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Comparison of a Semiautomated Commercial Repetitive-Sequence-Based PCR Method with Spoligotyping, 24-Locus Mycobacterial Interspersed Repetitive-Unit–Variable-Number Tandem-Repeat Typing, and Restriction Fragment Length Polymorphism-Based Analysis of IS*6110* for *Mycobacterium tuberculosis* Typing

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Fifty-two multidrug-resistant isolates of *Mycobacterium tuberculosis* representative of the currently predominant lineages in France were analyzed using repetitive-sequence-based PCR (rep-PCR) DiversiLab (DL), spoligotyping, 24-locus mycobacterial interspersed repetitive-unit–variable-number tandem-repeat typing (MIRU-VNTR), and restriction fragment length polymorphism of IS*6110* (IS*6110*-RFLP). DL, as opposed to MIRU-VNTR and IS*6110*-RFLP analysis, did not allow discrimination among half of the isolates, an indication of comparatively lower resolving power.

Multidrug-resistant (MDR) tuberculosis (TB) is a serious health threat that requires molecular procedures yielding results quickly to improve control of the diffusion of drug-resistant strains (1–4). Until recently, restriction fragment length polymorphism analysis of IS*6110* (IS*6110*-RFLP) (5) was considered the gold standard for *Mycobacterium tuberculosis* strain typing (5, 6). Two PCR-based methods, spoligotyping and mycobacterial interspersed repetitive-unit–variable-number tandem-repeat typing (MIRU-VNTR), which can be performed with very small quantities of crude DNA, have gradually supplanted RFLP. The signatures revealed by spoligotyping identify strains at the clade or subclade level (7, 8, 9), but a major limitation of this method is its inferior discriminatory power compared with that of IS*6110*-RFLP and complete 24-locus MIRU-VNTR (10). On the other hand, 24-locus MIRU-VNTR is fairly rapid and generates numerical values that can easily be compared in interlaboratory studies. Because of particular advantages over IS*6110*-RFLP and spoligotyping at the technical level and in discriminatory power, MIRU-VNTR is considered to be the new reference standard for molecular epidemiological studies (11).

Repetitive-sequence-based PCR (rep-PCR) is a rapid typing procedure using primers that bind to multiple noncoding repetitive sequences interspersed throughout the bacterial genome. The strain-specific band patterns generated by rep-PCR can be used to determine the similarity of bacterial isolates at the genomic level, as the repetitive sequences throughout the genome enable discrimination of interstrain variations on the basis of amplicon size and amount (12–17). However, rep-PCR typing is notorious for its susceptibility to minor variations in experimental conditions and reagents, resulting in poor reproducibility. The DiversiLab (DL) microbial typing system (bioMérieux, Marcy l'Etoile, France) (18) consists of a semiautomated highly standardized rep-PCR (18, 19). Studies reporting on DL performance in the analysis of mycobacteria are scarce and have been done with specific aims, e.g., rapid genotyping of nontuberculosis mycobacteria (20–24), analysis of *M. tuberculosis* microevolution within a patient (25), monitoring of TB outbreaks (1, 3, 26), and comparison of DL to other molecular techniques for *M. tuberculosis* typing (1, 19, 27, 28).

In this study, we provide a comprehensive evaluation of DL by comparing its ability to discriminate among MDR TB isolates for which epidemiological data are available.

Fifty-two *M. tuberculosis stricto sensu* clinical isolates and the H37Rv reference strain (French National Reference Center for Mycobacteria) were analyzed. All 52 were MDR isolates previously characterized by spoligotyping, 24-locus MIRU-VNTR, and IS*6110*-RFLP, including three East African-Indian (EAI) strains (one EAI3-IND and two EAI2-PHL) and six Beijing, five Haarlem (three H1 and two H3), six Latin American-Mediterranean LAM9, four URAL, three Cameroon (CAM), six S, and 19 T-related (seven T1, six T2, and six T2-T3) strains. The Beijing, Haarlem, LAM, and T families represent approximately 80% of the MDR strains circulating in France (10). The available epidemiological data are summarized in Table 1. For spoligotyping, MIRU-VNTR, and rep-PCR typing, DNA was extracted from a loopful (ca. 10 μl) of colonies grown on Lowenstein-Jensen agar, using the UltraClean microbial DNA isolation kit (Mo Bio Laboratories, Solana Beach, CA) according to the manufacturer's recommendations. The procedure and interpretation of IS*6110*-RFLP analysis were performed according to the standardized protocol recommended by van Embden et al. (6). RFLP patterns were

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TABLE 1 Epidemiological information concerning the isolates for which links were suggested by the molecular analysis

Lineage	Isolate no.	SIT no.	Epidemiological information	Country of birth	Yr of isolation
LAM9	S15	1106	Household contacts with S16 and S17	France	2007
	S16	1106	Same family as S17	Portugal	2007
	S17	1106	Same family as S16	France	2007
	S18	1106	NL[a]	Portugal	2011
T1	S38	53	Same family as S40	Guinea	2006
	S39	53	NL	Guinea	2006
	S40	53	Same family as S38	Guinea	2007
URAL	S12	262	NL	Romania	2006
	S13	262	NL	Romania	2008
	S14	262	NL	Romania	2006
T2	S35	52	NL	DR Congo[b]	2006
	S36	712	NL	DR Congo	2006
	S37	712	NL	DR Congo	2006
T1-Ghana	S41	53	NL but traveled to Africa	France	2009
	S42	53	NL	Ivory Coast	2006
	S43	53	NL	Ivory Coast	2006
S	S29	466	NL	Portugal	2008
	S30	466	NL	Algeria	2007
	S31	34	NL	France	2006
	S32	34	NL	Pakistan	2008
	S33	1063	NL	Algeria	2007
Haarlem-H3	S22	50	NL	France	2006
	S23	50	NL	Togo	2006
LAM9	S20	42	NL	Armenia	2007
	S21	42	NL	France	2006
Haarlem-H1	S9	62	NL	France	2006
	S10	47	NL	Unknown	2006
Beijing	S1	Beijing-like	NL	China	2007
	S2	1	NL	Unknown	2008
	S3	1	NL	Ukraine	2007
	S4	1	NL	Ukraine	2007
	S5	1	NL	France	2007
	S6	1	NL	Congo-Brazzaville	2006
T2-T3	S46	73	NL	DR Congo	2008
	S47	73	NL	Angola	2008
	S48	73	NL	DR Congo	2007
	S49	73	NL	DR Congo	2007
	S50	73	NL	Angola	2006
	S51	73	NL	DR Congo	2007

[a] NL, not linked by contact tracing, with differences in resistance profiles and resistance gene mutations.
[b] DR Congo, Democratic Republic of the Congo.

compared and analyzed using the computerized GelCompar software system (Applied Maths, Sint-Martens-Latem, Belgium) with the unweighted-pair group method using average linkages (UPGMA). Spoligotyping was performed as described by Abadia et al., with a Luminex microbead-based flow cytometry device (29). To determine the lineages of the 52 isolates, spoligotypes in binary format were converted to an octal code for comparison with the *M. tuberculosis* SpolDB4 database containing all spoligotype international types (SIT) (8). Standard 24-locus MIRU-VNTR typing was performed as previously described (11) with the MIRU-VNTR typing kit of GenoScreen. The 24 numerical values generated by MIRU-VNTR were compared with those existing in the MIRU-VNTRplus database (http://www.miru-vntrplus.org). Finally, DL was performed following the manufacturer's instructions. DNA fingerprint patterns were analyzed with the Web-based DiversiLab software, version 3.4, which uses the Pearson correlation coefficient and UPGMA for automatic comparison of the rep-PCR-based DNA fingerprints (18, 19). A percentage similarity set at ≥93% was used as the threshold in the cluster analysis (19).

Globally, based on a similarity cutoff of 93%, the results ob-

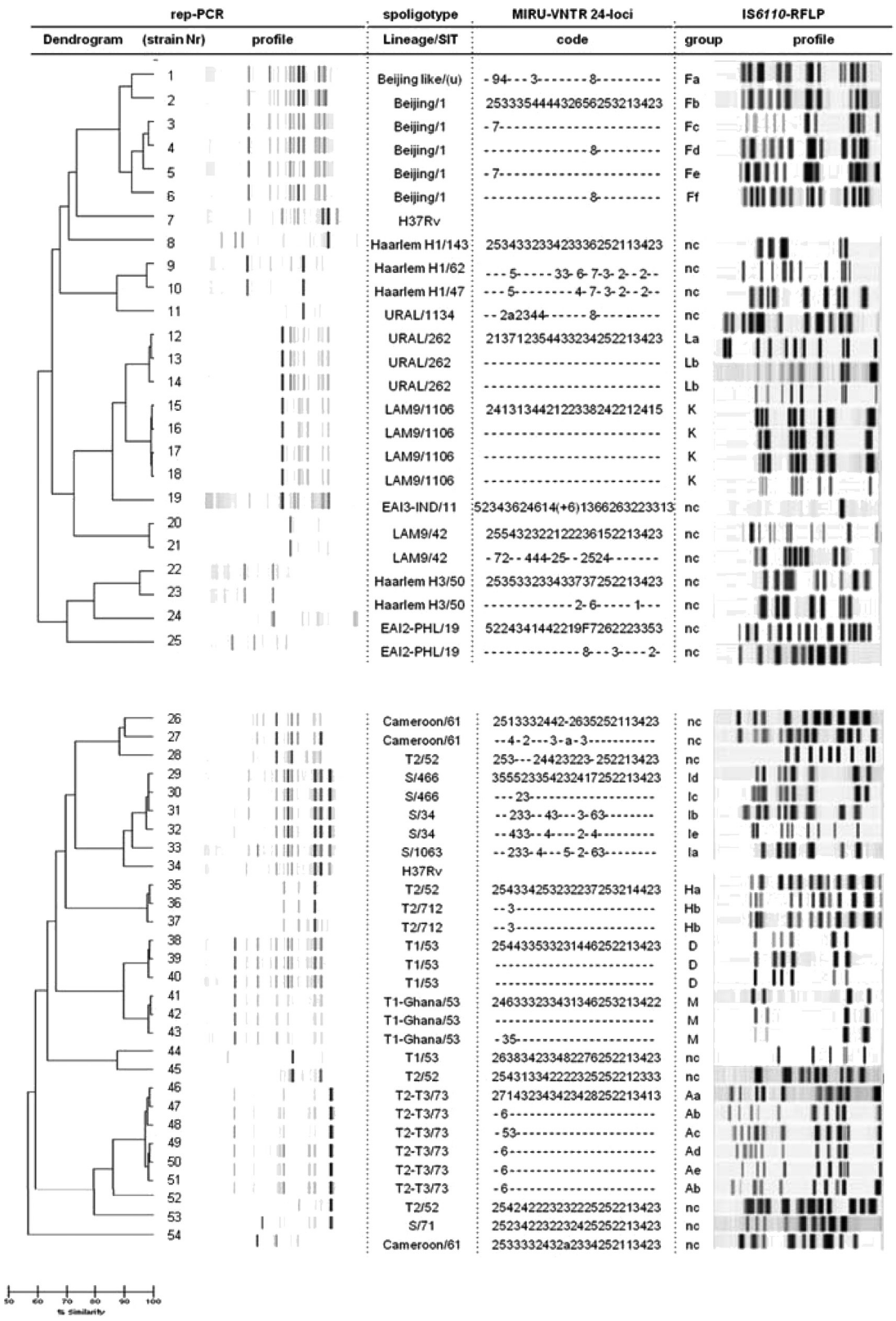

FIG 1 rep-PCR DiversiLab (DL), spoligotyping, 24-locus MIRU-VNTR, and IS*6110*-RFLP analysis of *Mycobacterium tuberculosis* isolates. First column, dendrogram and virtual band profiles generated using DL analysis. A scale of similarity (%) as determined using DL is shown at the bottom of the figure. Second column, spoligotyping-based lineage names and SIT numbers (u, unknown SIT). Third column, MIRU codes. Within each strain family, dashes indicate that the MIRU code does not differ from that written in full. Fourth column, RFLP cluster designation and RFLP profiles (nc, nonclustered profile).

tained with DL were in full agreement with those generated by the other typing methods for 27 (52%) of the 52 isolates analyzed. Seven of them, belonging to two groups, with epidemiological links and sharing identical SIT numbers, MIRU codes, and RFLP profiles within each group, were correctly allocated to two subsets of high similarity levels (>98%) by DL (the four LAM9 isolates S15 to S18 and the three T1 isolates S38 to S40) (Table 1 and Fig. 1). Likewise, seven isolates with identical SIT numbers, MIRU codes, and RFLP patterns but no obvious epidemiological relationships apart from the country of origin showed similarity of >98% within each of the three corresponding DL clusters (S12 to S14 in the URAL family, S36 and S37 in the T2 family, and S41 and S42 in the T1-Ghana family) (Table 1 and Fig. 1). Finally, 13 epidemiologically unrelated isolates (not shown in Table 1) had unique SIT, MIRU-VNTR, and RFLP profiles and were clearly not linked according to the DL results, which set them well apart from the remaining isolates: S11 (URAL); S8 (H1); S19, S24, and S25 (EAI); S53 (S); S26, S27, and S54 (CAM); S44 (T1); and S28, S45, and S52 (T2) (Fig. 1).

In contrast, and comparatively to spoligotyping, 24-locus MIRU-VNTR, and IS*6110*-RFLP, the rep-PCR results suggested false linkages in a significant proportion (13/52; 25%) of the isolates, including five S, two H1, two Beijing, two H3, and two LAM9 strains without epidemiological links. This finding is clearly illustrated considering the five isolates of the S family (S29 to S33; Table 1), which, using DL, were all grouped into a single high-similarity cluster (97% for S29 to S32 and 93% for S33) although they displayed different SIT, MIRU, and RFLP patterns (Fig. 1). Four further isolates of the same SIT but clearly distinct MIRU and RFLP patterns were also unexpectedly grouped into two clusters, i.e., H3 isolates S22 and S23 and LAM9 isolates S20 and S21, with DL pattern similarities of 95% and 98%, respectively (Table 1 and Fig. 1). Finally, four isolates with different SIT, MIRU, and RFLP patterns appeared unexpectedly linked using DL (cluster S9 and S10 in clade H1 and cluster S1 and S2 in the Beijing family, with 97% and 93% similarity, respectively) (Table 1 and Fig. 1).

The interpretation of the DL results was problematic for 12 (23%) isolates (four Beijing, six T2-T3, one T1, one T2). With respect to the Beijing strains S3 to S6 (SIT1) (Table 1), the two epidemiologically unrelated isolates S3 and S5 were considered linked using DL (97% similarity), which is concordant with the MIRU results (identical MIRU codes) but not with the RFLP patterns, which differed from one another by three bands (Fig. 1). Conversely, isolates S4 and S6 were not linked using DL (92% similarity, i.e., below the cutoff) despite their identical MIRU codes, but in accordance with the RFLP results showing significant differences between the corresponding patterns (changes in at least three bands) (Fig. 1). More strikingly, isolates S3 and S4, which differed in their MIRU and RFLP patterns, were closely linked according to DL (98% similarity) (Fig. 1). Jang et al. (27) previously suggested that DL discriminates efficiently among Beijing family strains with near-identical IS*6110*-RFLP patterns, in contrast with our data which rather suggest that DL is not reliable for analysis of Beijing strains. Finally, regarding the six epidemiologically unlinked T2-T3 (SIT73) isolates S46 to S51 (Table 1), the high similarity (97%) observed within the single cluster as found with DL was not consistent with the significant differences observed in their MIRU codes and especially their RFLP patterns (Fig. 1). Similarly, using DL, isolate S43 (T1) was tightly associated with S41 and S42 despite variations in two MIRU loci, and isolate S35 (T2) was linked to S36 and S37 despite their different SIT numbers, a single-locus MIRU variation, and changes in two bands in their IS*6110* fingerprints (Fig. 1). Taken together, these results suggest that DL might generate false linkages compared to MIRU-VNTR and RFLP, which have gold-standard status in molecular typing.

In conclusion, we observed here that DL did not allow discrimination in nearly half of the isolates which had been unquestionably differentiated by other techniques, indicating that rep-PCR has a lower resolving power than MIRU-VNTR and IS*6110* RFLP analysis in *M. tuberculosis* typing. These results are in disagreement with those of Cangelosi et al. (19), who reported that the discriminatory power of rep-PCR was at least as good as that of IS*6110*-RFLP for *M. tuberculosis*, but they are in agreement with those of Masala et al. (28), who suggested that the MIRU-VNTR and IS*6110*-RFLP typing methods have greater discriminatory power than rep-PCR DL. In light of the clear limitations highlighted in this study, confirmation by MIRU-VNTR and/or RFLP analysis is required to substantiate MDR TB transmission when clonal relationships are suggested using DL.

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