Prospective Universal Application of Mycobacterial Interspersed Repetitive-Unit–Variable-Number Tandem-Repeat Genotyping To Characterize *Mycobacterium tuberculosis* Isolates for Fast Identification of Clustered and Orphan Cases[▽]

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The use of molecular tools for genotyping *Mycobacterium tuberculosis* isolates in epidemiological surveys in order to identify clustered and orphan strains requires faster response times than those offered by the reference method, IS6110 restriction fragment length polymorphism (RFLP) genotyping. A method based on PCR, the mycobacterial interspersed repetitive-unit–variable-number tandem-repeat (MIRU-VNTR) genotyping technique, is an option for fast fingerprinting of *M. tuberculosis*, although precise evaluations of correlation between MIRU-VNTR and RFLP findings in population-based studies in different contexts are required before the methods are switched. In this study, we evaluated MIRU-VNTR genotyping (with a set of 15 loci [MIRU-15]) in parallel to RFLP genotyping in a 39-month universal population-based study in a challenging setting with a high proportion of immigrants. For 81.9% (281/343) of the *M. tuberculosis* isolates, both RFLP and MIRU-VNTR types were obtained. The percentages of clustered cases were 39.9% (112/281) and 43.1% (121/281) for RFLP and MIRU-15 analyses, and the numbers of clusters identified were 42 and 45, respectively. For 85.4% of the cases, the RFLP and MIRU-15 results were concordant, identifying the same cases as clustered and orphan (κ , 0.7). However, for the remaining 14.6% of the cases, discrepancies were observed: 16 of the cases clustered by RFLP analysis were identified as orphan by MIRU-15 analysis, and 25 cases identified as orphan by RFLP analysis were clustered by MIRU-15 analysis. When discrepant cases showing subtle genotypic differences were tolerated, the discrepancies fell from 14.6% to 8.6%. Epidemiological links were found for 83.8% of the cases clustered by both RFLP and MIRU-15 analyses, whereas for the cases clustered by RFLP or MIRU-VNTR analysis alone, links were identified for only 30.8% or 38.9% of the cases, respectively. The latter group of cases mainly comprised isolates that could also have been clustered, if subtle genotypic differences had been tolerated. MIRU-15 genotyping seems to be a good alternative to RFLP genotyping for real-time interventional schemes. The correlation between MIRU-15 and IS6110 RFLP findings was reasonable, although some uncertainties as to the assignment of clusters by MIRU-15 analysis were identified.

Molecular tools have been widely used to characterize *Mycobacterium tuberculosis* isolates, with the aim of better understanding the epidemiology of tuberculosis (TB) (1, 6, 8, 18, 23). This has enabled us to document suspected outbreaks (4, 28, 34), identify risk factors associated with TB transmission (13, 20, 36), and evaluate the efficiency of control programs by observing the dynamics of clustered cases (9, 12, 17, 22, 24).

Restriction fragment length polymorphism (RFLP) analysis based on the IS6110 sequence is the reference genotyping method for *M. tuberculosis* (35). However, its limitations (mainly response times) make its adaptation unsuitable for

real-time intervention epidemiological schemes. New genotyping techniques based on PCR have recently been developed and are more suitable for these purposes.

One of the most promising PCR-based methods is mycobacterial interspersed repetitive-unit–variable-number tandem-repeat (MIRU-VNTR) genotyping (21, 30–32). A novel format based on 15 loci has improved upon the initial 12-loci version. Its discriminatory power has been found to be equivalent to that of the standard approach on the basis of reference method, and its response time could be competitive. However, very few long-term analyses apply this technique universally in parallel to the reference method to identify advantages and pitfalls (1, 25, 27, 33).

In order to compare both techniques, we selected the province of Almería, in southeast Spain, because of the complexity of its socioepidemiological population profile, which challenges us to develop new and more-efficient methods of surveying TB transmission. In Almería, around 60% of the cases

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TABLE 1. Sizes and distributions of clusters

Method	No. (%) of:						
	Clustered cases	Clusters	Cases in each cluster	Autochthonous clusters	Immigrant, uninational clusters	Immigrant, multinational clusters	Mixed clusters
IS6110 RFLP	112 (39.9)	42	2–8	13	15	5	9
MIRU-15	121 (43.1)	45	2–8	14	17	5	9

involve immigrants who are dispersed throughout the province and who are highly mobile and difficult to access. Our group had already developed a new advanced system for studying clustered cases by active compilation of data through a newly developed computer application, GenContactTB, and standardized interviews of the patients on the basis of nominal and photographic identification (26). These aspects make Almería a suitable context for exploring novel, rapid *M. tuberculosis*-genotyping tools. Our study aimed to evaluate MIRU-VNTR genotyping with a set of 15 loci (MIRU-15) over a 39-month period by using a prospective design for most of the period (data for 2005 were retrospectively studied to increase analytical power), and the coverage of the population was universal (all *M. tuberculosis* isolates were included).

MATERIALS AND METHODS

Sample. The population covered by the study centers—health centers of the Servicio Andaluz de Salud (Regional Health Service) and the public network of mycobacteriology laboratories (Hospital de Poniente, C. H. Torrecárdenas, and Hospital La Inmaculada)—ranged from 565,310 inhabitants in 2003 to 665,099 inhabitants in 2008 (average, 617,547 inhabitants). Our sample was composed exclusively of patients with microbiological diagnoses of TB. The *M. tuberculosis* isolates were prospectively genotyped by RFLP analysis for the entire period and by MIRU-VNTR analysis for the 2006-to-2008 period. To increase the analytical power, we extended the MIRU-VNTR analysis to include the 2005 isolates, which were studied retrospectively.

Microbiological procedures. Clinical specimens were processed according to standard methods. Susceptibility testing was performed using a BacT/Alert 3D instrument (bioMérieux España SA, Madrid, Spain).

Genotyping procedures. *M. tuberculosis* isolates were genotyped by RFLP analysis (35); when the RFLP type contained fewer than six bands, spoligotyping (19) was used as a second-line genotyping method. Genotypes were analyzed using Bionumerics 4.6 (Applied Maths, Belgium).

For the retrospective period, MIRU-VNTR analysis was performed using the purified DNA preparations from RFLP analysis; for the prospective period, MIRU-VNTR analysis was performed directly on cultures grown in BacT/Alert liquid medium. One milliliter was centrifuged, and the pellet was boiled for 7 min in the presence of GenProbe lysis reagent (1:16 diluted; bioMérieux, Geneva, Switzerland). Five microliters of the crude extract (1:4 diluted) was used as a template for MIRU-VNTR multiplex PCR. MIRU-VNTR analysis was performed as described elsewhere (2, 30), although with some modifications for the MIRU-15 format: the final volume reaction mixture of 50 µl contained 1 µl (1 U) of *Taq* DNA polymerase (ROCHE) and 2 µl of dimethyl sulfoxide for Mix1 (580[MIRU4], 2996[MIRU26], and 802[MIRU40]) and Mix2 (960[MIRU10], 1644[MIRU16], and 3192[MIRU31]) and 6 µl for Mix3 (424[Mtub04], 577[ETRC], and 2165[ETRA]), Mix4 (2401[Mtub30], 3690[Mtub39], and 4156[QUB4156]), and Mix5 (2163b[QUB11b], 1955[Mtub21] and 4052[QUB26]). One microliter of the PCR products was mixed with 9 µl of formamide and 0.5 µl of GeneScan 2500 ROX size standard (Applied Biosystems). DNA fragments were separated by capillary electrophoresis using an ABI Prism 3100 genetic analyzer (Applied Biosystems). Run parameters were created from the GeneScan36 POP4 default module, with the run voltage changed from 15 to 11 kV and the run time set to 3,600 s. Sizing of the PCR fragments was done using GeneScan software (Applied Biosystems).

The MIRU-VNTR type was determined after the results for the 15 loci were combined in the following order: MIRU4, MIRU26, MIRU40, MIRU10, MIRU16, MIRU31, Mtub04, ETRC, ETRA, Mtub30, Mtub39, QUB4156, QUB11b, Mtub21, and QUB26.

For all cases in which either RFLP or MIRU-VNTR analysis offered discrepant results, both assays were repeated to confirm them.

Cluster analysis. Genotypic patterns were analyzed using Bionumerics 4.6 (Applied Maths, Belgium). Dendrograms were generated using the unweighted-pair group method with arithmetic averages and the Dice coefficient or the categorical coefficient for RFLP and MIRU-15 analyses, respectively.

RFLP clusters were defined for *M. tuberculosis* isolates sharing identical fingerprints. Isolates differing only in a low-molecular-weight band (<1.10 kb) of the RFLP pattern and sharing identical spoligotypes were also clustered. RFLP clusters defined by isolates with fewer than six RFLP bands were also required to share identical spoligotypes.

MIRU-VNTR clusters were defined for isolates sharing identical patterns.

Epidemiological survey. We analyzed clusters by using an advanced survey (26). Briefly, transmission of TB was investigated using two information sources: data obtained with the standard approach (based on conventional contact tracing) and those obtained by applying two interviews. The objective of the first standardized interview was to collect complete data and photographs from the patients. The second interview, performed only for the clustered cases, was an attempt to compile new data for them and to search for potential epidemiological links based on nominal/photographic recognition among the clustered cases.

RESULTS

Between January 2005 and March 2008, we obtained 343 *M. tuberculosis* isolates. Of these, 205 (59.8%) were from immigrants, mainly from North Africa (73/205; 36%) and Sub-Saharan Africa (56/205; 27.3%). For 297 (86.6%) isolates, an RFLP type was obtained, and for 312 (91%) isolates, a MIRU-VNTR type was obtained. The MIRU-VNTR type was obtained within a 2-week period for 57.1% of the isolates and within a 3-week period for 87.5% of the isolates. As for RFLP analysis, the shortest time for obtaining a genotype was 5 weeks. The response times were measured from the moment the cultures were received in the analysis center. Both RFLP and MIRU-VNTR types were available for 286 (83.4%) isolates. Five confounding cases were excluded from the analysis; two of them were clustered by both RFLP and MIRU-VNTR analyses but were linked to different cases by each of these methods, and the remaining three excluded cases comprised patients who were related to the two confounding cases. In total, 281 cases were included in the analysis.

RFLP analysis distributed the sample into 112 cases (39.9%) grouped in 42 clusters and 169 orphan strains, whereas MIRU-VNTR analysis distributed the sample into 121 cases (43.1%) grouped in 45 clusters and 160 orphan strains. The sizes and distributions of the clusters according to the nationalities of the included patients (autochthonous, uninational, multinational, or mixed) are shown in Table 1.

For 240/281 isolates (85.4%), the isolates identified as clustered and orphan by RFLP and MIRU-VNTR analyses were identical: 34.2% (96/281) of the isolates analyzed were clustered and 51.2% (144/281) were identified as orphan by both techniques. The results were discrepant for 14.6% of the isolates (Fig. 1): 16 cases were clustered by RFLP analysis but

a

Patient Code	COUNTRY of origin	RFLP type code	RFLP type	Discrepancies	MIRU-15 type															EPID. LINKS
					580	2996	802	960	1644	3192	424	577	2165	2401	3690	4156	2163b	1955	4052	
309-05	NIGERIA	155			2	5	3	3	3	3	2	4	4	2	X	2	5	3	5	YES
522-06	NIGERIA	155			2	5	3	3	3	3	2	4	4	2	X	2	5	3	5	YES
708-07	SENEGAL	155		SLV (1)	2	5	3	3	3	3	2	4	4	2	X	2	6	3	5	YES
566-06	SPAIN	28-28		6-7 loci	2	5	3	5	3	4	2	4	3	4	5	3	4	3	7	NO
630-07	ROMANIA	28-28		3-6 loci	2	5	4	5	3	3	2	3	3	4	3	3	6	3	4	NO
430-05	SPAIN	28-28		3-8 loci	2	5	4/3	6	3	3	2	3	3	4	4/3	3	6	2	6	NO
492-06	MOROCCO	28-28		3-7 loci	2	5	3	5	3	3	3	3	3	4	3	3	6	2	4	NO
286-05	MOROCCO	280		SLV (2)	2	6	4	4	3	3	3	4	2	1	2	3	2	3	5	YES
657-07	MOROCCO	280		SLV (2)	2	6	4	2	3	3	3	4	2	1	2	3	2	3	5	YES
343-05	ROMANIA	343		6 loci	2	5	3	6	3	4	2	3	2	4	3	3	4	3	7	NO
472-05	MOROCCO	343		6 loci	2	5	1	5	3	3	2	3	3	4	3	3	5	3	6	NO
594-06	SPAIN	514		5 loci	2	5	4	5	3	3	2	3	3	4	3	3	5	2	4	NO
514-06	BOLIVIA	514		5 loci	2	5	3	6	3	3	3	3	3	4	3	3	4	2	6	NO
704-07	ROMANIA	528		SLV (1)	2	5	3	5	2	3	2	3	3	4	3	3	3	3	7	n.a.
528-06	ROMANIA	528		SLV (1)	2	5	3	5	3	3	2	3	3	4	3	3	3	3	7	n.a.
284-05	SPAIN	99			2	5	2	2	3	2	3	4	3	2	3	2	4	2	6	YES
372-05	SPAIN	99			2	5	2	2	3	2	3	4	3	2	3	2	4	2	6	YES
457-05	GHANA	99		DLV	2	4	2	2	3	2	3	1	3	2	3	2	4	2	6	NO
327-05	SENEGAL	327			2	5	3	5	3	3	2	3	3	4	5	1	5	2	7	YES
682-07	SENEGAL	327			2	5	3	5	3	3	2	3	3	4	5	1	5	2	7	YES
673-07	SENEGAL	327		SLV (2)	2	5	3	5	3	3	2	3	3	4	3	1	5	2	7	YES
348-05	ROMANIA	28-217			2	5	3	5	3	3	2	3	3	4	3	3	7	4	6	YES
663-07	ROMANIA	28-217			2	5	3	5	3	3	2	3	3	4	3	3	7	4	6	YES
406-05	ROMANIA	28-217			2	5	3	5	3	3	2	3	3	4	3	3	7	4	6	YES
390-05	ROMANIA	28-217		3 loci	2	5	3	5	3	3	2	3	3	4	3	3	2	3	5	n.a.

FIG. 1. Discrepant cases. (a) Isolates clustered by RFLP analysis but identified as orphan by MIRU-VNTR analysis. (b) Isolates clustered by MIRU-VNTR analysis but identified as orphan cases by RFLP analysis. Shown are patient codes, countries of origin, RFLP and MIRU-VNTR types and corresponding codes, degrees of discrepancy (specified as percent similarity for RFLP data and number of loci with variations for MIRU-VNTR data), presence (YES) or absence (NO) of epidemiological links, and unavailability of epidemiological data (n.a.). DLV, double-locus variation; SLV (1), SLV based on differences in one repetition; SLV (2), SLV based on differences in two repetitions.

identified as orphan by MIRU-VNTR analysis, and 25 cases were clustered by MIRU-VNTR analysis but identified as orphan by RFLP analysis (Fig. 1). Therefore, the correlation between the findings for the two methods was good (kappa, 0.7).

We analyzed the discrepant cases (Fig. 1) to check the degree of genotypic difference supporting them. We independently evaluated the following discrepancies: (i) identification as clustered by RFLP analysis but as orphan by MIRU-VNTR analysis and (ii) identification as clustered by MIRU-VNTR analysis but as orphan by RFLP analysis. Among the 16 cases grouped by RFLP analysis in nine clusters and identified as orphan by MIRU-VNTR analysis, we identified 6 as sharing high similarity with the other representatives of the corresponding RFLP cluster (showing single-locus variations [SLVs], three with differences in one repetition and three with

differences in two repetitions for a single locus) (Fig. 1a). For the remaining cases, MIRU-VNTR analysis identified marked differences with the representatives of the same RFLP cluster (variations in two to eight loci). On the other hand, for the 25 cases grouped by MIRU-VNTR analysis in 14 clusters but identified as orphan by RFLP analysis, we identified 11 cases as showing high similarity (from 91.2% to 99%) with the other representatives in the cluster (Fig. 1b). If these low-degree discrepancies were tolerated, the number of discrepant cases would pass from 41 to 24 and the percentage of discrepancies would therefore fall from 14.6% to 8.6%.

With the aim of evaluating whether the extended set of 24 loci could clarify the cases which were clustered by MIRU-15 analysis but identified as orphan by RFLP analysis, we reanalyzed these cases by applying the 9 loci that are not included in the MIRU-15 format. Identical genotypes were obtained by

b

Patient Code	COUNTRY of origin	MIRU-15 type code	MIRU-15 type															% similarity	RFLP type	EPID. LINKS
			580	2996	802	960	1644	3192	424	577	2165	2401	3690	4156	2163b	1955	4052			
392-05	SPAIN	15-1	2	5	5	4	3	2	3	2	2	1	2	2	2	3	6	100%		YES
405-05	SPAIN	15-1	2	5	5	4	3	2	3	2	2	1	2	2	2	3	6	100%		YES
563-06	SPAIN	15-1	2	5	5	4	3	2	3	2	2	1	2	2	2	3	6	100%		YES
661-07	SPAIN	15-1	2	5	5	4	3	2	3	2	2	1	2	2	2	3	6	100%		YES
395-05	ECUADOR	15-1	2	5	5	4	3	2	3	2	2	1	2	2	2	3	6	92,3%		YES
292-05	SPAIN	292-1	2	5	2	3	4	3	2	4	2	2	3	2	4	2	5	90%		NO
627-06	SPAIN	292-1	2	5	2	3	4	3	2	4	2	2	3	2	4	2	5	90%		NO
776-08	MOROCCO	292-1	2	5	2	3	4	3	2	4	2	2	3	2	4	2	5	76,4%		NO
294-05	ROMANIA	294-1	2	5	3	5	3	3	2	3	3	4	4	3	5	3	7	73,7%		n.a.
705-07	ROMANIA	294-1	2	5	3	5	3	3	2	3	3	4	4	3	5	3	7	73,7%		NO
322-05	SPAIN	30-1	2	5	2	2	4	3	2	4	2	2	4	2	3	2	5	100%		YES
549-06	SPAIN	30-1	2	5	2	2	4	3	2	4	2	2	4	2	3	2	5	100%		YES
655-07	SPAIN	30-1	2	5	2	2	4	3	2	4	2	2	4	2	3	2	5	100%		YES
679-07	SENEGAL	30-1	2	5	2	2	4	3	2	4	2	2	4	2	3	2	5	100%		YES
800-08	GHANA	30-1	2	5	2	2	4	3	2	4	2	2	4	2	3	2	5	95,3%		YES
440-05	SPAIN	30-1	2	5	2	2	4	3	2	4	2	2	4	2	3	2	5	91,2%		NO
534-06	SPAIN	308-1	2	5	3	5	3	3	2	3	3	4	3	3	5	2	6	94,7%		n.a.
308-05	MOROCCO	308-1	2	5	3	5	3	3	2	3	3	4	3	3	5	2	6	94,7%		n.a.
792-08	ROMANIA	326-1	2	5	4	3	1	3	2	4	3	2	5	2	3	2	5	76,2%		YES
326-05	ROMANIA	326-1	2	5	4	3	1	3	2	4	3	2	5	2	3	2	5	76,2%		n.a.
500-06	SPAIN	353-1	2	4	1	4	2	3	2	4	2	1	2	2	2	3	4	100%		YES
573-06	SPAIN	353-1	2	4	1	4	2	3	2	4	2	1	2	2	2	3	4	100%		YES
353-05	SPAIN	353-1	2	4	1	4	2	3	2	4	2	1	2	2	2	3	4	86,7%		n.a.
425-05	SPAIN	353-1	2	4	1	4	2	3	2	4	2	1	2	2	2	3	4	95,2%		YES
363-05	SPAIN	363-1	2	5	3	5	3	2	2	3	3	3	3	3	2	3	5	92,3%		n.a.
511-06	SPAIN	363-1	2	5	3	5	3	2	2	3	3	3	3	3	2	3	5	92,3%		NO
386-05	MOROCCO	386-1	2	5	3	4	3	3	4	4	1	2	1	2	3	3	7	96,3%		n.a.
447-05	MOROCCO	386-1	2	5	3	4	3	3	4	4	1	2	1	2	3	3	7	96,3%		NO
450-05	SPAIN	450-1	2	5	3	5	3	3	2	3	3	4	4	3	3	3	7	100%		YES
634-07	SPAIN	450-1	2	5	3	5	3	3	2	3	3	4	4	3	3	3	6/7	100%		NO
807-08	SPAIN	450-1	2	5	3	5	3	3	2	3	3	4	4	3	3	3	7	87%		YES
797-08	ROMANIA	459-1	2	5	3	5	3	3	2	3	3	4	3	3	6	3	6	100%		n.a.
786-08	ROMANIA	459-1	2	5	3	5	3	3	2	3	3	4	3	3	6	3	6	100%		n.a.
720-07	SWITZERLAND	459-1	2	5	3	5	3	3	2	3	3	4	3	3	6	3	6	75%		NO
678-07	ECUADOR	660-1	2	5	3	5	3	3	2	3	3	4	3	3	5	3	6	75%		NO
660-07	MOROCCO	660-1	2	5	3	5	3	3	2	3	3	4	3	3	5	3	6	75%		NO
625-06	MOROCCO	625-1	2	5	4	3	1	3	2	4	3	2	5	2	4	2	5	88,9%		NO
771-08	MOROCCO	625-1	2	5	4	3	1	3	2	4	3	2	5	2	4	2	5	88,9%		NO
682-07	SENEGAL	327-1	2	5	3	5	3	3	2	3	3	4	5	1	5	2	7	100%		YES
327-05	SENEGAL	327-1	2	5	3	5	3	3	2	3	3	4	5	1	5	2	7	100%		YES
413-05	SENEGAL	327-1	2	5	3	5	3	3	2	3	3	4	5	1	5	2	7	99%		YES

FIG. 1—Continued.

MIRU-24 analysis for the discrepant cases for all but 3 of the 14 MIRU-15-defined clusters with some cases split by RFLP analysis, and for these 3 clusters, only subtle differences were observed (SLVs involving MIRU20 and MIRU23 for clusters 294-1 and 450-1 and a double-locus variation involving MIRU23 and VNTR2347 for cluster 450).

We used the advanced survey system implemented in Almería (based on standardized interviews and nominal/photographic recognition between the clustered cases) to check the existence of epidemiological links between the clustered cases as defined by RFLP and MIRU-15 analyses. Of the 135 cases clustered by any of the techniques, the advanced survey was performed with 105 cases: 74 of these cases were clustered by both techniques, with epidemiological links found for 62 of these cases (83.8%); 13 cases were clustered by RFLP analysis only, with epidemiological links found for 4 cases (30.8%); and 18 cases were clustered by MIRU-VNTR analysis only, with links found for 7 of these cases (38.8%).

We then evaluated whether the links found for the cases clustered by only one of the techniques and identified as orphan by the other corresponded to those cases that could also be clustered if subtle genotypic differences were tolerated. The four cases with links in the group clustered by RFLP analysis and identified as orphan by MIRU-VNTR analysis corresponded to cases split by MIRU-VNTR analysis on the basis of differences in one or two loci, whereas most of the remaining cases without links were those split by differences in a higher number of MIRU-VNTR loci (three to eight loci) (Fig. 1a). With regard to the other discrepancies, i.e., cases clustered by MIRU-VNTR analysis but identified as orphan by RFLP analysis, most of the links corresponded to cases that were clustered if subtle differences in RFLP types were tolerated, whereas most of the cases without links differed more markedly in their RFLP types (Fig. 1b).

DISCUSSION

Molecular tools for genotyping *M. tuberculosis* are being sought to improve standard epidemiology. Different systems have been developed to integrate molecular and epidemiological data to achieve more-efficient control of TB (23, 26, 29, 36). Our group is involved in the integration of molecular genotyping of *M. tuberculosis* in schemes that could allow intervention. Almería, in southeastern Spain, is the province with the highest percentage of TB among immigrants in Spain. Most of these immigrants are dispersed throughout the region outside the main cities and are highly mobile and difficult to access. We activated an advanced system to study clustered cases. This system was based on active compilation of data supported by a newly developed computer application, GenContactTB, and on standardized interviews using nominal and photographic identification (26). One of the main limitations of this design was the time required for identification of clusters by RFLP analysis, making it difficult to reveal potential transmission chains before ending the contact tracing, that is, the period when intervention is most possible. In a previous report (3), we evaluated MIRU-15 in Almería and showed good response times, discriminatory power, and correlation with RFLP findings, although the analysis was somewhat limited due to its retrospective design and to the fact

that the evaluation was restricted to cases clustered by RFLP analysis. It did not allow us to evaluate MIRU-VNTR genotyping for cases identified as orphan by RFLP genotyping (that is, most of the cases), considering that 36.8% of the cases in Almería were clustered in that study. The present study offers a more solid evaluation of MIRU-15 in a real-life context, as it was long-term (39 months) and prospective for most of the period (data for 2005 were analyzed retrospectively to increase analytical power), the coverage was universal (i.e., all *M. tuberculosis* isolates were included in the study), and the discrepancies were evaluated in all senses (identification as orphan by RFLP analysis and as clustered by MIRU-VNTR analysis or vice versa).

As our intention was to evaluate MIRU-VNTR genotyping in an intervention, we first compared the response times for obtaining a fingerprint. MIRU-VNTR type was obtained within a 2-week period for 57.1% of the isolates and within a 3-week period for 87.5% of the isolates. As for RFLP analysis, the shortest time for obtaining a genotype was 5 weeks. The response times were measured from the moment the fresh subcultures were received from Almería in the analysis center (Hospital Gregorio Marañón) until the genotype was obtained. This enabled us to evaluate the response times in a real day-to-day context so that the impact of the growth of the cultures received could be included in the analysis. In this sense, the MIRU-VNTR method enabled the samples to be analyzed on arrival, whereas with RFLP analysis, an incubation period was necessary before DNA extraction or, in cases with poor and slow growth, new subcultures, in order to guarantee the amount of bacterial load required to obtain definitive results. MIRU-VNTR analysis enabled a fingerprint pattern to be obtained in more cases than did RFLP analysis (91% versus 86.6%).

The next step was to evaluate the distribution of cases identified as clustered or orphan by each of the techniques evaluated. MIRU-VNTR genotyping has been reported to identify as many clustered cases as RFLP genotyping (27), which led MIRU-VNTR-based genotyping to be chosen for the epidemiological survey in different institutions (5, 8). However, in our study, MIRU-VNTR genotyping clustered isolates in a higher proportion than did RFLP genotyping. These data alert one to the existence of geographic settings in which there are high levels of heterogeneity among circulating strains and the behavior of RFLP genotyping is not equivalent to that of MIRU-VNTR genotyping. The increase in immigration means that contexts with an expected genetic heterogeneity of circulating *M. tuberculosis* strains are likely to be more frequent.

The percentage of multinational or mixed clusters reveals the frequency of cross-transmission between nationalities or between the immigrant and autochthonous populations (10, 18, 24, 26). The distributions among autochthonous, immigrant uniactional, immigrant multinational, and mixed clusters were equivalent for RFLP and MIRU-VNTR analyses. It is interesting that, whereas MIRU-VNTR genotyping split 16 cases from 6 of 20 immigrant clusters and 3 of 9 mixed clusters, it did not split any case among the 13 autochthonous clusters. Although highly speculative, this notion could suggest greater genetic stability for *M. tuberculosis* strains involved in transmission chains that are homogeneous in hosts (autochthonous) than for other strains that are more heterogeneous (multina-

tional and mixed) and in which MIRU-VNTR variants are more frequently found. However, not all the homogenous-host clusters in our study behaved in the same way, and MIRU-VNTR analysis also split isolates from uninational immigrant clusters.

The most relevant aspect of this evaluation was the analysis of the correlation between both techniques. If we had intended to switch to a rapid intervention scheme and base our analysis on the MIRU-VNTR method, we would first need to have known whether the observations would have differed from those obtained if RFLP genotyping had remained the preferred tool. Although the correlation between the findings for the two techniques was good (κ , 0.7), we still consider relevant the fact that some cases (14.6%) offered different results, depending on whether RFLP or MIRU-VNTR genotyping was used. Both tendencies—RFLP clusters split by MIRU-VNTR analysis and vice versa—were found.

A detailed analysis of the discrepancies shows that a percentage could be tolerated if we relaxed the clustering criteria, albeit minimally. Some studies (7, 16) have shown that applying slightly relaxed criteria for defining clusters by RFLP analysis allows epidemiologically related cases to be imported. When we tolerated SLVs and compared the results from both techniques, identifying those isolates with more than 90% similarity in their RFLPs as clustered, the percentage of discrepancies fell from 14.6% to 8.6%. In addition, five of the remaining discrepancies involved a genotype belonging to the Haarlem lineage (identified with the code 28 in this study), which has been found to be markedly split by MIRU-VNTR analysis, suggesting that RFLP analysis is unable to offer enough discrimination to analyze with precision some of the isolates within this lineage (3, 11, 27, 30, 31, 34). If we did not count cases involving these Haarlem strains, the number of discrepancies would fall to as low as 6.8%.

As a final step in the analysis, we used the GenContacTB standardized interview scheme in Almería to obtain information about the percentage of clustered cases for which epidemiological links could be identified. The clusters identified by both RFLP and MIRU-VNTR analyses appeared solid, as epidemiological links were found for most of the cases involved, reaching values that were similar only to other schemes applying refined surveys (83.8%) (14, 15, 27, 34). However, the opposite was found for cases clustered by RFLP or MIRU-VNTR analysis alone. We failed to find epidemiological links for most of these cases. For the few cases in which links were found in discrepant clusters, they seemed to correlate mainly with those cases showing discrepancies due only to subtle genotypic differences. Nevertheless, when an RFLP cluster was clearly split by MIRU-VNTR analysis or vice versa, epidemiological links were generally not found.

MIRU-15 analysis seems to fulfill the requirements of real-time interventional schemes. The correlation with RFLP results was reasonable. It is still necessary to identify some genotypes, as occurred with some of the Haarlem isolates in this study, in which MIRU-VNTR findings differed from RFLP findings. If the highest precision possible is sought, uncertainties as to the assignation of clusters by MIRU-15 analysis should be confirmed by RFLP analysis (and vice versa), as indicated by the fact that only cases clustered by both approaches were well supported by epidemiological links. Fur-

ther studies in challenging settings, such as those involving theoretically high genotypic heterogeneity among circulating strains, would demonstrate the advantages and uncertainties of switching our traditional genotyping schemes to other schemes that are better adapted to new requirements.

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