Qualitative Analysis To Ascertain Genotypic Identity of or Differences between *Mycobacterium tuberculosis* Isolates in Laboratories with Limited Resources

Fernanda Sislema-Egas,a Maria Jesús Ruiz-Serrano,a,b Emilio Bouza,a,b,c Darío García-de-Viedmaa,b,d

Servicio de Microbiología y Enfermedades Infecciosas, Hospital General Universitario Gregorio Marañón, Instituto de Investigación Sanitaria Gregorio Marañón, Madrid, Spain; CIBER Enfermedades Respiratorias (CIBERES06/0058), Spain; Departamento de Medicina, Facultad de Medicina, Universidad Complutense de Madrid, Madrid, Spain; CEI Campus Moncloa, UCM-UPM, Madrid, Spain

*Mycobacterium tuberculosis* is currently genotyped using mycobacterial interspersed repetitive-unit–variable-number tandem-repeat (MIRU-VNTR) typing, although the high cost of this technique restricts its implementation in resource-limited settings. We designed a MIRU-VNTR format, MLP3 (MIRU-VNTR length polymorphism triplex), that is based on the qualitative comparison of 5 nonfluorescent 3-band fingerprints in conventional electrophoresis and minimizes costs and technical demands. MLP3 successfully resolved cross-contamination alerts, discriminated reinfections from reactivations, clarified suspected microepidemics, and tracked transmission events of high epidemiological interest.

In recent years, the preferred genotyping strategy for fingerprinting *Mycobacterium tuberculosis* isolates has switched from IS6110 restriction fragment length polymorphism analysis to a PCR-based method, mycobacterial interspersed repetitive-unit–variable-number tandem-repeat (MIRU-VNTR) typing, which is faster, requires a lower bacterial load, facilitates data interchange with other laboratories, and offers equivalent discriminatory power (1–3). The 2 available formats of MIRU-VNTR, based on the use of 15 or 24 loci (MIRU-15 and MIRU-24, respectively), are the most-suitable approaches for epidemiological purposes, although the 15-locus format offers sufficient discriminatory power in most settings (4, 5). A high-throughput adaptation of MIRU-VNTR has also been developed. It requires fluorescent primers, multiplexing, and allele calling based on sizing of the amplified fragments by capillary electrophoresis (6). None of these alternatives is suitable for low-resource laboratories: the standard approach requires a high number of PCRs and access to image capture systems and specific software before allelic values can be assigned, and while the high-throughput approach reduces the number of PCRs, it increases equipment and software costs (3).

Our objective in this study was to evaluate an easier and less-expensive format of MIRU-VNTR analysis aimed at performing the standard scheme (i.e., sizing the MIRU PCR products to assign a strain-specific numerical code on which comparisons are based) but at performing a qualitative analysis to ascertain identities or differences between isolates. Although this strategy is of little use for universal molecular epidemiology programs, it might be able to solve many of the practical problems often faced by clinical mycobacteriology laboratories, namely, resolution of laboratory cross-contamination alerts (7–9), discrimination between reactivation and exogenous reinfection (10), analysis of suspected microepidemics (11, 12), and tracking of the spread of specific high-risk strains (9, 13–15).

We designed and evaluated a new approach, which we call MLP3 (MIRU-VNTR length polymorphism triplex). MLP3 is based on nonfluorescent triplex PCRs and separation of the amplified products using conventional agarose electrophoresis. This approach considers the electrophoretic mobility of the 3-band pattern itself as a fingerprint, without the need to assign allelic numerical values. We evaluated an MLP3 scheme adapted to the 15-locus format (5 triplex reactions), which has reasonably high discriminatory power (3, 4) and lower costs than the extended 24-locus format (16).

First, we tried to minimize the probability of finding overlaps in mobility between some of the amplification products coamplified in each of the 5 triplex PCRs. This step was not necessary in the distribution of loci assigned in the standard protocol, because the use of fluorescent primers enables independent detection of each product by capillary electrophoresis. Therefore, based on the theoretical sizes for the different allelic values and considering which of them were expected to be more frequent after analyzing the MIRU types in our database (1,390 MIRU types from cases from 37 different nationalities), we chose a new distribution of loci in mixes 1, 2, and 5 (Table 1). This new combination increased the probability that each of the loci coamplified in the same mix

| TABLE 1 Distribution of loci in the standard MIRU-VNTR and MLP3 master mixes |
|-----------------|-----------------|-----------------|
| Mix             | Loci in:         | MLP3 mix         |
|                 | Standard MIRU-VNTR mixa                      | MLP3 mix         |
| 1               | MIRU 4, MIRU 26, MIRU 40                      | MIRU 4,5 MIRU 16 |
| 2               | MIRU 10, MIRU 16, MIRU 31                      | MIRU 4,5 MIRU 10, MIRU 31 |
| 3               | MIRU 42, MIRU 43, ETRA                        | MIRU 42, MIRU 43, ETRA |
| 4               | MIRU 47, MIRU 52, MIRU 53                      | MIRU 47, MIRU 52, MIRU 53 |
| 5               | QUB-11B, QUB26, 1955                          | QUB-11B, QUB26, MIRU 26 |

a Genomic positions for the corresponding loci can be checked in reference 17.

b The final concentration of primers was 400 nM.
would lead to amplification products within a range of nonoverlapping mobilities.

This new distribution required some adjustments: the concentration of primers was increased from 200 to 400 nM in certain loci (Table 1), and PuReTaq Ready-To-Go PCR beads (GE Healthcare, United Kingdom) were added to ensure optimal and balanced amplification of the locus-sharing reaction (Fig. 1). PCRs were performed following an initial denaturation at 95°C for 15 min and then 30 cycles of 94°C for 60 s, 60°C for 60 s, and 72°C for 90 s. To minimize costs, the template used was crude lysate (5 μl) from the heat-inactivated MGIT culture, without further DNA purification.

**FIG 1** Different events resolved by MLP3. The gels show the patterns for the problem samples grouped by the five triplex reactions analyzed. Each gel is representative of one of the different events which are commonly required to be analyzed by genotyping (laboratory cross-contamination, recurrences, microepidemics, and surveys of recent transmission events of epidemiological interest). The MIRU-VNTR type of the isolate(s) involved is indicated at the bottom of each gel. Here are shown examples of cross-contamination alert confirmed (a), cross contamination alert ruled out (b), reactivation (three sequential episodes) (c), reinfection (two first episodes) and subsequent reactivation (third episode) (d), alert of microepidemic confirmed to involve three cases (e), cluster involving the Gran Canaria (GC) Beijing strain (lanes 14 correspond to the reference GC strain, lanes 15 to a case proved to be involved in the transmission, and lanes 16 to a case in which infection by GC strain was ruled out) (f), and cluster involving a rapidly transmitted strain (lanes 17 correspond to the reference transmitted strain, lanes 18 to 21 to four cases involved in recent transmission by the same strain, and lanes 22 to a case in which infection by the same strain was ruled out) (g).
Second, we evaluated the usefulness of MLP3 for providing the solutions to common practical problems that clinicians and epidemiologists refer to a mycobacteriology laboratory. We selected examples of laboratory cross-contamination alerts, recurrences, suspected microepidemics, and surveys of recent transmission events of interest (13). These events corresponded to real requests that were made to our laboratory (most from our own institution and some from Complejo Hospitalario Torrecárdenas, Almería, Spain) during the period from 2003 to 2009 and resolved after assignment of a complete genotype by applying standard MIRU-24 typing. Our purpose here was to retrospectively evaluate whether those demands could also have been solved by MLP3 analysis—if it had been available—without requiring the refinement and costs associated with standard multiplex MIRU typing.

Of the 5 cross-contamination alerts analyzed, MLP3 correctly confirmed 2 (identical patterns between the isolates that had been managed simultaneously in the laboratory) and ruled out cross-contamination in the remaining 3, in which the differences observed between the MLP3 patterns indicated that both isolates were true positives (Fig. 1a and b).

The application of MLP3 analysis to the 9 recurrences (involving 6 cases with 2 episodes, 2 with 3, and 1 with 4) allowed us to discriminate between 5 reactivations caused by the same strain (Fig. 1c), 3 cases of exogenous reinfection by a strain other than that involved in the first episode, and 1 mixed event with 3 episodes in which a reinfection was followed by a reactivation (Fig. 1d). An overlap between 2 fragments in mix 2—seen in the gel in Fig. 1d as a band that was thicker than usual—was observed in 2 recurrences, although it did not hamper correct assignment of reactivation (both isolates shared the thick band) or reinfection (the number of differences in other loci was sufficient for assignment).

Regarding microepidemics, MLP3 confirmed the involvement of the same strain in 4 events, including 1 case with 2 patients, 2 with 3 patients (Fig. 1e), and 1 with 4 patients. In the remaining 2 suspected cases, the differences between the MLP3 patterns of the cases included ruled out a microepidemic.

Finally, we evaluated whether the technique could track the spread of strains whose epidemiological features justified specific surveillance because they were involved in extensive clusters that included a high number of cases (2 clusters) or in fast-growing clusters in which the number of related cases increased rapidly (2 clusters) or they involved the high-risk Beijing lineage (2 clusters) (13). Our scheme of analysis was to compare the MLP3 pattern for the problem case surveilled with that of the reference strain known to be involved in the cluster. In all clusters of epidemiological interest, MLP3 succeeded in discriminating between cases infected by the strain of interest and others infected by different strains (Fig. 1f and g).

Again, mix 2 led to an overlap of 2 amplified products, although it did not affect the correct assignment of transmission (Fig. 1g).

Conventional agarose gel electrophoresis of the products of 5 nonfluorescent triplex reactions carried out in the proposed MLP3 format can be implemented in low-resource settings. The decision to apply the 15-locus format was correct, as all the events that had been resolved by applying standard MIRU-24 were now resolved by interrogating only the 15 discriminatory loci. Therefore, we observed (i) no negative impact on the efficiency of our MIRU-15-based design to solve the problems analyzed and (ii) a positive impact due to a reduction in costs from performing only 5 triplex PCRs instead of the 8 multiplex reactions required in the 24-locus format. Equivalent efforts have recently been made to develop low-cost versions for MIRU typing (16, 18), although they used allelic sizing to fingerprint the M. tuberculosis isolate. Our strategy, on the other hand, proved its efficiency in solving key issues using only the triplex PCR pattern as a qualitative feature for each strain, without having to assign allelic values for the loci analyzed. Overlaps between 2 bands were observed in only 3 cases, all of which involved mix 2, and they did not interfere with the resolution of the problem in any of the 3 cases. In the few situations in which a single overlap occurred, a simplex PCR for the locus involved could be performed, if needed, without delaying the analysis.

In low-resource settings, MLP3 could be implemented as a first-line step in laboratories with minimal equipment and could provide a quick answer for practical issues. This approach must be complemented with analyses by a well-equipped national reference laboratory if universal genotyping with epidemiological purposes is required.

ACKNOWLEDGMENTS
F.S.-E. was supported by a fellowship from AECID and by a grant for International Cooperation Projects by Instituto de Investigación Sanitaria Gregorio Marañón (FIBHGM-PRMGDV-2013). The work was supported by Fondo de Investigaciones Sanitarias (FIS PI 509/02205, PI12/02800) and AECID (Ref A1/036088/11).

We thank Miguel Martínez Lirola from Complejo Hospitalario Torrecárdenas for allowing us to use some of his microbiological problems as material to evaluate the test. We are grateful to Thomas O’Boyle for proofreading the manuscript.

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