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Evaluation of Spoligotyping in a Study of the Transmission of *Mycobacterium tuberculosis*

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Spoligotyping (for spacer oligotyping) is an easy, economical, and rapid way of typing *Mycobacterium tuberculosis* complex strains with the DR spacer markers (J. Kamerbeek et al., J. Clin. Microbiol. 35:907–914, 1997; D. van Soolingen et al., 33:3234–3248, 1995). The stability of the markers was demonstrated by showing that all the *Mycobacterium bovis* BCG strains tested gave the same spoligotyping pattern. None of the 42 atypical mycobacterial strains tested gave a spoligotyping signal, indicating the specificity of the technique for *M. tuberculosis* complex. The utility of the spoligotyping method was demonstrated by analyzing 106 isolates of *M. tuberculosis* obtained over 1 year in three Paris hospitals. The results obtained by this technique were compared to those obtained by Torrea et al. (G. Torrea et al., J. Clin. Microbiol. 34:1043–1049, 1996) by IS*6110*-based restriction fragment length polymorphism (RFLP) analysis. Strains from patients with epidemiological relationships that were in the same IS*6110*-RFLP cluster were also in the same spoligotyping group. Spoligotyping was more discriminative than RFLP analysis for strains with one or two copies of IS*6110*. RFLP analysis did not discriminate between the nine strains with one or two IS*6110* bands with no known epidemiological relation, whereas spoligotyping distinguished between eight different types. IS*6110*-RFLP analysis split some of the spoligotyping clusters, particularly when the IS*6110* copy number was high. Therefore, we propose a strategy for typing *M. tuberculosis* strains in which both markers are used.

Tuberculosis remains a major worldwide health problem, with approximately 3 million cases recorded each year, although the World Health Organization suggests that more than 8 million cases may arise annually worldwide (1). An estimated 88 million new cases of tuberculosis, of which 8 million will be associated with human immunodeficiency virus infection, will occur in the world during the 1990s; 30 million people are predicted to die of tuberculosis during this period (3).

The discovery of active antituberculosis antibiotics, starting with streptomycin in 1944 to rifampin in 1965, raised hopes of being able to eradicate this disease. Although efficacy is evident for individual cases, their efficiency at the community level has been disappointing. Because of the socioeconomic difficulties in many parts of the world, many patients are not able to follow their treatment. In the worst cases, this results in the selection of drug-resistant strains. The emergence of AIDS has further complicated this situation. The increasing rate of new cases of tuberculosis around the world appears to be a consequence of these factors. New strategies to fight tuberculosis are urgently needed, and a better understanding of the dynamics of transmission of the bacilli would help define the epidemiological group where the disease must be fought. The identification of outbreaks and tracing the transmission of particular strains of *Mycobacterium tuberculosis* are also important in the control of tuberculosis.

Since the discovery of polymorphic DNA in *M. tuberculosis*, strain differentiation has become a valuable tool in the study of the epidemiology of tuberculosis (2).

Various genetic elements contribute to DNA polymorphism in *M. tuberculosis*, and of these genetic elements the insertion sequence IS*6110* (16) has been studied most intensively (13, 15, 17). IS*6110* is a transposable element. Its value is that it can be used to differentiate between almost all epidemiologically unrelated *M. tuberculosis* strains (4, 20), and thus can be used to suggest previously unsuspected epidemiological links or confirm the identities of strains coming from patients with known epidemiological links.

The classical way of studying this marker is by the restriction fragment length polymorphism (RFLP) method (19). Unfortunately, the RFLP procedure requires growing the organism, extraction of genomic DNA, and digestion of the DNA with a restriction enzyme for Southern blot analysis. This method is useful for retrospective epidemiological studies, but because of the time needed to obtain the results, it has limitations concerning disease management and rapid response to outbreaks. It is also technically too demanding to be easily used in studies of large cohorts.

Other techniques have been developed with the aim of obtaining a reliable identification of strains without the need for large amounts of intact, pure genomic DNA. Various PCR approaches have been described. For example, Plikaytis et al. (14) described an amplification method used to measure the variable distances between IS*6110* elements and copies of a major polymorphic tandem repeat sequence of *M. tuberculosis*.

A technique called spoligotyping (for spacer oligotyping) was recently described (7, 21). Spoligotyping analyzes a single locus to characterize tuberculosis strains. The method relies on the in vitro amplification of the DNA sequence of the highly polymorphic direct repeat (DR) locus in the chromosome of

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the mycobacteria of the *M. tuberculosis* complex (7, 21). This region was described as flanking an IS*6110* sequence (4, 11). It has a characteristic organization, with conserved 36-bp DR sequences interspersed with variable spacers. The polymorphism is carried by these spacers, which are variable in length (35 to 41) (5) and sequence. The number of DR spacers is also variable (49 in BCG and 39 in *M. tuberculosis* H37Rv). Although these spacers vary, each is common to a group of strains. This technique tests which spacers from a spacer catalog are present in the strains studied and allows each strain to be characterized by its spacers. The study described in this report compared spoligotyping and IS*6110*-RFLP typing.

MATERIALS AND METHODS

Patients and strains. The spoligotyping technique and IS*6110*-RFLP analysis were compared by using 106 clinical *M. tuberculosis* isolates collected during 1993 by the bacteriological laboratories of three Paris hospitals. These strains have been described previously (18). Mycobacteria were kept on Löwenstein-Jensen medium at 4°C or in Youmans solution at −20°C.

Seven *M. bovis* BCG strains were tested: Montreal, Russian, Japanese 172, Prague, Glaxo 1077, Danish 1331, and the "Pasteur BCG" 1173P2 strain.

Forty-two atypical mycobacterial strains were tested by spoligotyping: *M. agri*, *M. alvei*, *M. asiaticum*, *M. aurum*, *M. austroafricanum*, *M. avium*, *M. celatum*, *M. chelonae*, *M. chitae*, *M. confluentis*, *M. diernhoferi*, *M. farcinogenes*, *M. flavescens*, *M. fortuitum*, *M. gastri*, *M. gordonae*, *M. flavescens*, *M. haemophilum*, *M. intracellulare*, *M. kansasii*, *M. komossense*, *M. malmoense*, *M. marinum*, *M. moriokalense*, *M. nonchromogenicum*, *M. paratuberculosis*, *M. porcinum*, *M. poriferae*, *M. pulveris*, *M. rhodesiae*, *M. scrofulaceum*, *M. senegalense*, *M. shimodei*, *M. simiae*, *M. smegmatis*, *M. sphagni*, *M. szulgai*, *M. terrae*, *M. tokaiense*, *M. triviale*, *M. vaccae*, and *M. xenopi.*

Spoligotyping. The spoligotyping method relies on the in vitro amplification of the DNA of the highly polymorphic DR locus in the *M. tuberculosis* chromosome (7, 21). The DNA in supernatants obtained from strains diluted in TE (Tris-EDTA) and warmed at 80°C for 20 min was used for amplification. Two microliters of each sample was suspended in a final volume of 50 μl containing 7 mM $MgCl_2$; 0.4 mM (each) dATP, dGTP, and dCTP, dTTP (Boehringer Mannheim GmbH, Mannheim, Germany); 67 mM Tris-HCl (pH 8); 16.6 mM $(NH_4)_2SO_4$; 0.75 mM EGTA; 5% glycerol; 0.02% Tween 20; 0.5 U of *Tth* DNA polymerase (Eurobio, Les Ulis, France); and 5 pmol of each primer. The primers were DRa (5′-CCG AGA GGG GAC GGA AAC-3′) and Drb (5′-GGT TTT GGG TCT GAC GAC-3′). Primer Drb was biotinylated at the 5′ end. The cycling temperatures were 3 min at 96°C and then 1 min at 96°C, 1 min at 55°C, and 30 s at 72°C for 30 cycles. The amplified DNA was tested for hybridization with a set of 43 oligonucleotides derived from the spacer sequences of *M. tuberculosis* H37Rv and *M. bovis* BCG P3 (the GenBank accession no. for the sequence of *M. tuberculosis* H37Rv is Z48304, and that for *M. bovis* BCG P3 is X57835). The multiple synthetic spacer oligonucleotides were covalently bound to a nylon membrane (Biotrans; ICN), in parallel lines (8). Hybridization was performed in a 45-lane blotter (Miniblotter 45; Immunetics, Cambridge, Mass.) by using 20 μl of the PCR products in 150 μl of 2× SSPE (1× SSPE is 0.15 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.4]–0.1% sodium dodecyl sulfate at 60°C for 1 h. The membrane was washed twice in 250 ml of 2× SSPE–0.5% sodium dodecyl sulfate for 10 min at 60°C. Bound fragments were revealed by chemiluminescence after incubation with horseradish peroxidase-labeled streptavidin (Boehringer Mannheim). The membrane was incubated for 2 min in 30 ml of ECL detection liquid and then exposed to ECL hyperfilm (Amersham).

Computer-assisted analysis of fingerprints. Gel Compar software (Applied Maths, Kortrijk, Belgium) was used to compare hybridization patterns. Each positive spot was defined as a band. Then, the software clustered strains with the same spoligotyping patterns. The software also recorded the picture of the typical pattern for each strain. The strain patterns and clustering results were checked by direct visual inspection. A transmission scanner (150 dots per in.) was used to record the autoradiographic images.

RESULTS AND DISCUSSION

Spoligotyping and IS*6110*-RFLP techniques were compared by using 106 clinical *M. tuberculosis* isolates collected over 1 year (1993) by the bacteriological laboratories of three Paris hospitals. The published RFLPs and inter-IS*6110* PCR data (called also rep-PCR) were used (18).

Each patient who provided an isolate with a fingerprint identical to that of another isolate in the study underwent rigorous epidemiological investigation. The 106 isolates studied were from 101 patients (95% of the isolates and 96% of the patients described by Torrea et al. [18]). A total of 57 different spoligotypes were obtained (Fig. 1), confirming the high degree of diversity of the markers and suggesting that the DR spacer markers in the strains from Paris are sufficiently polymorphic to type strains by spoligotyping. Twenty spoligotypes contained more than one isolate: 10 spoligotypes of 2 isolates each, 3 of 3 isolates each, 2 of 4 isolates each, 2 of 5 isolates each, 2 of 6 isolates each, and 1 of 10 isolates.

A group of nine isolates from four patients with relapses or from different anatomical sites on the same patient (isolates 107 and 108, 58 and 59, 62 to 64, and 72 and 73) were spoligotyped. There was no difference between the spoligotyping patterns for the different isolates from a given patient.

The eight groups of isolates with identical IS*6110*-RFLP profiles, designated by the letters A to H in the previous study (18), were also clustered by spoligotyping (we use the word "cluster" to indicate that strains share the same pattern, as determined by the marker and the technique used).

The two patients giving strains of group A were relatives. The isolates had only two copies of IS*6110* and identical spoligotyping patterns.

The IS*6110*-RFLP group B isolates (from two relatives) also had the same spoligotype (along with four other strains with a different IS*6110*-RFLP fingerprint).

Group C isolates, from three patients who were friends and seropositive intravenous drug users (two of them were living in the same house), were all of the same spoligotype.

IS*6110*-RFLP clusters D and E (excluding strain 104, which was not spoligotyped), both from laboratory cross-contamination, gave a single spoligotype that was the same as that for six other strains that had different IS*6110*-RFLP patterns; this was the largest spoligotype in this study. These six strains and strains in clusters D and E clearly have different IS*6110*-RFLP patterns, even if some of them shared many bands.

The strains in the last three clusters, IS*6110*-RFLP clusters F, G, and H, were similarly grouped by spoligotyping (with a fourth strain placed in the F cluster by spoligotyping). These results confirm the identities of genetic markers of the strains in each group, even though no clear epidemiological relation was found between the patients. These results agree with those obtained for other markers (DR-RFLP and polymorphic GC-rich-repetitive sequence RFLP) by Torrea et al. (18).

The last IS*6110*-RFLP cluster contained nine strains with one or two bands (one of the two bands had the same length as the single band of the seven strains with one IS*6110* copy). Strains with only a few IS*6110* copies cannot be satisfactorily distinguished by RFLP analysis. Spoligotyping split this group into eight different spoligotypes, one of which included two isolates (strain 86 with one copy of IS*6110* and strain 77 with two copies of IS*6110*). Thus, IS*6110*-RFLP analysis failed to discriminate between nine strains with one or two IS*6110* bands, whereas spoligotyping clearly distinguished between eight different types.

Overall, 6 clusters were the same by spoligotyping and IS*6110*-RFLP analysis, and 12 spoligotyping clusters were split by the IS*6110* marker (5 spoligotyping clusters had 2 strains, 2 had 3 strains, 3 had 4 strains, 2 had 5 strains, 1 had 6 strains, and the largest cluster had 10 strains). In contrast, one IS*6110*-RFLP cluster, with few copies of IS*6110* (two or fewer in this case), gave several distinct spoligotyping patterns (despite many spacers being common). Other than for strains with a low IS*6110* copy number, spoligotyping never split IS*6110*-RFLP groups. The clinical epidemiological data confirmed the results, demonstrating the relevance of this approach to typing.

The extensive polymorphism of the spoligotyping patterns suggests that the spacers might be unstable, and spoligotyping

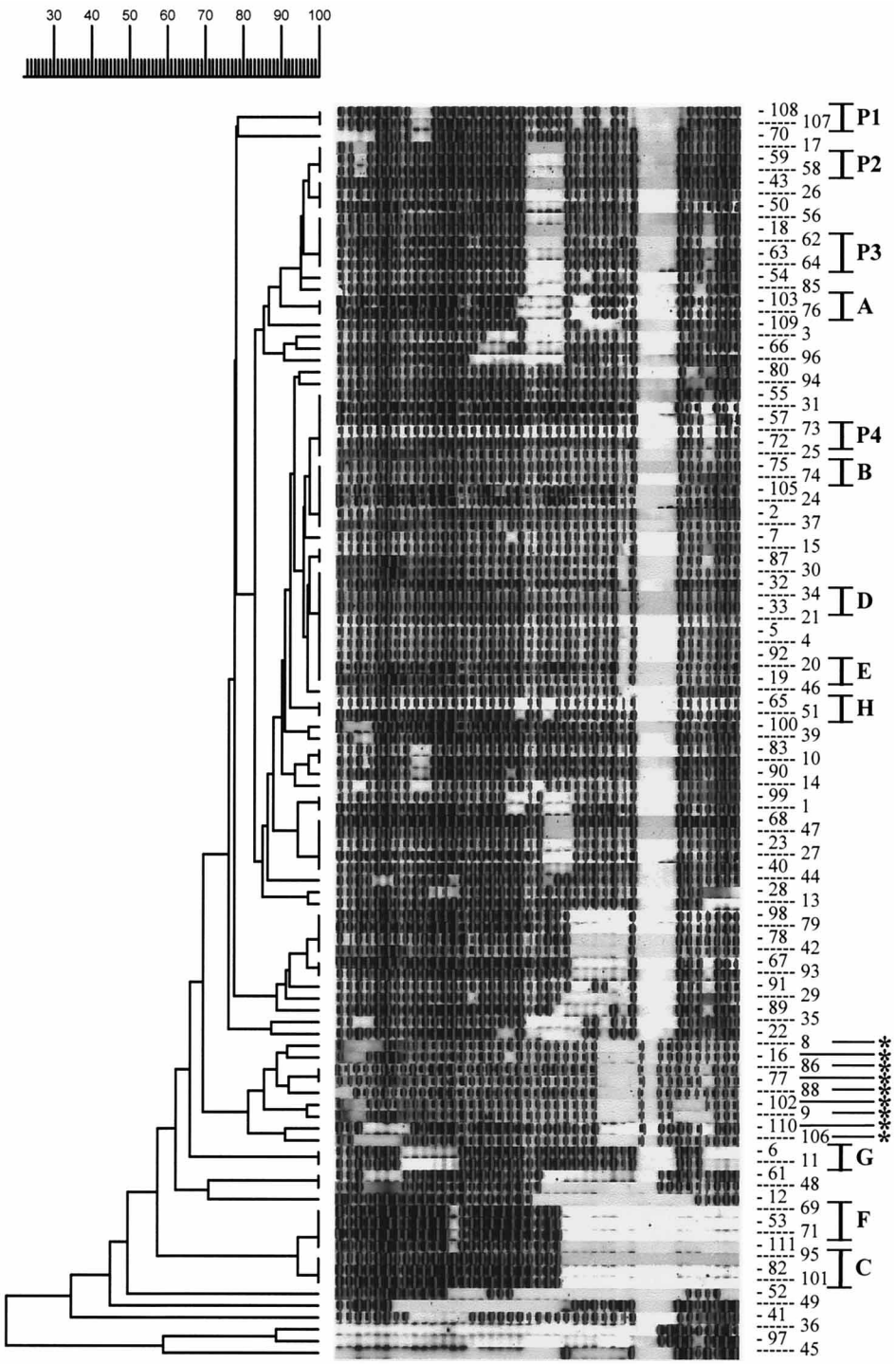

FIG. 1. Spoligotype dendrogram generated by 106 *M. tuberculosis* strains from three Paris hospitals and corresponding patterns after computer analysis. The two right sets of numbers correspond to isolates from tuberculous patients. The patterns obtained after computer processing with the GelCompar software are presented before these numbers. The letters correspond to the clusters described in the text. Asterisks indicate strains with one or two IS*6110* bands and clustered by IS*6110*-RFLP analysis. The letter P indicates the nine isolates obtained from four patients (arbitrarily named P1 to P4) with relapses or from different anatomical sites of the same patient. The scale on the left indicates the band-based similarity coefficients as described by Jaccard (6) [$N_{ab}/(N_a + N_b - N_{ab})$, where N_{ab} is the number of bands common to strains *a* and *b*, N_a is the total number of bands in strain *a*, and N_b is the total number of bands in strain *b*].

is based on a region with many repeats that could lead to genomic rearrangements. However, our results demonstrate that the DR spacer markers are stable enough for spoligotyping to be used as a tool for molecular epidemiology.

Multiple isolates from the same patient (nine strains from four patients) corresponding to relapses or infections at different sites gave the same spoligotyping pattern in all cases.

The higher number of isolates which were clustered by spo-

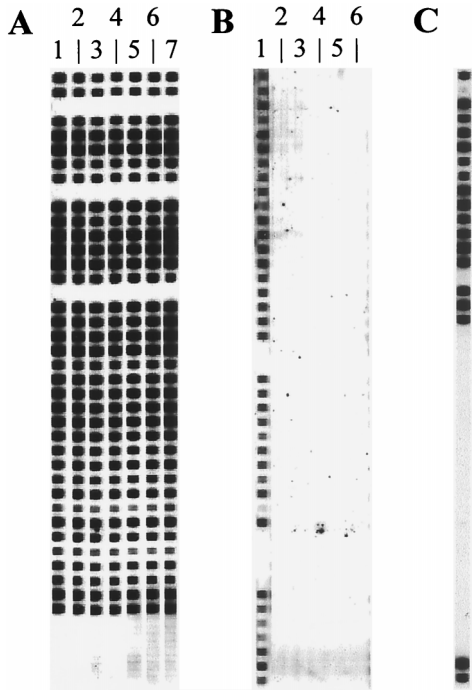

FIG. 2. Examples of spoligotypes. (A) Spoligotypes of seven BCG strains: Montreal (lane 1), Russian (lane 2), Japanese 172 (lane 3), Pasteur 1173P2 (lane 4), Prague (lane 5), Glaxo 1077 (lane 6), and Danish 1331 (lane 7). (B) Example of results obtained with atypical mycobacterial strains compared with the spoligotype obtained for reference strain H37Rv on the same day. Lanes: 1, H37Rv; 2, *M. scrofulaceum*; 3, *M. szulgai*; 4, *M. flavescens*; 5, *M. gordonae*; and 6, *M. terrae*. (C) Strain from Asia without IS*6110*.

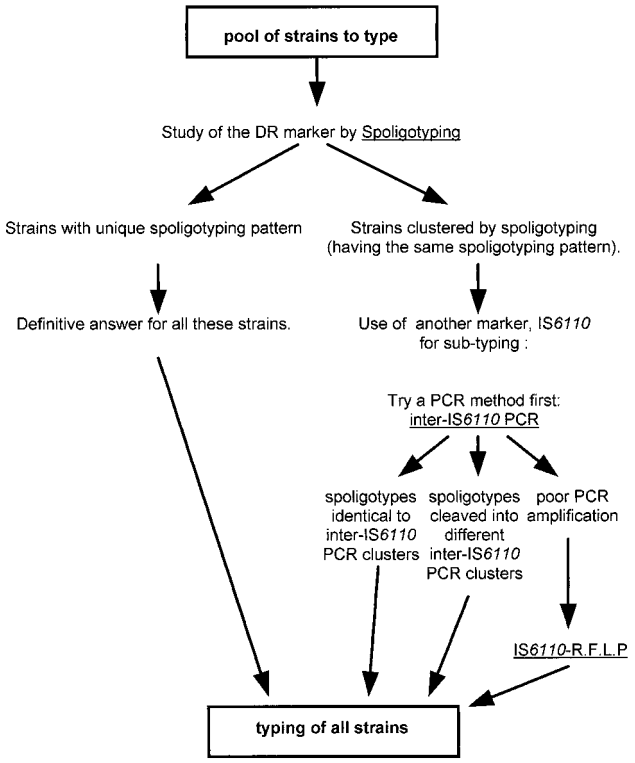

FIG. 3. Strategy used to type a large number of *M. tuberculosis* complex strains. The spoligotype is determined for each isolate in the undifferentiated pool of strains. Spoligotyping clusters and differentiated strains are then obtained. The spoligotyping clusters are checked, in a second step, with the IS*6110* marker by a PCR technique. As a last step, IS*6110*-RFLP analysis could be used for confirmation of the results (for instance, if some inter-IS*6110* PCR did not give an amplification product), but with a small number of strains.

ligotyping than by IS*6110*-RFLP analysis (57 spoligotypes against 81 IS*6110*-RFLP patterns) suggests that the DR spacer markers may evolve slightly more slowly. The diversities of the two markers, however, are of the same magnitude. Even though the two markers are independent, strains of the same spoligotype that could be differentiated by IS*6110*-RFLP analysis had similar RFLP patterns.

The stability of the DR spacer markers was checked by studying the spoligotyping patterns of the BCG daughter strains used in different countries. Seven BCG strains were tested (Fig. 2A). The precautions taken since the 1960s to avoid mutation are likely to have kept the strains almost unchanged. Before this date, the conservation techniques allowed some variation in the BCG strains from one country to another (9, 12). Analysis of the genomes of the different BCG daughter strains by pulsed-field gel electrophoresis showed major differences (22). The spoligotyping patterns obtained from the seven different BCG strains were exactly the same.

The spoligotyping method is suitable for initial screening. It is an easy, economical, and rapid way of typing *M. tuberculosis* complex strains since one person can test about 40 strains in a day. Furthermore, because it is a PCR technique, only a very small amount of DNA is required, avoiding the problems associated with the systematic slow growth of these bacteria. This point is important and simplifies experiments and laboratory organization. In addition, the speed of the method is very valuable when a rapid answer is needed, such as in an outbreak. This also allows comparison of stored strains that are not able to grow again (for example, in the case of relapses, when we need to compare a new strain from a patient to an old one which is no longer viable).

The DR region has been described as flanking an IS*6110* sequence (5, 11). However, our study of three isolates with no copy of IS*6110* shows that even these strains can be studied by spoligotyping (Fig. 2C). The DR region that had been investigated is present in the genomes of all *M. tuberculosis* strains. This sequence is thus a potentially valuable target for detecting *M. tuberculosis* in clinical specimens. Moreover, amplification products generated by amplifying the DR region can be used to type the detected mycobacterial strains (10). This technique could permit the rapid identification of community outbreaks, nosocomial infections, or laboratory cross contamination.

We also tested 42 atypical mycobacterial strains by spoligotyping (the results for some of these are presented in Fig. 2B). None of these strains gave an amplification product detectable by hybridization with the 43 spacers tested. The DR spacer markers seem to be specific to *M. tuberculosis* complex strains. It was found in all the *M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti* strains tested (data not shown). These markers are present in multiple copies and are thus valuable targets for the detection, identification, and typing of *M. tuberculosis* complex strains.

This report demonstrates the usefulness of the spoligotyping method. IS*6110*-RFLP analysis split some of the spoligotyping clusters defined in a study of 106 isolates obtained from three Parisian hospitals in 1 year. We therefore propose a strategy for typing *M. tuberculosis* strains in which both markers are used (Fig. 3). Spoligotyping could be performed first and will give a definitive answer for many isolates with a unique spoli-

gotyping pattern. The other isolates will be grouped into spoligotyping clusters. Each of these clusters can then be analyzed by IS*6110* typing. Because of the advantages of the amplification technology, we suggest that another PCR method be used for the small number of strains clustered by spoligotyping, such as the inter-IS*6110* PCR method used by Torrea et al. (18). This inter-IS*6110* PCR could confirm the clusters or discriminate between the isolates that they contain. If needed, for instance, if some inter-IS*6110* PCR did not give any amplification product, IS*6110*-RFLP analysis could be used for confirmation, but this time as a last step, with a small number of strains.

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