Utility of New 24-Locus Variable-Number Tandem-Repeat Typing for Discriminating *Mycobacterium tuberculosis* Clinical Isolates Collected in Bulgaria^{∇†}

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The present study evaluated new markers for molecular typing of *Mycobacterium tuberculosis* with a collection of strains circulating in Bulgaria. A study sample included 133 strains from epidemiologically unlinked patients from different regions of the country. Spoligotyping was used as a primary typing tool; it subdivided these strains into 37 types, including 15 clusters and 22 singletons. Traditional IS6110-restriction fragment length polymorphism (RFLP) typing and novel 24-locus variable number tandem-repeat (VNTR) typing methods were applied to the selection of 73 strains. Discriminatory power (Hunter-Gaston index [HGI]) of these methods was found to be 0.983 and 0.997, respectively. The 73 strains were subdivided into 66 types by a 24-locus mycobacterial interspersed repetitive unit (MIRU)-VNTR scheme, 62 types by a classical 12-locus MIRU-VNTR scheme, 51 types by IS6110-RFLP typing, and 31 types by spoligotyping. A combination of the five most polymorphic loci (MIRU40, Mtub04, Mtub21, QUB-11b, and QUB-26) was shown to achieve a high discrimination (HGI = 0.984). To conclude, a complete 24-locus scheme excellently differentiated strains in our study, whereas a reduced 5-locus set provided a sufficiently high differentiation and may be preliminarily suggested for the first-line typing of *M. tuberculosis* isolates in Bulgaria.

Molecular typing of *Mycobacterium tuberculosis* complex isolates is a useful tool for epidemiological studies at different levels. In the last decade, the application of different DNA fingerprinting techniques has contributed significantly to our understanding of the transmission of tuberculosis. The most widely applied and standardized molecular typing method for *M. tuberculosis* complex isolates is IS6110-restriction fragment length polymorphism (RFLP) typing, which is based on the detection of the insertion sequence IS6110 found in most isolates of the *M. tuberculosis* complex (19, 20). The method is considered to be the most discriminatory and has therefore remained as the ultimate tool for identifying epidemiological clusters (10, 19, 20). Unfortunately, this method is cumbersome, time-consuming, and requires large quantities of DNA, and so a simpler and discriminatory alternative typing method is needed. More recently, spoligotyping, a PCR-based reverse-hybridization technique targeting the genetic diversity of the direct repeat (DR) locus, has been proven useful for the clinical laboratory and for molecular epidemiology and evolutionary and population genetics (9, 20). Compared to IS6110 typ-

ing, spoligotyping is (i) more cost-effective, (ii) portable, and (iii) easier to perform, although less discriminating.

In recent years, various novel DNA typing methods have been developed that are faster and easier to perform than the IS6110-RFLP method. Among them, variable-number tandem-repeat (VNTR) typing is probably the most popular approach. This method is based on the VNTRs of mycobacterial interspersed repetitive units (MIRU-VNTR) scattered throughout the genome, and each isolate is typed based on the number of copies of repeated units (16). Implementation of the large number of loci is expected to achieve a high discrimination. This relatively new method, which requires only basic PCR and agarose electrophoresis equipment, was shown with different strain samples to possess a higher discriminatory power than that of spoligotyping and only slightly below that of IS6110-RFLP typing (16). The apparent advantage of the VNTR approach (compared to the IS6110 typing) is its portability due to easy digitalization of the generated profiles and hence easy interlaboratory exchange, as well as easy creation and maintenance of the databases. Since 1998, the VNTR typing of *M. tuberculosis* has undergone a remarkable improvement. Whereas the initial scheme used only six exact tandem-repeat loci (4), a more recently developed and already classical MIRU set involved 12 loci (16). The most recently proposed new format for MIRU typing includes 24 loci (15).

Our previous study evaluated a spoligotype-defined population structure of *M. tuberculosis* in Bulgaria that appears to be sufficiently heterogeneous and dominated by several worldwide distributed and Balkan specific spoligotypes (18). In the present study, different typing methods, including IS6110-RFLP and MIRU-VNTR, were applied to *M. tuberculosis* strains from Bulgaria. The objective was to assess new versus

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traditional molecular markers for epidemiological studies of *M. tuberculosis* in Bulgaria.

MATERIALS AND METHODS

Mycobacterial isolates. A total of 133 *M. tuberculosis* isolates were randomly selected among *M. tuberculosis* strains isolated from newly diagnosed, adult, pulmonary tuberculosis patients in different regions of Bulgaria from December 2004 to March 2006. The DNA of the studied strains was extracted from 4- to 6-weeks Löwenstein-Jensen medium culture using the recommended method (19).

Genotyping. Extraction of DNA from mycobacterial strains and DNA fingerprinting using IS6110 as a probe was performed according to an internationally standardized protocol as described previously (19). PvuII-digested total DNA of reference strain 14323 was included in each Southern blot experiment as an external size standard and was used for quality control of IS6110-RFLP experiments.

Spoligotyping of isolates was performed as described by Kamerbeek et al. (9). In short, PCR-amplified biotin-labeled DR locus is hybridized against an array of 43 different immobilized DR spacers in a Miniblotter MN45 apparatus. The resulting hybridization signals are revealed by chemiluminescence and are visualized as a profile of discrete dots. The profiles were entered into Excel spreadsheets and compared to SITVIT2, an international spoligotype database at the Institut Pasteur de Guadeloupe, which is a most recent version of the published SpolDB4 database (2).

Each of the 24 MIRU-VNTR loci was amplified individually with primers specific for sequences flanking the MIRU units as described by Supply et al. (15, 16). The amplicons were evaluated on the 1.5% standard agarose gels by using a 100-bp DNA ladder (GE Healthcare). The H37Rv strain was run as an additional control of the performance of the method. Size analysis of the PCR fragments in 1.5% agarose gels and assignment of the VNTR alleles were done by using TotalLab TL100 software (Nonlinear Dynamics, Ltd., United Kingdom) and by comparison with correspondence tables kindly provided by Philip Supply. Some PCRs were repeated, and allele scoring was done by independent analysis by two technicians.

Analysis of the IS6110 element specific for LAM genetic family was done as described previously (11). In brief, a 205-bp band indicates a LAM strain by the presence of an IS6110 element in a specific site in genome, whereas a 141-bp band indicates a non-LAM strain lacking the IS6110 element in this site.

To minimize the risk of laboratory cross-contamination during PCR amplification, each procedure (preparation of the PCR mixes, the addition of the DNA, the PCR amplification, and the electrophoretic fractionation) was conducted in physically separated rooms. Negative controls (water) were included to control for reagent contamination.

Statistical analysis. The Hunter-Gaston index (HGI) was calculated as described previously (6) and was used to evaluate the discriminatory power of the typing methods and the allelic diversity of the VNTR loci. The mean HGI was calculated as a mean value of HGI values of the 24 loci.

TotalLab TL100 software (Nonlinear Dynamics, Ltd., United Kingdom) was used to calculate the molecular weight of the fragments in the IS6110-RFLP profiles; the resulting molecular weight matrix was used by Taxotron package (5) to build an unweighted pair-group method for arithmetic averages dendrogram. PAUP* 4.0 package (17) was used to reconstruct the most parsimonious dendrogram of the VNTR digital profiles treated as categorical variables.

RESULTS

A total of 133 *M. tuberculosis* isolates recovered from 133 different patients have been examined. The patients were permanent Bulgarian residents and were proven to be unlinked on the basis of a standard epidemiological investigation.

On the basis of spoligotyping, the 133 strains were subdivided into 37 distinct spoligotypes (see Table S1 in the supplemental material). Twenty-two spoligotypes represented single isolates; the other 111 isolates were grouped into 15 clusters containing from 2 to 33 isolates. The HGI was 0.893. The distribution of the major spoligotypes was plotted to the map of the country; this also demonstrated a geographic diversity of our collection (Fig. 1).

FIG. 1. Regional distribution of the major spoligotypes and clades identified in 133 *M. tuberculosis* strains from Bulgaria. Circle size is roughly proportional to the number of strains from an area (exact number of strains is shown after the city's name). The data on Turkey and Greece are based on the SITVIT2 database at Institut Pasteur de Guadeloupe.

Application of the published rules for definition of the major spoligotype clades (3) and comparison with SITVIT2 global database permitted us to assign most of the 133 strains to the known spoligotype families (see Table S1 in the supplemental material). At the same time, spoligotypes ST4, ST125, and ST1280 were classified as LAM/S since the absence of spacers 21-24 and 33-36 is specific for the LAM family, whereas the absence of spacers 9-10 and 33-36 is specific for the S family (3). We additionally used a recently proposed PCR approach to the definition of the LAM family (11) and found that ST4, ST125, and ST1280 strains did not harbor a LAM-specific IS6110 insertion (not shown). A phylogenetic position of these strains is discussed below in the light of the VNTR data.

Seventy-three strains had a sufficient quantity of DNA for traditional IS6110-RFLP typing. Accordingly, this subsample served to compare all three methods in the present study: spoligotyping, IS6110-RFLP typing, and the newly proposed 24-locus VNTR scheme (15). One should note that a reduction in the sample size did not decrease either genetic diversity (spoligotyping $HGI_{133} = 0.893$ versus $HGI_{73} = 0.939$) or geographical representativeness ("city of isolation" based $HGI_{133} = 0.838$ versus $HGI_{73} = 0.873$) of the collection as a whole.

Table 1 shows a comparison of the discriminatory capacities of the different VNTR sets and IS6110-RFLP typing. The IS6110 fingerprinting subdivided 73 *M. tuberculosis* isolates into 39 unique types and 12 clusters. The IS6110 copy number varied between 2 and 13 copies per profile, although it was generally high (Fig. 2 and Table 1). Only three strains in the present study exhibited a low copy number of <5, making an outgroup in the IS6110-RFLP tree (strain 46 and cluster XII in Fig. 2).

The 24 published MIRU-VNTR loci (15) were further analyzed in the present study (Table 1). Examples of different

TABLE 1. Discriminatory power of the genotyping methods evaluated with 73 strains

Method ^a	No. of types	No. of unique isolates	No. of clustered isolates	No. of clusters	Cluster size (range)	HGI
MIRU-VNTR 24 loci	66	61	12	5	2–3	0.997
MIRU-VNTR 15 loci	65	59	14	6	2–3	0.996
MIRU-VNTR 12 loci	62	55	18	7	2–4	0.994
MIRU-VNTR 5 loci	45	28	45	17	2–4	0.984
IS6110-RFLP	51	39	34	12	2–7	0.983
Spoligotyping	31	18	55	13	2–14	0.939

^a MIRU-VNTR 12 loci correspond to the former set defined by Supply et al. (16), while MIRU-VNTR 15 and 24 loci correspond to the optimized formats defined by Supply et al. (15). The five-locus scheme includes five MIRU-VNTR loci found to be the most polymorphic in this study: MIRU40, Mtub04, Mtub21, QUB-11b, and QUB-26.

alleles for the most polymorphic loci are shown in Fig. S1 in the supplemental material. The allelic diversity differed significantly among the VNTR loci (Table 2). The highest allelic diversity among all strains was observed for QUB-26 (0.827), and null allelic diversity was found for the monomorphic MIRU24. The lowest diversity (HGI $\sim$ 0.1) was found for six loci: MIRU20, MIRU27, MIRU31, MIRU39, ETR-B, and Mtub34.

A comparison of different combinations of the VNTR loci revealed that the use of the “old/classical” 12-locus combination was the least discriminatory; it identified 55 unique and 18 clustered strains (HGI = 0.994). A better resolution with 59 unique and 14 grouped strains (0.996) was observed by using the 15-locus MIRU-VNTR system. Finally, a use of the full set of the 24 loci permitted us to identify 61 unique and 12 clustered isolates (HGI = 0.997). Compared to the 15-locus scheme, the 24-locus scheme differentiated within a cluster of strains 17 and 25 due to the difference in the Mtub34 locus; it may be noted that these two strains were identical in other VNTR loci, as well as in their IS6110-RFLP and spoligoprofiles (Fig. 2). Otherwise, except for the above example, a use of the moderately polymorphic (Table 2) 9 auxiliary loci of the 24-locus scheme did not contribute to the additional differentiation of strains compared to the 15-locus scheme.

We further tested various combinations of VNTR loci in order to find one based on a reduced number of loci and close in discrimination to the 24-locus typing. The applied criteria were the number of alleles and the individual diversities of the loci assessed as HGI (not shown). Finally, the most obvious combination of the five most polymorphic loci (HGI > 0.6) was shown to achieve a good discrimination, although below that of the 15-locus scheme, but still higher than that for IS6110-RFLP typing (Table 1).

DISCUSSION

Today, a large number of genotyping tools for *M. tuberculosis* fingerprinting exists, of which spoligotyping, IS6110-RFLP, and MIRU-VNTR typing are the most commonly used. The development and application of MIRU-VNTR typing for *M. tuberculosis* became an important methodological achievement toward a better understanding of the molecular epidemiology of tuberculosis. The first study on the new 24-locus format dealt with a mainly cosmopolitan, geographically diverse set of strains (15). Although it made a critically important step in evaluating a wide array of loci and selecting those most

appropriate, more common in-field studies are carried out, by definition, in geographically limited settings with possibly biased local population structures of the circulating strains. A population-based study in Hamburg, Germany, concluded that 15- and 24-locus VNTR typing combined with spoligotyping represents the first PCR-based method with operating parameters (specificity and sensitivity) comparable to those of the “gold standard” IS6110 fingerprinting and thus can be used as a stand-alone approach to study tuberculosis transmission (13). Since then, this new 24-locus set has been evaluated in four other studies (1, 7, 8; I. Mokrousov, O. Narvskaya, A. Viazovaya, J. Millet, T. Otten, B. Vishnevsky, and N. Rastogi, unpublished data). Three of them were carried out in specific world areas (Japan, China, and Russia) with “biased” population structures of *M. tuberculosis*, i.e., dominated by a single and homogeneous clonal group, the Beijing genotype. In fact, two of these studies focused exclusively on the Beijing genotype strains (8; Mokrousov et al., unpublished). Finally, a large-scale 3-year population-based study in the Brussels-Capital Region in Belgium included strains of highly diverse origins, in particular, 76% patients were foreign-born from 69 countries, the majority being from Africa (1).

Therefore, the general interest of our study was to evaluate the performance of the newly proposed 24-locus standard (i) in the relatively heterogeneous *M. tuberculosis* population, (ii) circulating in the setting of a single country, and (iii) devoid of the significant influx of the foreign-born population.

A detailed analysis of the spoligotype-defined population structure of *M. tuberculosis* in Bulgaria was presented in our previous publication (18). The population structure of *M. tuberculosis* in Bulgaria appears to be both sufficiently heterogeneous (HGI_{133 strains} = 0.893) and dominated by two spoligotypes, ST125 (19%) and ST53 (25%), whose distribution patterns differ strikingly. Spoligotype ST53 is found in similar and rather high proportions in neighboring Greece and Turkey and is almost equally distributed across different regions of Bulgaria (Fig. 1). In contrast, ST125 is not found elsewhere (18) and is specific to Bulgaria; furthermore, it appears to be mainly confined to the southern part of that country (Fig. 1). ST125 and the related spoligotypes ST4 and ST1280 were classified as LAM/S in the SITVIT2 database. However, LAM-specific IS6110 insertion (11) was found to be absent from these strains in our study. Interestingly, strains of these three spoligotypes were grouped closely in the 24-locus-based VNTR dendrogram and together with ST34 that is a prototype of the S family (a cluster marked by an asterisk in Fig. S2 in the

TABLE 2. Allelic diversity of 24 VNTR loci in *M. tuberculosis* strains from Bulgaria and other locations^a

VNTR locus combination or parameter	VNTR locus	VNTR alias	Diversity in Bulgarian strains			HGI			
			No. of alleles	No. of repeats (range)	HGI	Global set (n = 494)	Japan, non-Beijing types (n = 54)	Japan, Beijing genotype (n = 181)	China, Beijing genotype (n = 72)
Discriminatory loci	580	MIRU4	5	0–4	0.557	0.38	0.469	0.086	0.120
	960	MIRU10	4	2–5	0.532	0.74	0.794	0.419	0.144
	1644	MIRU16	4	1–4	0.524	0.53	0.610	0.310	0.068
	2996	MIRU26	7	1–7	0.422	0.75	0.739	0.383	0.353
	3192	MIRU31	3	2–4	0.106	0.72	0.537	0.322	0.169
	802	MIRU40	6	1–6	0.806	0.73	0.752	0.327	0.194
	424	Mtub04	4	1–4	0.655	0.71	0.471	0.459	0.306
	1955	Mtub21	5	1–5	0.669	0.76	0.599	0.393	0.556
	2401	Mtub30	3	1–4	0.430	0.62	0.580	0.403	0.068
	3690	Mtub39	5	2–6	0.465	0.69	0.735	0.186	0.171
	2165	ETR-A	4	1–4	0.554	0.75	0.554	0.147	0.232
	577	ETR-C	4	2–5	0.419	0.69	0.230	0.022	0.094
	2163b	QUB-11b	7	1–7	0.773	0.82	0.748	0.772	0.651
	4052	QUB-26	9	2–11	0.827	0.84	0.798	0.741	0.518
	4156	QUB-4156	5	0–4	0.447	0.67	0.665	0.611	0.395
Additional loci	154	MIRU2	3	1–3	0.249	0.16	0.105	0	0
	2059	MIRU20	2	1–2	0.129	0.30	0.226	0.022	0.014
	2531	MIRU23	5	3–8	0.399	0.65	0.597	0.176	0.014
	2687	MIRU24	1	1	0	0.35	0.105	0	0
	3007	MIRU27	3	1–4	0.106	0.25	0.036	0.115	0.014
	4348	MIRU39	2	1–2	0.153	0.45	0.497	0.221	0.119
	2461	ETR-B	3	1–3	0.106	0.44	0.370	0.033	0.014
	2347	Mtub29	4	2–5	0.204	0.48	0.262	0.043	0.119
	3171	Mtub34	3	2–4	0.154	0.27	0.036	0.065	0.014
Mean					0.404	0.573	0.480	0.260	0.181
Reference						15	7	7	8

^a The five most polymorphic loci in this study are indicated in boldface.

supplemental material). It appears that spoligotypes ST125, ST4, and ST1280 may indeed belong to the S family, although further studies targeting VNTR loci in strains of these spoligotypes from other world regions are needed to clarify their phylogenetic clade position.

In the present study, of the three methods used spoligotyping, not unexpectedly, showed the lowest discrimination. At the same time, it should be noted that, similar to the German study (13), spoligotyping in our setting, albeit least discriminatory, contributed to the subdivision within two of five 24-locus VNTR clusters (clusters A and C in Fig. S2 in the supplemental material). In contrast, spoligotype ST41 makes the most apparent example of the slower evolution of the DR locus compared to the VNTR haplotypes or IS6110-RFLPs: the ST41 strains differ in 7 of 24 loci, although they indeed remained weakly related and located in the same part of the 24-VNTR dendrogram (see Fig. S2 in the supplemental material). An interesting finding of the present study is that a “gold standard” IS6110-RFLP appeared even less variable marker than the classical 12-locus MIRU scheme (Fig. 2 and Table 1).

Most of the IS6110 clusters in the Fig. 2 were completely or partially differentiated by the use of the 24-VNTR set. On the other hand, all three VNTR clusters included strains with identical RFLP profiles (not shown). A remarkable evolutionary stability of some IS6110-RFLP profiles is especially manifested in the ST125/ST4 cluster of strains, the largest cluster in the present study (see cluster I in Fig. 2). Perhaps, mapping of the IS6110 insertions in the genome in these strains would help elucidate this intriguing situation.

It should be also noted that addition of the VNTR typing to the IS6110-RFLP allowed for more precise tracing of the local clones at the city level. For example, an IS6110 cluster IV (spoligotype ST2905) was further subdivided by VNTR typing: three strains from Pleven remained identical and differed in three loci from a strain from Shumen (Fig. 2). Previous studies on the 24-locus format showed a general congruence of IS6110 and 24-VNTR results, while the latter method was suggested to be more accurate overall for cluster analysis (13, 15). In the Belgian study, of 23 IS6110-RFLP clusters with high copy numbers 20 were found to be completely identical by MIRU-

FIG. 2. IS6110-RFLP based dendrogram of *M. tuberculosis* strains from Bulgaria compared to their 24-locus VNTR digital haplotypes, 43-signal spoligoprofiles. SIT, spoligotype international type (2). IS6110-RFLP clusters in the dendrogram are designated with Roman numerals from I to XII. “A” designates 11 repeat units in a VNTR locus. VNTR profiles of the strains included in the IS6110-RFLP clusters are in boxes; minor variable alleles within these clusters are in bold (confirmed by repeating PCR experiments, see Materials and Methods section).

VNTR typing. Of the three remaining IS6110 RFLP clusters, two were fully subdivided both by four to seven MIRU-VNTR loci (1). In this sense, our result of the superior discrimination achieved by the 24-locus VNTR scheme compared to IS6110 fingerprinting is not so surprising.

Various sets of MIRU-VNTR loci demonstrated different levels of discriminatory power (Table 1). Only one locus, MIRU24, was monomorphic, a finding in agreement with the previous observation that this locus is phylogenetically conserved and discriminates between large ancestral and modern *M. tuberculosis* lineages with or without the TbD1 genome region (14). Compared to the IS6110-RFLP typing, a 12-locus MIRU scheme already showed a good discrimination, but indeed the addition of the new VNTR loci, mainly those from the discriminatory 15-locus set, improved a discrimination by reducing the number of clusters and clustered isolates (Table 1).

A further closer look at the individual diversities of the loci (Table 2) showed that loci found to be the most polymorphic in Bulgaria were also among the most polymorphic loci in the global set of strains (15). At the same time, some globally variable loci were low polymorphic in the Bulgarian collection, e.g., MIRU31. Altogether and not unexpectedly, the mean per-locus diversity was higher for the global set of strains (Table 2). Comparison with available data from other published studies in Japan and China (Table 2) reconfirmed a strong phylogeographical structure of *M. tuberculosis* that appeared to have a direct impact on the observed diversity of the VNTR loci. Both China and Japan are dominated by the Beijing genotype strains (7, 8, 12, 21), a closely related clonal group of strains; this led to low mean per-locus diversity, as well as a low diversity of the most VNTR loci, including those from the 15-locus "discriminatory" set (Table 2). A lower mean HGI for Beijing genotype samples (Japan and China) versus non-Beijing genotype samples (global set, Japan, Bulgaria) may result from a stronger clonality in the Beijing genotype strains compared to the much more diverse strains of other genotypes. On the other hand, a lower mean HGI in the Beijing genotype samples in China versus Japan may be explained by the much smaller sample size in the Chinese study. Further, although non-Beijing strains are likely to be of the diverse origins, these origins still differ and depend on the area of isolation. Nevertheless, individual and mean HGI values in the Bulgarian collection were similar to the respective values for the non-Beijing subsample from Japan and the global collection (Table 2). This observation is made on the geographically very distant locations, such as Bulgaria and Japan, and it apparently gives additional support to the new 24-locus format of *M. tuberculosis* genotyping. Indeed, the mean HGI value in the non-Beijing sample from Japan is higher than it was in Bulgaria, especially due to the low-polymorphic (in our collection) loci MIRU31, MIRU39, and ETR-B. A general explanation, albeit speculative, may lie in the different levels of clonality and/or more or less recent dissemination of the strains in a survey area or more diverse origins of the circulating strains. These factors may be additionally influenced by human population size and the level of urbanization.

An increase of a number of the targeted VNTR loci is expected to result in an increased discrimination. Nevertheless, it also makes such multilocus schemes rather time-consuming

and expensive in settings with relatively limited resources. It appears that a primary typing may be reasonably limited to a few loci if they still achieve a sufficiently high discrimination. Since the population structures of circulating *M. tuberculosis* strains vary across different world regions, these first-line typing schemes may be country dependent and could include different loci. The five most polymorphic (in our study) loci used together allowed the achievement of an HGI higher than that of IS6110-RFLP typing (Table 1). In this view, an apparent utility of the newly proposed 24-locus format (15) has been manifested by the fact that four of five loci of the "Bulgaria-specific" reduced set represented these new loci (Mtub04, Mtub21, QUB11b, and QUB26), while only one locus was retained from the earlier 12-locus scheme (MIRU40). Accordingly, this leads us to preliminarily suggest these five loci (Table 1) for use in the first-line typing of the *M. tuberculosis* strains in Bulgaria, although further studies are undoubtedly required to test the proposed provisional scheme.

To conclude, the present study demonstrated MIRU-VNTR typing to be the most discriminatory tool compared to spoligotyping and IS6110-RFLP typing of *M. tuberculosis*. Consequently, a new 24-locus MIRU-VNTR standard (15) appears to be taking an increasingly leading position as the primary method for *M. tuberculosis* epidemiological typing. A reduced five-locus set provided sufficiently high differentiation and may be used for first-line typing of *M. tuberculosis* isolates in Bulgaria, although further studies are needed to validate this scheme. At the same time, a comprehensive secondary subtyping of clustered isolates should target all 15 loci of the discriminatory set of Supply et al. (15), at least for the time being.

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