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Characterization of Ancestral *Mycobacterium tuberculosis* by Multiple Genetic Markers and Proposal of Genotyping Strategy

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Sixty-eight ancestral *Mycobacterium tuberculosis* isolates were previously identified by using the tuberculosis-specific deletion 1 (TbD1) PCR and mycobacterial interspersed-repetitive-unit–variable-number tandem repeat (MIRU-VNTR) typing (Y. J. Sun, R. Bellamy, A. S. G. Lee, S. T. Ng, S. Ravindran, S.-Y. Wong, C. Locht, P. Supply, and N. I. Paton, J. Clin. Microbiol. 42:1986–1993, 2004). These TbD1$^+$ ancestral isolates were further characterized and typed in this study by IS*6110* restriction fragment length polymorphism (RFLP) typing, VNTR typing using exact tandem repeats (VNTR-ETR), and spoligotyping of the direct-repeat region. To our knowledge, this is the first characterization of this genogroup by multiple genetic markers based on a fairly large sample size. In this genogroup, all spoligotypes were characterized by the absence of spacers 29 to 32 and 34. In addition, VNTR-ETR typing could add further resolution to the clustered isolates identified by MIRU-VNTR, and the combination of MIRU-VNTR and VNTR-ETR, called MIRU-ETR, showed the highest discriminatory power for these strains compared to IS*6110* RFLP typing and spoligotyping alone. However, MIRU-ETR appeared to still cluster some probably epidemiologically unrelated strains, as judged by IS*6110* RFLP divergence. Therefore, a typing strategy based on stepwise combination of MIRU-ETR and IS*6110* RFLP is proposed to achieve maximal discrimination for unrelated TbD1$^+$ strains. This typing strategy may be useful in areas where TbD1$^+$ ancestral strains are prevalent.

Tuberculosis remains a major infectious disease and causes high morbidity and mortality worldwide. A better understanding of *Mycobacterium tuberculosis* epidemiology and phylogeny will be helpful for containment of the malady. DNA fingerprinting of *M. tuberculosis* has gained increased acceptance as a useful tool for epidemiological (40) and phylogenetic (4, 18, 30, 35, 44) investigations of *M. tuberculosis*. During the past decade, a large number of DNA-fingerprinting methods based on various genetic markers have been developed (40). As no single method has so far defined all unique isolates, multistep typing strategies have been proposed and evaluated in a number of studies (2, 3, 7, 13, 15, 21, 28, 30–32, 45, 46).

IS*6110* restriction fragment length polymorphism (RFLP) typing based on the variability in numbers and chromosomal positions between strains (6) is thus far the most commonly applied method and also the most discriminatory method at the population level (39). Identical IS*6110* RFLP patterns often suggest recent transmission within a community (26, 29). This is not always true, however, because of the insufficient discriminatory power of IS*6110* typing for *M. tuberculosis* strains having few (≤5) IS*6110* copies, especially for one- or zero-IS*6110*-copy strains (2, 7, 15, 20, 22, 24). Even for strains having multiple (>5) copies of IS*6110*, identical patterns may still be found in epidemiologically unrelated strains (24, 46).

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Therefore, another genetic typing method is needed for assessing IS*6110*-defined epidemiologically unrelated clustered strains. Of the methods evaluated for this purpose, spoligotyping, which is based on the analysis of polymorphisms in the direct-repeat region (19, 41); typing based on mycobacterial interspersed repetitive units and variable numbers of tandem repeats of genetic elements (MIRU-VNTR) at 12 independent minisatellite loci scattered throughout the *M. tuberculosis* genome (24, 34); and VNTR typing using exact tandem repeats (VNTR-ETR) at five minisatellite loci (14) have been demonstrated to add further resolution to IS*6110*-defined clusters (1–3, 15, 21, 22, 24, 36, 46). Compared to IS*6110* RFLP, these PCR-based methods are highly reproducible, less labor-intensive, and faster; require much less DNA; and produce genotypes that are more comparable between different laboratories. MIRU-VNTR typing has been adapted to high-throughput analysis (7, 33, 36). Moreover, in several studies, MIRU-VNTR typing showed high discriminatory power, close to that of IS*6110* RFLP typing (7, 16, 24, 36).

Using both the tuberculosis-specific deletion 1 (TbD1) (4) and MIRU-VNTR (24, 34), 68 TbD1$^+$ ancestral (also called East Africa-Indian [EAI]) *M. tuberculosis* isolates, which accounted for ~23% of the tuberculosis cases in Singapore, have been identified (33). Preliminary analysis indicated that a substantial proportion of these TbD1$^+$ strains contain only a single IS*6110* element, and the discriminatory power of IS*6110* RFLP typing is therefore insufficient for them (4, 20). MIRU-VNTR typing has been shown to be highly discriminative for

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this genogroup (33, 36). In this study, we further examined the ancestral isolates by IS*6110* RFLP typing, spoligotyping, and VNTR-ETR typing. Based on our results, a stepwise genotyping strategy is proposed to achieve maximal discrimination among these ancestral isolates.

MATERIALS AND METHODS

Mycobacterial isolates. *M. tuberculosis* clinical isolates were collected from the Central Tuberculosis Laboratory in Singapore between August and December 1994. The incidence of tuberculosis in 1994 was 49 per 100,000 population (Communicable Disease Surveillance Report 1994; Department of Clinical Epidemiology, Tan Tock Seng Hospital, Singapore). DNA was extracted from *M. tuberculosis* cultures using the protocol of van Soolingen et al. (42). Sixty-eight ancestral *M. tuberculosis* isolates identified previously by TbD1 PCR and MIRU-VNTR typing (33) were analyzed.

IS*6110* RFLP typing. The recommended international standard protocol for IS*6110* RFLP (38) was followed. The IS*6110* RFLP patterns were analyzed by using Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium). Similarities between RFLP patterns were calculated by using the Dice coefficient, and the dendrogram was produced with the unweighted pair group method using arithmetic averages algorithm.

Spoligotyping. Spoligotyping was performed using a commercial kit (Isogen Bioscience BV, Maarssen, The Netherlands) according to the instructions of the manufacturer. The spoligotypes were presented by using pseudo-original patterns or 15 octal codes, as described by Dale et al. (8). For the 15 octal codes, briefly, the 43 spacers were recorded as 43 binary digits (where 1 indicates the presence of a spacer and 0 indicates the absence of a spacer). From spacers 1 to 42, every three digits were converted to an octal code (000 = 0, 001 = 1, 010 = 2, 011 = 3, 100 = 4, 101 = 5, 110 = 6, and 111 = 7), with the final digit remaining either 1 or 0. This yields a 15-digit octal designation.

VNTR-ETR typing. Three ETR loci (ETR-A, ETR-B, and ETR-C) described by Frothingham and Meeker-O'Connell (14) were used in the VNTR-ETR typing scheme. ETR-D and ETR-E were previously incorporated as MIRU loci in the MIRU-VNTR typing system based on the 12 loci (24, 34). The PCR primers and amplification conditions for these loci described by Frothingham and Meeker-O'Connell (14) were followed. The numbers of tandem-repeat units were determined by estimating the sizes of the amplicons on agarose gels.

Calculation of discriminatory power. The Hunter-Gaston Discriminatory Index (HGDI) described by Hunter and Gaston (17) was used as a numerical index for the discriminatory power of each typing method and strategy. The HGDI was calculated using the following formula:

$$HGDI = 1 - \left[\frac{1}{N(N-1)}\sum_{j=1}^{s} n_j(n_j - 1)\right]$$

where N is the total number of strains in the typing scheme, s is the total number of distinct patterns discriminated by each typing method and strategy, and n_j is the number of strains belonging to the jth pattern.

RESULTS

IS*6110* typing of ancestral *M. tuberculosis*. A previous MIRU-VNTR analysis showed that the ancestral isolates were the most heterogeneous genogroup among the genotypes identified in Singapore (33). This genetic heterogeneity of ancestral isolates was also reflected in the IS*6110* RFLP patterns. As shown in Fig. 1A and 2, IS*6110* RFLP similarities between these isolates were from 0 to 100%, and the number of IS*6110* copies among these isolates ranged from 0 to 15 copies. Most of the ancestral isolates belonged to two distant branches, a high-IS*6110*-copy branch (≥6 copies with one exception) and a low-IS*6110*-copy branch (≤4 copies with one exception), and only ~20% similarity was shown between the two branches. Despite the high heterogeneity, IS*6110* RFLP typing was less discriminative in typing these ancestral isolates than MIRU-VNTR typing (Fig. 1) because of the high frequency of low-IS*6110*-copy isolates, especially one-copy isolates, which accounted for 34% of the 68 ancestral strains (Fig. 2). IS*6110* RFLP defined four cluster patterns and 43 unique patterns; the cluster sizes ranged from 2 to 18 isolates (Fig. 1A). Three of the four clusters consisted of one- or zero-IS*6110*-copy isolates, and they accounted for 23 of the 25 clustered isolates (92%). The HGDI of IS*6110* RFLP typing was 0.931.

Spoligotypes of ancestral *M. tuberculosis*. Figure 1B shows the pseudospoligotypes of the 68 ancestral isolates. Unlike the IS*6110* RFLP patterns, the spoligotypes appeared to be more homogeneous. A typical characteristic of all the spoligotypes was the absence of spacers 29 to 32 and 34. This characteristic was also seen in the spoligotypes of other East Africa-Indian strains, as shown in previous studies (4, 9, 12, 30, 39). Forty-one distinct spoligotypes were detected, 36 of which were unique patterns; five cluster spoligotypes were shared by 32 isolates. The two largest clusters correspond to the shared patterns EAI2 and EAI3 in a spoligotype database (12). The isolates with unique spoligotypes accounted for 53% of the 68 isolates. The HGDI of spoligotyping was 0.923. Unlike the clusters defined by MIRU-VNTR, which contained only high-IS*6110*-copy isolates, three of the five spoligotype-defined clusters contained one-IS*6110*-copy isolates (isolates 43, 44, 47, 49, 50, 54 to 57, and 61) (Fig. 1), suggesting the poor discriminative ability of spoligotyping of one-IS*6110*-copy isolates compared to that of MIRU-VNTR typing.

Increase of MIRU-VNTR discriminatory power by addition of ETR loci. To investigate the discriminatory power of VNTR locus-based typing after addition of the three ETR loci to the 12 MIRU loci, all isolates were subjected to VNTR-ETR typing of ETR loci A, B, and C. VNTR-ETR typing using these ETR loci yielded 24 distinct patterns, 13 of which were unique patterns and 11 of which were cluster patterns. The sizes of clusters ranged from 2 to 17 isolates (Fig. 1D). Our results showed that VNTR-ETR typing was able to add further resolution to the clusters previously defined by MIRU-VNTR, reducing the number of clusters from five to three and the number of clustered isolates from 19 to 10 (Table 1), which increased distinct patterns, unique patterns, and the HGDI from 54, 49, and 0.978 (33) to 61, 58, and 0.994, respectively. We regard this combination of VNTR methods as an independent single typing method, like IS*6110* RFLP and spoligotyping, and in this work the combination of the 12-locus-based MIRU-VNTR typing and the 3-locus-based VNTR-ETR typing is referred to as MIRU-ETR.

Genotyping results by combinations of methods. Among the individual methods, MIRU-ETR was the most discriminative; its HGDI was 0.994 compared to 0.931 for IS*6110* RFLP typing and 0.923 for spoligotyping. Hence, MIRU-ETR is the most powerful first-line method for typing ancestral isolates in our setting. All of the isolates clustered on the basis of MIRU-ETR showed from zero to five band differences when their IS*6110* RFLP patterns were compared within each of the three clusters (Table 1 and Fig. 1).

To further discriminate the MIRU-ETR-defined clustered isolates, spoligotyping and IS*6110* RFLP typing were examined as secondary typing methods, and the results of resolving MIRU-ETR-defined clusters by spoligotyping or IS*6110* RFLP typing are also summarized in Table 1. Spoligotyping added marginal resolution to MIRU-ETR-defined clusters, with re-

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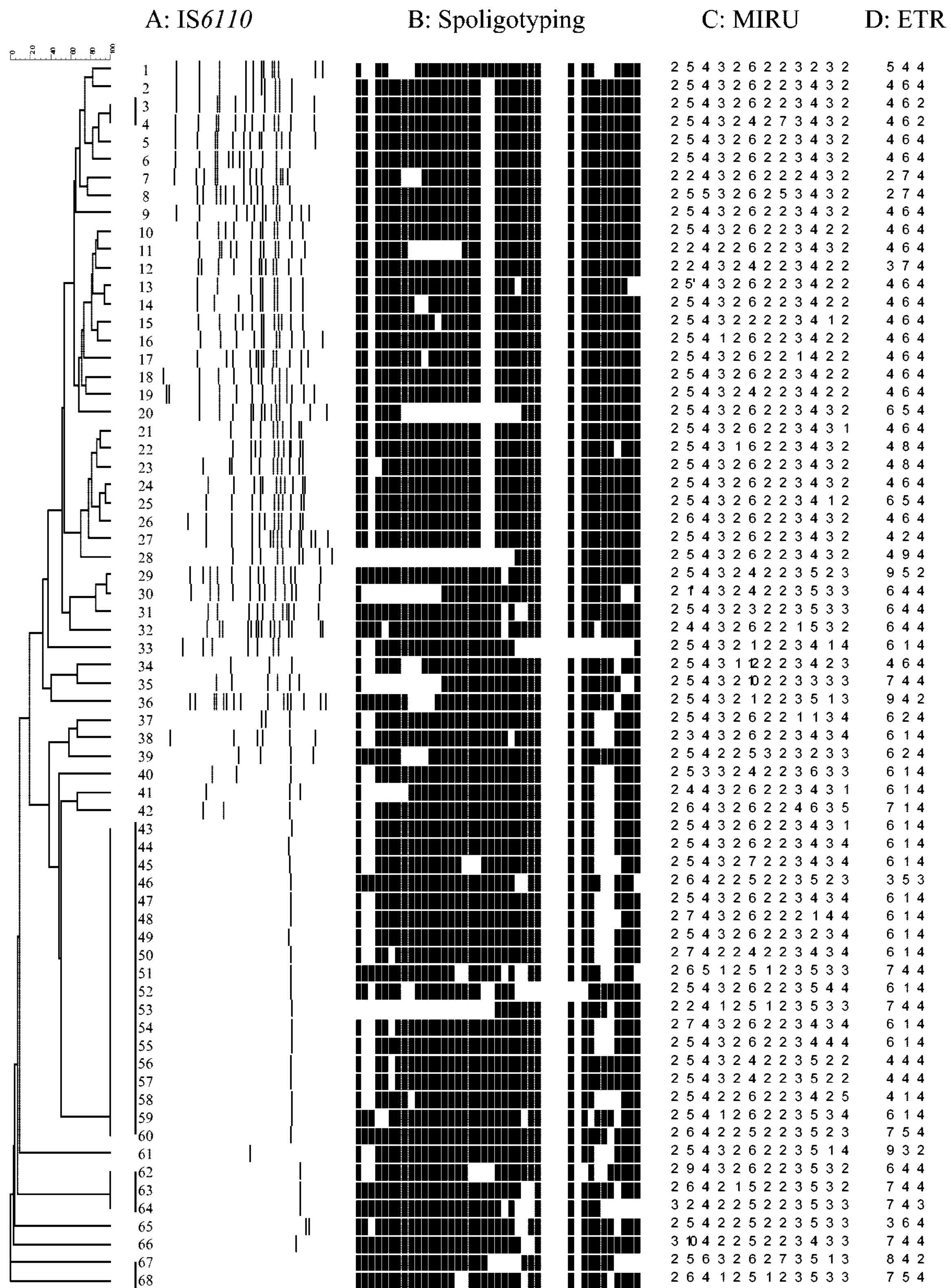

FIG. 1. Dendrogram and fingerprints of the 68 ancestral *M. tuberculosis* strains. (A) Dendrogram based on IS*6110* RFLP patterns. (B) Spoligotypes. (C) MIRU-VNTR patterns (33). (D) Patterns of VNTR-ETR loci A, B, and C. Isolates clustered by IS*6110* RFLP are labeled by vertical bars.

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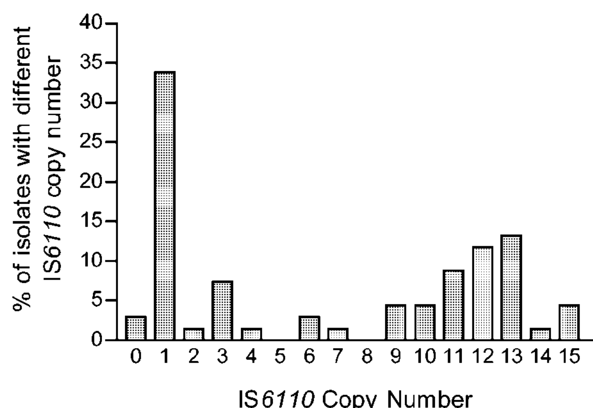

FIG. 2. Distribution of IS*6110* copy numbers of the 68 TbD1$^+$ ancestral *M. tuberculosis* isolates. One-copy isolates accounted for ~34% of the total isolates.

TABLE 2. Discriminatory power of each individual typing method and combinations of typing methods

Method or combination of methods	No. of distinct patterns	No. of unique isolates	No. of clusters	No. of clustered isolates	HGDI
IS*6110* RFLP	47	43	4	25	0.931
Spoligotyping	41	36	5	32	0.923
MIRU-ETR	61	58	3	10	0.994
IS*6110* RFLP and spoligotyping	62	59	3	9	0.995
IS*6110* RFLP and MIRU-ETR	67	66	1	2	1.000
MIRU-ETR and spoligotyping	62	59	3	9	0.995
MIRU-ETR and IS*6110* RFLP	67	66	1	2	1.000
Spoligotyping and MIRU-ETR	62	59	3	9	0.995
Spoligotyping and IS*6110* RFLP	62	59	3	9	0.995
All three methods	67	66	1	2	1.000

duction of clustered isolates only from 10 to 9, whereas IS*6110* RFLP typing appeared to achieve maximum discrimination of MIRU-ETR-defined clusters, leaving only two isolates clustered (isolates 44 and 47). This cluster is most likely a "true cluster," because all the genetic markers tested in the two isolates in the cluster were identical, strongly suggesting their epidemiological link.

Other combinations of typing methods were also examined. The HGDIs for the individual methods and combinations are shown in Table 2. Both MIRU-ETR typing and spoligotyping were able to greatly further discriminate IS*6110*-defined clusters, but MIRU-ETR typing performed better. Similarly, both IS*6110* RFLP typing and MIRU-ETR typing could further resolve many spoligotype-defined clusters.

DISCUSSION

In this study, we compared the VNTR typing system based on two sets of loci (14, 24, 34) with the other two widely accepted and used typing methods, IS*6110* RFLP typing and spoligotyping, using 68 ancestral isolates and analyzed the discriminative abilities of various combinations of the three methods. When used alone, the 12-locus-based MIRU-VNTR typing (24, 34) is already the most discriminative method for the ancestral isolates compared to IS*6110* RFLP typing or spoligotyping (reference 33 and this study). Furthermore, this discriminative ability could be enhanced by addition of the VNTR loci ETR-A, -B, and -C. Therefore, incorporating loci ETR-A, -B, and -C into the 12-locus MIRU-VNTR typing system to form a MIRU-ETR typing system based on 15 loci is useful for improving the definition of clustered isolates.

MIRU-ETR typing defined three clusters which contained a total of 10 isolates. Two isolates (44 and 47) in a cluster were likely epidemiologically linked, as all the genetic markers tested were identical. However, the mutual similarities of IS*6110* RFLP of the remaining eight isolates suggest that at least some of them were not epidemiologically related, although we do not have epidemiological data to further assess this issue. These isolates differed by from zero to five bands. Epidemiologically related isolates generally have identical

TABLE 1. Resolution of MIRU-VNTR-defined clusters by other methods[a]

MIRU-VNTR	No. of isolates	VNTR-ETR	No. of isolates	Spoligotype (Octal)	No. of isolates	Resolution by IS*6110* RFLP
254326223434 (44, 47)	2	614 (44, 47)	2	477777777413071 (44, 47)	2	Clustered pattern
254326223432 (2, 3, 5, 6, 9, 20, 23, 24, 27, 28)	10	464 (2, 5, 6, 9, 24)	5	677777477413771 (2, 5, 6, 9, 24)	5	10 unique patterns
		494	1	000000007413771	1	
		484	1	637777477413771	1	
		462	1	677777477413771	1	
		654	1	674000003413771	1	
		424	1	677777477413771	1	
254326223431 (21, 43)	2	614	1	477777777413071	1	2 unique patterns
		464	1	677777477413771	1	
254326223422 (10, 14, 18)	3	464 (10, 14, 18)	3	677777477413771 (10, 18)	2	3 unique patterns
				677177477413771	1	
264225223523 (46, 60)	2	754	1	777777777413671	1	2 unique patterns
		353	1	777777771413470	1	

[a] Numbers in parentheses indicate clustered isolates.

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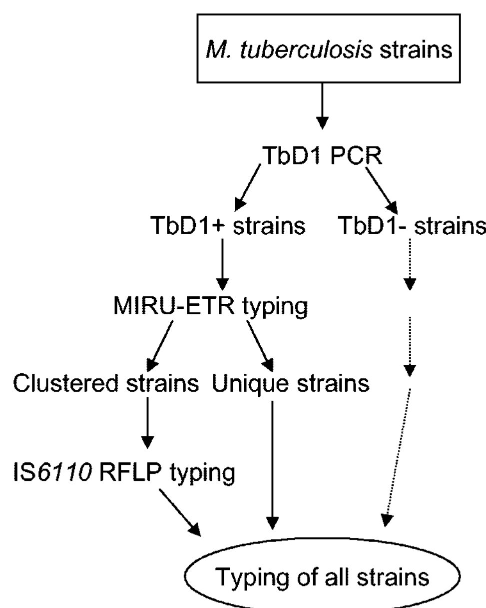

FIG. 3. Schematic representation of a genotyping strategy for ancestral *M. tuberculosis*. TbD1 PCR is used to dichotomize unknown strains; TbD1$^+$ strains are then typed by MIRU-ETR, which discriminates most TbD1$^+$ strains into unique strains. Strains clustered by MIRU-ETR are then subtyped by IS*6110* RFLP. The strategy indicated by solid arrows is for TbD1$^+$ strains. The typing strategy for TbD1$^-$ strains remains open and is indicated by dashed arrows.

IS*6110* RFLP patterns (2, 26, 29, 37, 44, 46–48), although changes of one to four RFLP bands have been observed in up to 29% of such isolates in some studies (5, 25, 43). In addition, three of the eight isolates clustered by MIRU-ETR were split into two clustered isolates and one unique isolate by spoligotyping. Therefore, MIRU-ETR likely still overestimated true clustering, and additional markers are thus needed to further improve resolution. PCR-based spoligotyping added only marginally to the discrimination of these clusters, and only IS*6110* RFLP typing could provide sufficient discrimination for the isolates.

On the other hand, two isolates (3 and 4) clustered by IS*6110* RFLP, which had a high number of IS*6110* copies (12 copies), were discriminated as unique isolates by MIRU-VNTR: their MIRU-VNTR patterns were different in MIRU loci 23 and 26. Moreover, of three pairs of isolates (13 versus 14, 24 versus 25, and 29 versus 30) which had only one band difference within each pair, two pairs of isolates had both different MIRU-ETR patterns and different spoligotypes; the remaining pair of isolates had the same spoligotypes but different MIRU-ETR patterns. This might be due to either overdiscrimination by MIRU-ETR or too stable IS*6110* fingerprints.

In the present setting, the combination of MIRU-ETR and IS*6110* RFLP was the most powerful combination. However, as an individual typing method, MIRU-ETR was much superior to IS*6110* RFLP or spoligotyping in discriminatory power for the ancestral isolates. We therefore propose a stepwise genotyping strategy for ancestral *M. tuberculosis* isolates, using MIRU-ETR as the main screening method (Fig. 3). Unknown *M. tuberculosis* isolates could be divided first by TbD1 PCR (4, 33) into TbD1$^+$ and TbD1$^-$ groups, and MIRU-ETR could then be used to type TbD1$^+$ isolates. The majority of TbD1$^+$ isolates would have unique patterns at this step, whereas isolates clustered by MIRU-ETR could be further discriminated by IS*6110* RFLP. This typing combination has been proposed for defining ongoing-transmission clusters (28). Alternatively, as a substantial majority (96%) of ancestral strains contained more than one repeat at MIRU locus 24 and 100% of modern strains contained one repeat at this locus, locus 24 can also be used as a genetic marker to distinguish ancestral and modern strains (33, 36), but at the risk of misclassifying ~4% of ancestral strains as modern strains. Such a genotyping strategy would be applicable in Singapore and probably in other countries of Southeast Asia (10, 23, 27, 49) and South India (11), where ancestral *M. tuberculosis* strains are also prevalent.

Studies by us and by others have suggested that the *M. tuberculosis* genotyping strategy may change depending on the population structure of *M. tuberculosis*, but the present tendency is that VNTR typing will likely prevail as a first-line method regardless of the population structure. For TbD1$^+$ strains, neither IS*6110* RFLP typing nor spoligotyping is sufficiently discriminatory, because they cluster one-IS*6110*-copy isolates. Using the PCR-based, highly discriminative MIRU-ETR typing as the screening method in the typing strategy for TbD1$^+$ ancestral *M. tuberculosis* will greatly reduce the numbers of strains that need subtyping by IS*6110* RFLP. This will speed up the typing process.

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