indian (EAI) lineage; and among these, EAI3-IND sublineage predominated ($n = 61$ strains). The EAI3-IND sublineage strains were further split into 7 subclusters: a major subcluster that belonged to EAI3-IND prototype SIT11 ($n = 48/61$) with high phylogeographical specificity for India [4], and six other SITs ($n = 13/61$). Interestingly, three out of the nine MDR cases belonged to SIT11/EAI3-IND lineage strains.

12-loci MIRUs could differentiate this ancestral prototype into 15 distinct patterns and three orphans (Fig. 1). Out of the 15 patterns observed, eight patterns corresponded to "newly created shared-type" in the database (two or more strains belonging to a new pattern detected within this study, or due to a match with an orphan in the database). The analysis showed that 45% (21/48) of the SIT11 strains belonged to MIT69, which was discriminated using ETR-A, B and C in 16/21 strains with ETR pattern 614 (copy numbers for ETR A, B & C, respectively), followed by 2/16 isolates with ETR pattern 624, and a single isolate each with ETR patterns 514, 664 and 622, respectively.

Calculations of the Hunter Gaston Discriminatory Index (HGDI) for the study sample ($n = 168$) vs. SIT11/EAI3-IND strains ($n = 48$) are summarized in Table 1. The HGDI values for the global dataset ($n = 168$) by different typing formats were as follows: (i) spoligotyping alone, 0.9113; (ii) 15 VNTRs, 0.9735; and (iii) spoligotyping + 15 VNTRs, 0.9904.

The discriminatory power of 12 loci MIRU set for SIT11 sublineage was found to be 0.7819, with individual loci ranking in the order: MIRU4 (0.37) > MIRU10 (0.23) > MIRU31 (0.20). The ETRs showed a global HGDI of 0.5523 for SIT11/EAI3-IND strains, followed by individual values of 0.339 (ETR-B) > 0.199 (ETR-A) > 0.196 (ETR-C). Interestingly, the same values calculated for the global sample population were much higher: 0.738 (ETR-A) > 0.674 (ETR-B) > 0.617 (ETR-C).

Ali et al. has reported the efficient use of MIRUs for the discrimination of CAS1 strains isolated from Pakistan [11]. Their study showed MIRU-4 to have the least HGDI in CAS1 strains while the present study shows that it has maximal discrimination for the strains. A recent study from Poland showed the efficient use of 15 and 19 loci MIRU-VNTR for further discrimination of spoligotype clusters [12]. In their study population, MIRU-40 exhibited maximum polymorphism. In India, Sharma et al. has shown the efficient use of MIRU typing for better discrimination of spoligotype of MTB isolates from north India. In their study population, MIRU-26 showed

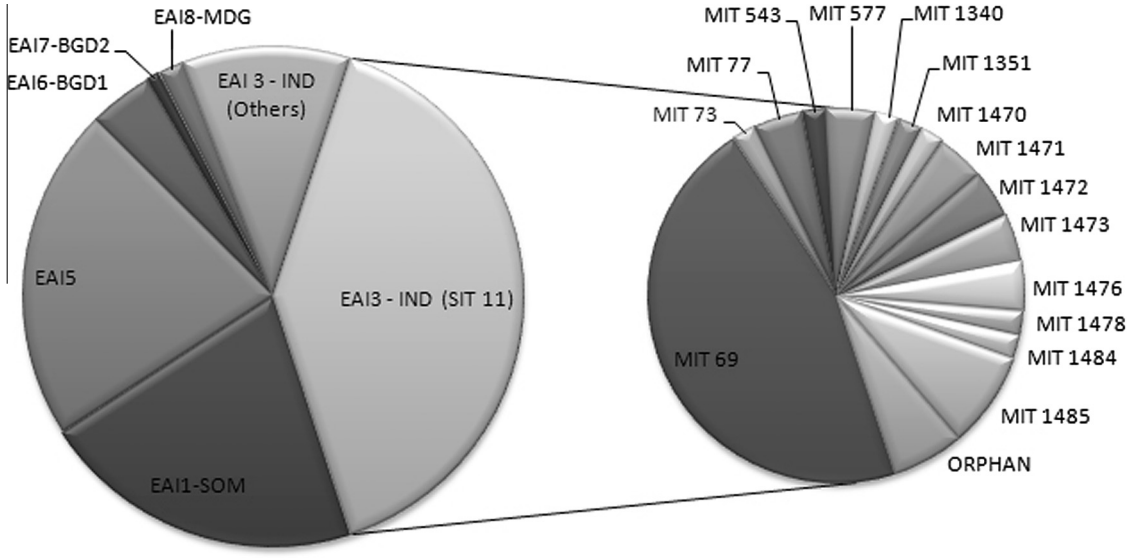

Fig. 1 – Discrimination of SIT11/EAI3-IND genotype of *Mycobacterium tuberculosis* isolates from Kerala based on MIRU-VNTRs & ETRs.

Table 1 – Allelic diversity of SIT11/EAI3–IND ancestral genotype of *Mycobacterium tuberculosis* isolates from Kerala based on MIRU-VNTRs and ETRs.

VNTRs	Copy no											SIT11		168 Isolates	
	0	1	2	3	4	5	6	7	8	9	10	HGDI	Rank	HGDI	Rank
MIRU 2	0	0	48	0	0	0	0	0	0	0	0	0	9	0.035	12
MIRU 4	0	0	0	1	4	38	4	0	1	0	0	0.3661	1	0.595	4
MIRU 10	0	0	0	3	42	3	0	0	0	0	0	0.2314	2	0.426	7
MIRU 16	0	0	2	46	0	0	0	0	0	0	0	0.0816	7	0.231	9
MIRU 20	0	0	47	1	0	0	0	0	0	0	0	0.0417	8	0.07	11
MIRU 23	0	0	0	0	2	0	44	2	0	0	0	0.1596	4	0.477	5
MIRU 24	0	2	46	0	0	0	0	0	0	0	0	0.0816	7	0.434	6
MIRU 26	0	0	48	0	0	0	0	0	0	0	0	0	9	0.379	8
MIRU 27	0	0	2	45	1	0	0	0	0	0	0	0.1215	6	0.093	10
MIRU 31	0	0	0	2	43	2	0	1	0	0	0	0.1977	3	0.652	2
[a] MIRU 39	0	0	1	44	2	0	0	0	0	0	0	0.124	5	0.629	3
MIRU 40	0	0	1	45	2	0	0	0	0	0	0	0.1215	6	0.656	1
ETR-A	0	0	2	1	0	1	43	0	0	1	0	0.1986	2	0.738	1
ETR-B	0	39	6	0	2	0	1	0	0	0	0	0.3289	1	0.674	2
ETR-C	0	0	2	3	43	0	0	0	0	0	0	0.1959	3	0.617	3

a Total number of isolates considered for MIRU 39 locus is 47 as one isolate showed two bands corresponding to three copies and four copies.

maximum discriminatory power of 0.896 [13]. A recent study from the nearby State of Tamil Nadu showed that MIRU-39 showed a better discriminatory power (0.58 for EAI) [14] compared with the earlier reports (HGDI for MIRU-39 = 0.4 by Supply et al. [15]). In this study, HGDI of MIRU-39 was 0.692 for the complete data set, and only 0.124 for EAI-3/IND strains. Thus, apparently, there is a wide diversity among MTB isolates from different parts of the world, and even within nearby geographical regions in India.

These observations underline that none of the available formats may be truly considered as being universal for optimal fingerprinting and epidemiological analysis of MTB. One therefore needs to adopt a typing method that best suits the study population. In this study, MIRU-VNTRs resulted in a better discrimination of MTB isolates from Kerala than IS*6110* and spoligotyping, and are therefore suitable to type low IS*6110* copy isolates that prevail in this part of the world.

Conflict of interest statement

The authors declare no conflict of interests.

Author contributions

S.M., R.A.K. conceived and designed the experiments; B.V.J., SS performed the experiments; B.V.J., V.H., N.R. analyzed the data; B.V.J., N.R. wrote the manuscript.

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