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Optimization of Standard 24-Locus Variable-Number Tandem-Repeat Typing of *Mycobacterium tuberculosis* Isolates: a Multicenter Perspective

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De Beer et al. (1) report that the percentages of instantly complete 24-locus variable-number tandem-repeat (VNTR) genotypes obtained in their hands rose from 72.3% when using the triplex-based Genoscreen kit until November 2011 to 84.7% afterwards using a modified version of our original triplex-based method (2).

In order to provide an independent multicenter assessment, seven national tuberculosis (TB) reference laboratories were contacted, representing typical permanent kit users in Europe and Asia. Their percentages of instantly complete genotypes routinely obtained when using the triplex-based kits until the beginning of 2012 are listed in Table 1, as reported to us. They averaged 84.5% with a total of 3,964 isolates evaluated, well above the kit-based percentage reported by de Beer et al. It is particularly noteworthy that none of the laboratories used column-based DNA purification, in contrast to de Beer et al. Their DNA isolation procedures included just crude DNA extraction after heat inactivation ($n = 6$) or DNA preparation as for restriction fragment length polymorphism (RFLP) typing ($n = 1$), fully compatible with the kit and our original protocols (2, 3).

The triplex-based kit was replaced by a 4-plex version 2 years ago. The associated technical optimization is obvious: a gain of 25%, considering the numbers of multiplex PCRs and of DNA analyzer capillaries per isolate (i.e., 6 versus 8), consumables utilized, and hands-on time for DNA amplification and fragment analysis. The 4-plex-based success rates at the first PCR round averaged 85.8% with a total of 3,076 isolates evaluated by the 7 laboratories, again well above the kit-based results reported by de Beer et al. (Table 1).

De Beer et al.'s lower performances, despite the use of highest-purity DNA, mostly suggest the particular impact of accessory issues in their initial study period, rather than subsequent optimization. The variable influence of such factors is not revealed by their crude comparison of success rates at the first PCR attempt, even with statistical analysis, especially since this comparison was not done in parallel and does not consider distinct situations.

For instance, when the alleles of a complete multiplex are missing at the first PCR round for a sample, a second analysis round performed under the same conditions most often results in a complete result (obviously excluding problematic DNA preparations) (see Fig. S1A in the supplemental material). Such a situation basically reflects occasional PCR-independent problems related, e.g., to (multi)pipetting, capillary electrophoresis, or organizational aspects inherent in large-scale analyses. In contrast, the analyses of single, truly missing alleles, identified by using the other amplicons of the multiplex as internal controls to exclude independent technical problems (Fig. S1B), are more meaningful. Such results, e.g., for locus 2163b and/or the physically close marker 2165b (part of a distinct multiplex) (Fig. S1B), suggest primarily the loss of the corresponding genomic region, known to be vulnerable to deletion (4). Missing alleles in one or both of these loci represent the most frequent cases, representing about 30% of the truly incomplete genotypes among the users on average. Analysis of a genetic tree based on a sample data set including genotypes from 442 isolates from different genetic lineages shows that isolates with such missing alleles tend to be grouped, as expected for genomic deletions in recent common ancestors with vertical transmission to the respective derived clones (Fig. S2). We used whole-genome sequence analysis to demonstrate the occurrence of such suspected genomic deletions, which happened for instance in a *Mycobacterium bovis* BCG Pasteur-derived isolate (Fig. S3). Of course, the change in PCR primer sequences for locus 2163b made by De Beer et al. has no impact in such cases, and trying to force amplification might then just favor a falsely positive result.

Along the same lines, the change introduced in the primer sequence of locus 4052, mostly to avoid single nucleotide polymorphisms (SNPs) specific to the target region of exceptional *Mycobacterium canettii* isolates (5), has probably little-to-no significant impact beyond the typing of these very rare strains. In addition, insertion of an insertion sequence (IS) element can also occur in certain markers, obstructing amplification and allelic determination regardless of PCR conditions (2). Finally, competition between multiplexed markers can be specifically suspected only under certain conditions, typically when a ladder of so-called stutter peaks is observed without detection of a clear stronger peak or band representing the actual allele at the extremity of the ladder (see, e.g., page 61 of the mycobacterial interspersed repetitive unit [MIRU]-VNTR typing manual freely accessible under Background/Protocols at www.miru-vntrplus.org [6], or as explained in MIRU-VNTR typing kit manuals from Genoscreen). However, on the whole, such situations of truly missing alleles represent at most only a small percentage of the cases encountered by the typical users listed in Table 1.

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TABLE 1 Percentages of instantly complete 24-locus VNTR types obtained by using Genoscreen typing kits for routine typing activities

Location of TB reference center	% of instantly complete 24-locus MIRU-VNTR types (no. of isolates evaluated[a]) by the:	
	Triplex-based typing kit	Quadruplex-based typing kit
Stockholm, Sweden	94.9 (512)	92.2 (266)
Golnik, Slovenia	88.5 (887)	81.1 (249)
Brussels, Belgium	80.5 (619)	85.1 (644)
Dublin, Ireland	76.8 (500)	76.8 (500)
Copenhagen, Denmark	73.0 (446)	84.2 (417)
Singapore	83.6 (500)	85.2 (500)
Paris, France	94.0 (500)	95.8 (500)
Avg (total no. of isolates)	84.5 (3,964)	85.8 (3,076)

[a] Excluding positive controls.

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