Vol. 46, No. 10

Discordance between Mycobacterial Interspersed Repetitive-Unit–Number Tandem-Repeat Typing and IS6110 Restriction Fragment Length Polymorphism Genotyping for Analysis of *Mycobacterium tuberculosis* Beijing Strains in a Setting of High Incidence of Tuberculosis[▽][†]

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IS6110 restriction fragment length polymorphism (RFLP) genotyping is the most widely used genotyping method to study the epidemiology of *Mycobacterium tuberculosis*. However, due to the complexity of the IS6110 RFLP genotyping technique, and the interpretation of RFLP data, mycobacterial interspersed repetitive-unit–variable-number tandem-repeat (MIRU-VNTR) genotyping has been proposed as the new genotyping standard. This study aimed to determine the discriminatory power of different MIRU-VNTR locus combinations relative to IS6110 RFLP genotyping, using a collection of Beijing genotype *M. tuberculosis* strains with a well-established phylogenetic history. Clustering, diversity index, clustering concordance, concordance among unique genotypes, and divergent and convergent evolution were calculated for seven combinations of 27 different MIRU-VNTR loci and compared to IS6110 RFLP results. Our results confirmed previous findings that MIRU-VNTR genotyping can be used to estimate the extent of recent or ongoing transmission. However, molecular epidemiological linking of cases varied significantly depending on the genotyping method used. We conclude that IS6110 RFLP and MIRU-VNTR loci evolve independently and at different rates, which leads to discordance between transmission chains predicted by the respective genotyping methods. Concordance between the two genotyping methods could be improved by the inclusion of genetic distance (GD) into the clustering formulae for some of the MIRU-VNTR loci combinations. In summary, our findings differ from previous reports, which may be explained by the fact that in settings of low tuberculosis incidence, the genetic distance between epidemiologically unrelated isolates was sufficient to define a strain using either marker, whereas in settings of high incidence, continuous evolution and persistence of strains revealed the weaknesses inherent to these markers.

Over the past 2 decades, molecular genotyping methods have enhanced our understanding of the epidemiology of tuberculosis (TB) in numerous geographical settings. These methods have enabled geo-temporal tracking of *Mycobacterium tuberculosis* strains with the view to identifying source cases responsible for TB outbreaks (3), tracking of recent and ongoing disease transmission (31), distinguishing between reinfection and relapse (28), evaluating the effectiveness of direct observed therapy short-course-based TB control programs (5, 16), and identifying global genetic lineages (7). Ideally, molecular genotyping tools should be inexpensive, highly discriminative, deliver rapid results, be straightforward to perform, and produce easily interpretable results that allow for accurate interlaboratory comparisons (universally comparable databases).

Three genotyping methods are currently widely used in molecular epidemiological studies of TB: IS6110 restriction fragment length polymorphism (RFLP) genotyping (27), spoligotyping (14), and mycobacterial interspersed repetitive-unit–variable-number tandem-repeat (MIRU-VNTR) genotyping (21, 22). Currently, IS6110 RFLP genotyping is the most widely used genotyping method (27). However, this method is time-consuming, laborious, and complex. Furthermore, differences in application can make interlaboratory comparisons difficult, and the data generated may have limitations (i.e., comparison of strains with high versus low IS6110 copy numbers). More recently, the validity of the calculation of IS6110 RFLP clustering, as a surrogate for transmission, has been questioned, as the IS6110 banding pattern may change during transmission (33, 35). A nearest genetic distance model has been evaluated to incorporate IS6110 banding changes into the calculation of ongoing transmission (24). The term "cluster" has also been questioned in studies which have compared contact tracing data with IS6110 RFLP data (4, 26). In response, numerous studies have been conducted to try to identify alternative methods that have the ability to accurately describe epidemiological events in different settings at a similar

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discriminatory level to that of IS6110 RFLP genotyping. One of the most promising methods is MIRU-VNTR genotyping, a PCR-based method for detecting the number of tandem repeats at a given genetic locus. Supply et al. (21) defined a set of 15 MIRU-VNTR loci for molecular epidemiological investigations and a set of 24 MIRU-VNTR loci for phylogenetic analysis of M. tuberculosis strains worldwide. In support of this, another study concluded that this "real-time" MIRU-VNTR genotyping approach was highly applicable for population-based studies (18). This view was reinforced by a study conducted in the Brussels region, where the authors concluded that a standardized MIRU-VNTR genotyping method could be a new reference for epidemiological and phylogenetic screening of M. tuberculosis strains (2).

A study from Japan (10) investigated the differentiation power of the proposed 15- and 24-loci MIRU-VNTR genotyping methods for strains with the Beijing genotype and concluded that the analyses of these loci were of limited use for discriminating strains of this genotype. In their study they showed that VNTR loci 3820, 3232, and 4120 were highly polymorphic in Beijing genotype strains and thus proposed the use of these loci to enhance the discriminatory power of the proposed 15-MIRU-VNTR genotyping method. However, other studies have excluded these loci due to difficulties associated with the reproducibility of PCR amplification (15, 21, 36).

Subsequently, a study in Hong Kong, which also examined strains of the Beijing genotype, showed that a different combination of 12 VNTR and QUB (Queen's University of Belfast) loci gave a Hunter-Gaston discriminatory index value which was almost equal to that obtained in IS6110 RFLP genotyping (12, 13). However, this was refuted by a more recent study from China which suggested that MIRU-VNTR genotyping may overestimate transmission in isolates with the Beijing genotype (11). Collectively, these findings suggest that the selection of MIRU-VNTR loci for optimal differentiation of M. tuberculosis requires further validation in different geographical settings. To date, the performance of the MIRU-VNTR genotyping method has not been evaluated in an epidemic setting, nor has it been tested within the context of a robust M. tuberculosis phylogeny.

In this study the discriminatory power of different MIRU-VNTR locus combinations was determined as previously described (8, 10, 21, 22) and compared to the IS6110 RFLP genotyping method by using a collection of Beijing genotype M. tuberculosis strains with a well-established phylogenetic history (9). The results are discussed in the context of concordance between the different genotyping methods in their abilities to define a strain and to accurately describe the epidemiology of TB in a high-incidence setting.

MATERIALS AND METHODS

Study population. Sputum samples were collected during the period from January 1993 to December 2004 from new and retreatment TB patients who were resident and attending health care clinics in an epidemiological field site in Cape Town, South Africa (31). This study is part of a larger, long-term molecular epidemiological project which has been approved by the ethics committee of Stellenbosch University.

IS6110 RFLP genotyping. M. tuberculosis isolates were cultured on MGIT (Becton Dickinson) or Löwenstein-Jensen medium, and DNA was extracted as previously described (32). Each isolate was classified by IS6110 RFLP genotyping

(27) and spoligotyping (14) using internationally standardized protocols. IS6110 RFLP patterns were analyzed using Gelcompar II (Applied-Maths, Sint-Martens-latem, Belgium) with tolerance settings allowing a 5% shift in lane position and a 0.6% variation in individual band position to compensate for minor technical errors. Isolates were assigned as members of the Beijing genotype if they had the characteristic Beijing spoligotype (30). Only the first M. tuberculosis isolate from each case was included for subsequent analysis. Each Beijing isolate was grouped into one of seven phylogenetic sublineages according to 40 different genetic markers, as previously described (9).

DNA sequencing. The DNA sequence of the katG, rpoB, embB, and rrs genes of isolates classified as members of the Beijing sublineage 5 were determined as previously described (19, 25).

MIRU-VNTR typing. Twenty-seven MIRU-VNTR loci were amplified by PCR as described previously (8, 10, 21, 22). The number of repeats at each genomic locus was calculated according to the electrophoretic mobility of the corresponding PCR product (23). Alleles were assigned numerical values according to the number of repeats present in that genomic locus. Isolates were genotypically classified according to seven different MIRU-VNTR locus combinations (Table 1).

Analytical calculations. (i) Estimation of clustering. A cluster (representing either recent or ongoing transmission or a <2-year interval) was defined as a series of isolates having the same genotype (IS6110 RFLP or MIRU-VNTR), while isolates with unique IS6110 RFLP or MIRU-VNTR genotypes were considered to represent reactivation or influx of disease into the study community (20). Secondary analyses which incorporated the concept of evolution during transmission were done using data sets (genotypes according to IS6110 RFLP or a particular MIRU-VNTR locus combination) in which isolates separated by a single evolutionary event were combined into transmission chains with a genetic distance of 1 (24).

(ii) Estimation of genetic diversity. The genetic diversity for each individual MIRU-VNTR locus, each of the seven MIRU-VNTR locus combinations (Table 1), and the IS6110 RFLP fingerprints was calculated as $h = 1 - \sum x_i^2[n/(n-1)]$, where x_i is the frequency of the ith allele at the locus, n is the number of isolates in the sample, and the term $n/(n-1)$ is a correction for bias in small samples (17).

(iii) Estimation of matching and mismatching concordance. Concordance between the IS6110 RFLP genotypes and the respective MIRU-VNTR genotypes was calculated as follows: each isolate was paired with every other isolate in the data set, and their genotypes (IS6110 RFLP and MIRU-VNTR) were scored as either a match (identical) or mismatch (nonidentical). Matching concordance between the respective genotyping methods was calculated according to the number of paired isolates having a match for both of the methods as a proportion of the total number of pairs having matching IS6110 RFLP genotypes. This is a measure of agreement between two methods as to whether any two isolates form part of the same transmission chain. Mismatching concordance was calculated as the number of paired isolates having nonmatching genotypes for both of the methods as a proportion of the total number of pairs having nonmatching IS6110 RFLP genotypes. This is a measure of agreement between two methods for any two isolates that do not form part of the same transmission chain.

(iv) Estimation of concordance among unique genotypes. Concordance between uniquely occurring IS6110 RFLP genotypes and the MIRU-VNTR genotypes was calculated as the proportion of isolates having unique IS6110 RFLP genotypes that also had unique MIRU-VNTR genotypes.

(v) Estimation of number of convergent events. Convergent evolution was identified by drawing connecting lines between each IS6110 RFLP genotype and each MIRU-VNTR genotype for which isolates were found to have that particular genotype combination (Fig. 1). Convergent evolution was defined, conservatively, as the existence of isolates representing each of the four possible combinations of two IS6110 RFLP genotypes (e.g., IS_1 and IS_2) and two MIRU-VNTR genotypes (e.g., M_1 and M_2) (Fig. 1). This scenario would only be possible if one of the MIRU-VNTR genotypes had evolved more than once, assuming that the chance of IS6110 RFLP genotype convergence was significantly lower than that of MIRU-VNTR genotype convergence. The validity of this method was confirmed by plotting the IS6110 RFLP genotypes onto a phylogenetic tree constructed using the MIRU-VNTR data in combination with a neighbor-joining algorithm (data not shown) (34).

(vi) Estimation of number of divergent events. A divergent evolutionary event was scored for each MIRU-VNTR genotype which existed in combination with only one IS6110 RFLP genotype and where this IS6110 RFLP genotype was found in combination with more than one MIRU-VNTR genotype (Fig. 2). This implies that the MIRU-VNTR genotype arose subsequent to the IS6110 RFLP genotype. A divergent event was also added for each convergent event, since a convergent event implies a prior divergent event.

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TABLE 1. MIRU-VNTR locus combinations

Locus	Locus included in MIRU-VNTR combination[a]						
	12-MIRU[b]	12-MIRU + ETR A, B, C[c]	12-MIRU + hypervariable loci[d]	15-MIRU-VNTR[e]	15-MIRU-VNTR + hypervariable loci[f]	24-MIRU-VNTR[e]	24-MIRU-VNTR + hypervariable loci[d]
MIRU02	●	●	●			●	●
MIRU04	●	●	●	●	●	●	●
MIRU10	●	●	●	●	●	●	●
MIRU16	●	●	●	●	●	●	●
MIRU20	●	●	●			●	●
MIRU23	●	●	●			●	●
MIRU24	●	●	●			●	●
MIRU26	●	●	●	●	●	●	●
MIRU27	●	●	●			●	●
MIRU31	●	●	●	●	●	●	●
MIRU39	●	●	●			●	●
MIRU40	●	●	●			●	●
VNTR1955				●	●	●	●
VNTR2165/ETR-A		●		●	●	●	●
QUB11b				●	●	●	●
QUB26b				●	●	●	●
VNTR0424				●	●	●	●
VNTR2401				●	●	●	●
VNTR4156				●	●	●	●
VNTR3690				●	●	●	●
ETR-C		●		●	●	●	●
VNTR2347						●	●
ETR-B		●				●	●
Mtub 34						●	●
QUB3232			●		●		●
VNTR3820			●		●		●
VNTR4120			●		●		●

[a] ●, locus included.
[b] According to reference 22.
[c] According to reference 8.
[d] From this study.
[e] According to reference 21.
[f] According to reference 10.

(vii) Sensitivity and specificity calculations. The sensitivity and specificity (and positive and negative predictive values) of the IS*6110* RFLP and respective MIRU-VNTR genotyping methods were calculated using GraphPad Prism 5 software (La Jolla, CA) based on their ability to correctly identify an independently genotyped drug-resistant cluster.
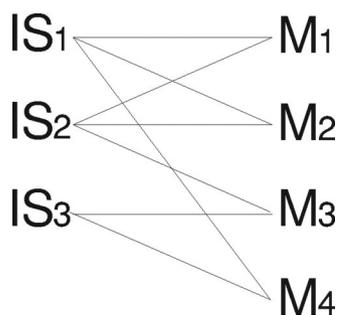

FIG. 1. An example of MIRU-VNTR (M_x) and IS*6110* RFLP (IS_x) genotypes. The connecting lines represent the MIRU-VNTR and IS*6110* RFLP genotype combinations observed in *M. tuberculosis* isolates in the study setting. M_1 and M_2 are both linked to IS_1 and IS_2 and therefore represent a convergent event. Neither M_3 nor M_4 share common connections to more than one IS_x with any other M_x. Their connecting lines therefore indicate simple, linear evolution.

RESULTS

IS*6110* RFLP genotyping identified 74 different strains among the 321 isolates with the Beijing spoligotype collected over a 12-year period (Table 2). Of these strains, 272 were grouped into 25 clusters (containing between 2 and 100 isolates), and 49 were unique strains. The overall percent clustering was calculated to be 84.7% using the n/T formula (1). Each isolate was subsequently genotyped with 27 MIRU-VNTR loci and analyzed according to seven different MIRU-VNTR locus combinations (Table 1; see also data sets in the supplemental material). The performance of these locus combinations, in relation to the IS*6110* RFLP genotyping method, was determined either over a 12-year period (Table 2) or over six consecutive 2-year periods (Table 3). In both analyses the traditional 12-MIRU loci genotyping method underestimated the number of genotypes (strains) identified and thereby overestimated the percentage of clustering (Tables 2 and 3). The inclusion of exact tandem repeat (ETR) alleles A, B, and C to the 12-MIRU loci set did not significantly improve the number of strains detected or the estimate of clustering (Tables 2 and 3). Analysis of the isolates using the newly proposed 15- and 24-MIRU-VNTR locus combinations increased the number of strains identified; however, the discriminatory power of these locus combinations remained lower than that observed using
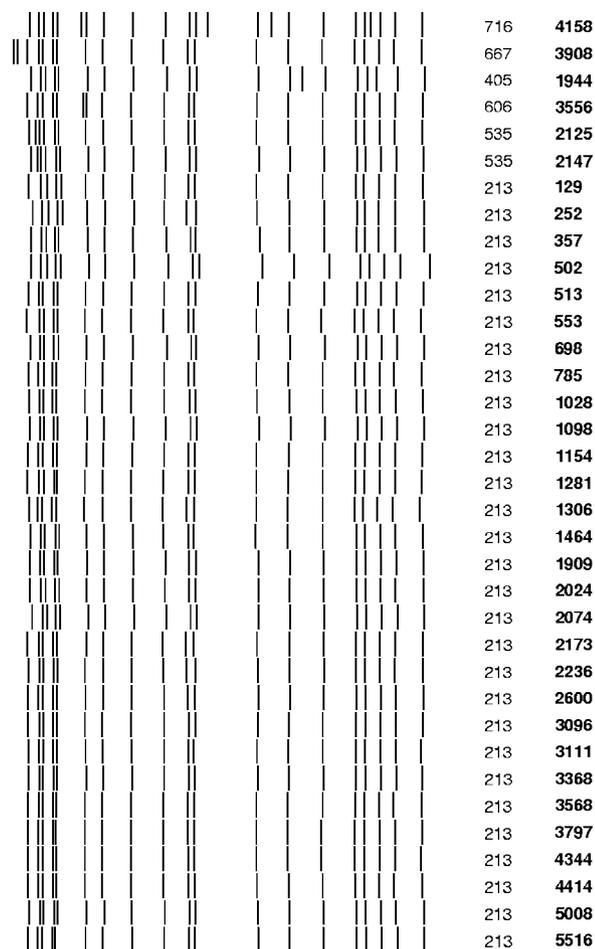
	716	**4158**
	667	**3908**
	405	**1944**
	606	**3556**
	535	**2125**
	535	**2147**
	213	**129**
	213	**252**
	213	**357**
	213	**502**
	213	**513**
	213	**553**
	213	**698**
	213	**785**
	213	**1028**
	213	**1098**
	213	**1154**
	213	**1281**
	213	**1306**
	213	**1464**
	213	**1909**
	213	**2024**
	213	**2074**
	213	**2173**
	213	**2236**
	213	**2600**
	213	**3096**
	213	**3111**
	213	**3368**
	213	**3568**
	213	**3797**
	213	**4344**
	213	**4414**
	213	**5008**
	213	**5516**

FIG. 2. IS*6110* RFLP banding patterns of Beijing sublineage 5 isolates sharing identical *katG*, *rpoB*, *embB*, and *rrs* gene mutations. The isolate numbers are indicated in bold, while the IS*6110*-RFLP cluster numbers are indicated in standard text (these numbers correspond to the numbers given in data set S2 of the supplemental material).

IS*6110* RFLP genotyping (Tables 2 and 3). Consequently, these locus combinations overestimated clustering. The addition of the VNTR loci 3232, 4120, and 3820 to the 12-, 15-, and 24-MIRU-VNTR locus combinations increased the number of strains detected and thereby produced clustering estimates similar to or slightly lower than that of IS*6110* RFLP genotyping (Tables 2 and 3). This implies that some MIRU-VNTR locus combinations could be selected as epidemiological markers to estimate the extent of both recent (<2-year interval) and ongoing (unrestricted interval) transmission in settings with a high incidence of strains with the Beijing genotype.

To determine whether a correlation existed between the definitions of a strain according to IS*6110* RFLP or MIRU-VNTR genotyping methods, the respective genotypes were compared. From the results shown in Table 2 it is evident that a strain classified as a cluster according to IS*6110* RFLP genotyping may in some instances be classified as unique according to the different MIRU-VNTR locus combinations, or vice versa. Using a pair-wise analysis, we estimated the degree of matching concordance between the IS*6110* RFLP and MIRU-VNTR genotyping methods to range between 39% and 68%

depending on the locus combinations used (Tables 2 and 3). The inclusion of additional MIRU-VNTR loci decreased the degree of matching concordance, as a result of an increased rate of divergence caused by more rapid evolution, with the hypervariable loci having the greatest effect. Conversely, the inclusion of additional loci increased the degree of mismatching concordance, as well as concordance between strains identified as having unique genotypes according to both genotyping methods (IS*6110* RFLP and MIRU-VNTR). A consequence of more rapid evolution was the increased risk of convergent evolutionary events (Table 2).

To determine whether concordance between the respective genotyping methods could be improved, the analysis was repeated to allow for a genetic distance of 1, i.e., evolution of single MIRU-VNTR loci or single-band changes in the IS*6110* pattern within the definition of a cluster. The results showed that the inclusion of genetic distance had a significant influence on the MIRU-VNTR definition of a cluster, collapsing many of the genotypes (Table 2; see also data set S1 in the supplemental material). This was less pronounced for IS*6110* RFLP analysis (Table 2; see also data set S1 in the supplemental material). Matching concordance was improved by allowing for evolution of the MIRU-VNTR genotypes; however, mismatching concordance was concomitantly reduced for genotypes based on the 12-MIRU loci combinations. This may be explained by the loss of discriminatory power as a result of the collapsing of genotypes, which is associated with a low rate of evolution. In contrast, mismatching concordance was improved for 15- and 24-MIRU-VNTR combinations due to the higher evolutionary rates of these markers. However, the concordance among unique genotypes remained low (Table 2).

To establish which of the genotyping methods provided the most accurate description of ongoing transmission in the study setting, the largest group of drug-resistant isolates (found within sublineage 5) was selected, based on identical mutations conferring resistance to isoniazid, rifampin, ethambutol, and streptomycin (see data set S2 in the supplemental material). These isolates represent the continuing spread of a previously described multidrug-resistant TB outbreak (29). A total of 35 isolates were identified with the *katG*315 AGC to ACC, *rpoB*531 TCG to TTG, *embB*306 ATG to ATA, and *rrs*513 CAG to CCG mutations, forming a single drug resistance-based cluster (Fig. 2). The sensitivity, specificity, and positive and negative predictive values related to the abilities of the different markers to identify the drug-resistant cluster are given in Table 4. While the sensitivities of all the markers were high, with some of those based on MIRU-VNTR loci outperforming IS*6110* RFLP, the specificity of all MIRU-VNTR markers was substantially lower than that of IS*6110* RFLP. The inclusion of genetic distance (single events) within the definition of a cluster appeared to improve the sensitivity of most of the markers but concomitantly decreased the specificity of the MIRU-VNTR markers. The specificity of IS*6110* was not affected by the inclusion of genetic distance. Positive predictive values were not significantly affected by allowing for evolution of the markers; however, with the exceptions of IS*6110*, which increased, and the 24 MIRU and three hypervariable loci, which remained unchanged, the negative predictive values for all markers were reduced to zero.

TABLE 2. Comparison between molecular epidemiological data generated over a 12-year interval using IS*6110* RFLP and MIRU-VNTR genotyping methods

Method	No. of genotypes	% Clustering	Genetic distance of 0				% Pair-wise matching concordance	% Pair-wise mismatching concordance	% Concordance between unique strains
			Unique IS*6110* genotype ($n = 49$) with:		Clustered IS*6110* genotype ($n = 272$) with:				
			Unique MIRU-VNTR genotype (n)	Clustered MIRU-VNTR genotype (n)	Unique MIRU-VNTR genotype (n)	Clustered MIRU-VNTR genotype (n)			
IS*6110* RFLP	74	84.7	NA[g]	NA	NA	NA	100	NA	100
MIRU-VNTR locus combinations									
12-MIRU[a]	27	95.0	6	43	10	262	68	69	20.4
12-MIRU + ETR A, B, C[b]	39	91.2	8	41	18	254	60	72	22.4
12-MIRU + hypervariable loci[c]	67	84.1	14	35	38	234	53	71	28.6
15-MIRU-VNTR[d]	47	89.4	12	37	22	250	67	68	34.7
15-MIRU-VNTR + hypervariable loci[e]	83	78.8	19	30	49	223	51	71	40.8
24-MIRU-VNTR[d]	57	87.2	12	37	28	244	52	73	40.8
24-MIRU-VNTR + hypervariable loci[c]	91	77.3	19	30	54	218	39	81	42.9

[a] According to reference 22.
[b] According to reference 8.
[c] According to this study.
[d] According to reference 21.
[e] According to reference 10.
[f] According to reference 15.
[g] NA, not applicable.

To determine whether MIRU-VNTR genotyping could be used as a method to phylogenetically group strains with the Beijing genotype, the correlation between MIRU-VNTR genotype and Beijing sublineage was quantified. As sublineages 3 and 4 and sublineages 5 and 6 were distinguished solely on the basis of IS*6110* in our data set, these two pairs of sublineages were combined for the purposes of this analysis. Table 2 shows that the respective MIRU-VNTR locus combinations correctly grouped >96% of the isolates according to their sublineage designation, in comparison to 100% with IS*6110* RFLP genotyping. The incorporation of genetic distance reduced the ability of genotyping methods based on the 12-MIRU locus combinations to correctly group isolates (Table 2).

TABLE 3. Comparison between molecular epidemiological data generated over six consecutive 2-year intervals by IS*6110* RFLP and MIRU-VNTR genotyping methods

Method	Avg value (range)					
	No. of strains	No. of clusters	% Clustering	% Pair-wise matching concordance	% Pair-wise mismatching concordance	% Concordance between unique strains
IS*6110* RFLP	17.9 (7–20)	6.1 (4–10)	74.5 (55.4–86.7)	100	NA[f]	100
MIRU-VNTR locus combinations						
12-MIRU[a]	9.9 (5–14)	4.6 (2–7)	85.1 (60.0–93.1)	69.7 (60–80)	92.8 (72–99)	19.1 (6.1–60.0)
12-MIRU + ETR A, B, C[b]	10.9 (7–14)	4.9 (3–7)	83.1 (60.0–91.7)	63.7 (44–72)	95.0 (84–99)	25.7 (15.6–60.0)
12-MIRU + hypervariable loci[c]	15.4 (8–22)	4.0 (2–6)	73.1 (60.0–77.8)	52.7 (40–58)	94.0 (77–99)	42.2 (27.0–66.7)
15-MIRU-VNTR[d]	12.0 (5–17)	3.7 (2–5)	80.3 (66.7–93.1)	68.2 (45–78)	92.3 (70–99)	31.3 (16.7–60.0)
15-MIRU-VNTR + hypervariable loci[e]	16.6 (7–23)	3.6 (2–6)	70.7 (57.6–76.4)	51.0 (22–63)	94.0 (76–100)	50.9 (32.0–66.7)
24-MIRU-VNTR[d]	13.6 (7–19)	4.6 (3–6)	77.8 (60.0–89.7)	55.0 (44–69)	95.2 (84–99)	35.4 (24.0–60.0)
24-MIRU-VNTR + hypervariable loci[c]	18.1 (9–25)	4.3 (3–7)	67.7 (54.5–75.0)	40.2 (22–51)	95.0 (84–99)	51.3 (32.0–66.7)

[a] According to reference 22.
[b] According to reference 8.
[c] According to this study.
[d] According to reference 21.
[e] According to reference 10.
[f] NA, not applicable.

TABLE 2—*Continued*

Genetic distance of 0			Genetic distance of 1				% Beijing sublineage discrimination	
No. of converged genotypes	No. of diverged genotypes	Diversity index[f]	No. of genotypes	% Pair-wise matching concordance	% Pair-wise mismatching concordance	% Concordance between unique strains	GD = 0	GD = 1
NA	NA	0.85	40	100	NA	100	100	99
3	14	0.63	3	99	2	0	96	1
5	26	0.67	4	99	3	4	96	1
5	47	0.7	11	96	8	4	99	3
3	27	0.63	11	99	80	15	99	99
5	58	0.7	20	96	82	30	99	99
7	38	0.72	15	99	83	22	99	99
9	68	0.78	27	96	88	41	99	99

DISCUSSION

IS*6110* RFLP genotyping is the most widely used genotyping method for investigating and understanding the epidemiology of *M. tuberculosis* (27). However, studies comparing IS*6110* RFLP molecular epidemiological and contact tracing data have questioned the validity of the definition of transmission (4, 26). In order to address these concerns MIRU-VNTR genotyping using either 15- or 24-MIRU-VNTR loci combinations have been extensively evaluated as the new genotyping standard for molecular epidemiological studies of *M. tuberculosis* (21). Concordance between MIRU-VNTR genotyping and contact tracing data was found to be superior to that of IS*6110* RFLP in settings of low incidence (2, 18). However, these MIRU-VNTR locus combinations have not been fully tested in geographical regions of TB endemicity or within a robust *M. tuberculosis* phylogeny. Our results confirm previous findings (2, 10, 18, 21) which suggested that MIRU-VNTR genotyping, using carefully selected locus combinations, could be used to estimate the extent of recent or ongoing transmission. The inclusion of the three hypervariable loci improved the discriminatory power of the MIRU-VNTR genotyping method in this Beijing lineage, thereby supporting a previous suggestion for their inclusion (10). However, the use of these loci needs further evaluation in other evolutionary lineages, as difficulties associated with amplification reproducibility have been reported (15, 21, 36).

We conclude that the PCR-based MIRU-VNTR genotyping method could be applied as an epidemiological tool to measure the performance of a TB control program over time in a defined geographical setting. However, the observed concordance in the estimate of recent and ongoing transmission when using the IS*6110* RFLP or MIRU-VNTR genotyping methods was only coincidental. A subsequent analysis of the MIRU-VNTR data, in comparison to the IS*6110* RFLP genotyping data, revealed that the classification of a strain according to its genotype differed significantly depending on the genotyping method used. Accordingly, our study showed that the degree of matching and mismatching concordance as well as concordance among unique strains was low. This led to discordance between the transmission chains predicted by the respective genotyping methods. Matching concordance increased when genetic distance was incorporated into the clustering calculation for all of the MIRU-VNTR combinations. However, this effect was offset in the case of 12-MIRU-based markers by the concomitant reduction in mismatching concordance, which was not the case for the 15- and 24-MIRU-VNTR combinations. From this, it is apparent that the additional loci included in the 15- and 24-MIRU-VNTR combinations (with or without the addition of the hypervariable loci) improved the overall concordance of MIRU-VNTR with respect to IS*6110* RFLP. This may be due to these loci being inherently less stable and therefore more informative. However, a caveat to the inclusion of genetic distance in the clustering formula is that epidemiologically unrelated cases may be incorrectly linked within a transmission chain.

Our analysis of the drug-resistant cluster to elucidate which of the genotyping methods provided the most accurate reflection of the epidemiology highlighted shortcomings of both the IS*6110* RFLP and MIRU-VNTR genotyping methods. This analysis supported a previous study which demonstrated that ongoing transmission was characterized by the evolution of variant IS*6110* RFLP genotypes while simultaneously preserving existing genotypes (33). A similar observation was found when using the different MIRU-VNTR locus combinations. This could be explained by the fact that the evolution of different loci could take place both convergently and divergently. Together, these results substantiate previous findings which have suggested that the definition of ongoing transmission according to IS*6110* RFLP or MIRU-VNTR genotyping should include closely related genotypes (18, 24, 35). However,

TABLE 4. Sensitivity, specificity, PPV, and NPV values for IS6110 RFLP and MIRU-VNTR genotyping methods based on correct identification of an independently genotyped drug-resistant cluster characterized by unique mutations in the katG, rpoB, embB, and rrs genes

Method	Value (95% CI) when GD = 0[f]				Value (95% CI) when = 1[f]			
	Sensitivity	Specificity	PPV	NPV	Sensitivity	Specificity	PPV	NPV
IS6110 RFLP	0.83 (0.66–0.93)	1.00 (0.48–1.00)	1.00 (0.88–1.00)	0.45 (0.17–0.77)	0.94 (0.81–0.99)	1.00 (0.48–1.00)	1.00 (0.89–1.00)	0.71 (0.29–0.96)
MIRU-VNTR locus combinations								
12-MIRU[a]	1.00 (0.90–1.00)	1.00 (0.48–1.00)	0.90 (0.76–0.97)	1.00 (0.03–1.00)	1.00 (0.72–1.00)	0.00 (0.00–0.54)	0.88 (0.72–0.95)	ND
12-MIRU + ETR A, B, C[b]	0.97 (0.85–1.00)	0.20 (0.01–0.72)	0.89 (0.75–0.97)	0.50 (0.01–0.99)	1.00 (0.72–1.00)	0.00 (0.00–0.54)	0.88 (0.72–0.95)	ND
12-MIRU + hypervariable loci[c]	0.80 (0.63–0.92)	0.20 (0.01–0.72)	0.88 (0.71–0.96)	0.13 (0.00–0.53)	0.97 (0.85–1.00)	0.00 (0.00–0.52)	0.87 (0.73–0.96)	0.00 (0.00–0.98)
15-MIRU-VNTR[d]	0.91 (0.77–0.98)	0.40 (0.05–0.85)	0.91 (0.77–0.98)	0.40 (0.05–0.85)	1.00 (0.72–1.00)	0.00 (0.00–0.537)	0.88 (0.72–0.95)	ND
15-MIRU-VNTR + hypervariable loci[e]	0.74 (0.57–0.88)	0.40 (0.05–0.85)	0.90 (0.73–0.98)	0.18 (0.02–0.52)	0.94 (0.81–0.99)	0.00 (0.00–0.52)	0.87 (0.72–0.96)	0.00 (0.00–0.84)
24-MIRU-VNTR[d]	0.91 (0.77–0.98)	0.40 (0.05–0.85)	0.91 (0.77–0.98)	0.40 (0.05–0.85)	1.00 (0.72–1.00)	0.00 (0.00–0.54)	0.88 (0.72–0.95)	ND
24-MIRU-VNTR + hypervariable loci[c]	0.74 (0.57–0.88)	0.40 (0.05–0.85)	0.90 (0.73–0.98)	0.18 (0.02–0.52)	0.74 (0.57–0.88)	0.40 (0.05–0.85)	0.90 (0.73–0.98)	0.18 (0.02–0.52)

[a] According to reference 22.
[b] According to reference 8.
[c] According to this study.
[d] According to reference 21.
[e] According to reference 10.
[f] Abbreviations: ND, not determined; PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval; GD, genetic distance.

when allowing for single MIRU-VNTR changes within the definition of a cluster, the MIRU-VNTR genotyping method collapsed many of the sublineage 5 isolates into a limited number of clusters. As a result, most of the isolates were grouped as resistant, giving the method a high sensitivity, but in doing so, compromising specificity. In contrast, the identification of isolates within the drug-resistant cluster was largely retained by IS6110 RFLP analysis despite the inclusion of genetic distance. This suggests that IS6110 RFLP analysis in combination with genetic distance provides a more accurate reflection of ongoing transmission of this multidrug-resistant TB outbreak in this setting. This finding is important for the interpretation of molecular epidemiological data in settings where contact tracing is extremely difficult. However, we acknowledge that the concordance between IS6110 RFLP findings and transmission needs further investigation in different settings and in *M. tuberculosis* strains with different genetic backgrounds.

Our results differ from previous studies (2, 18), which demonstrated a close correlation between IS6110 RFLP and MIRU-VNTR genotyping. These studies were conducted in settings in western Europe with a low incidence of TB and where the TB epidemic is primarily driven by reactivation and immigration (6). In these settings, efficient TB control programs would largely prevent recent and ongoing transmission and the subsequent generation of closely related clonal variants. Thus, genetic diversity is predicted to be preserved. In most instances, this would imply that the strains cultured from TB cases would be genetically distantly related and thus would not share either IS6110 RFLP banding patterns or MIRU-VNTR genotypes. Accordingly, MIRU-VNTR genotyping would discriminate strains at a level similar to that of IS6110 RFLP genotyping. In contrast, our setting of high TB incidence has promoted the evolution of a large number of genetically closely related strains which are maintained within the host population. The genetic distance between these strains is often of such a nature that strains either have identical IS6110 RFLP genotypes and variant MIRU-VNTR genotypes or vice versa. Accordingly, we hypothesize that the degree of discordance between IS6110 RFLP and MIRU-VNTR genotyping is dependent on the genetic distance between isolates. This is supported by the observation that distantly related isolates from the different Beijing sublineages have evolved distinct IS6110 RFLP and MIRU-VNTR genotypes.

In summary, we conclude that both IS6110 RFLP and MIRU-VNTR genotyping methods have limitations in defining chains of transmission of Beijing genotype *M. tuberculosis* strains in this setting of high incidence.

ACKNOWLEDGMENTS

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