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Molecular Characterization of *Mycobacterium tuberculosis* Strains with TB-SPRINT

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Abstract. We evaluated Tuberculosis-Spoligo-Rifampicin-Isoniazid Typing (TB-SPRINT), a microbead-based method for spoligotyping and detection of rifampicin and isoniazid resistance in *Mycobacterium tuberculosis*. For that, 67 *M. tuberculosis* complex strains were retrospectively selected. Membrane-based spoligotyping, restriction fragment length polymorphism, DNA sequencing/pyrosequencing of *rpoB*, *katG*, and *inhA* promoter, TB-SPRINT, and SNP typing were performed. Concordance between spoligotyping methods was 99.6% (2,785/2,795 spoligotype data points). For most of the discordant cases, the same lineage was assigned with both methods. Concordance between phenotypic drug susceptibility testing and TB-SPRINT for detecting rifampicin and isoniazid resistance was 98.4% (63/64) and 93.8% (60/64), respectively. Concordance between DNA sequencing/pyrosequencing and TB-SPRINT for detecting mutations in *rpoB*, *katG*, and *inhA* were 98.4% (60/61), 100% (64/64), and 96.9% (62/64), respectively. In conclusion, TB-SPRINT is a rapid and easy-to-perform assay for genotyping and detecting drug resistance in a single tube; therefore, it may be a useful tool to improve epidemiological surveillance.

During the last decades, emergence and spread of drug-resistant strains of *Mycobacterium tuberculosis* have posed challenges to tuberculosis (TB) control.[1] Molecular genotyping methods have been used to detect transmission chains and outbreaks for local TB control, and to study the genetic diversity of the *M. tuberculosis* strains disseminated worldwide.[2] A reference method for *M. tuberculosis* genotyping has been IS*6110*-restriction fragment length polymorphism (RFLP), which presents a high discriminatory power but is slow and laborious.[3] Another widely used method is spoligotyping (*spacer oligonucleotide* typing), which is based on a polymerase chain reaction (PCR) amplification of the clustered regularly interspaced short palindromic repeats (CRISPR) locus and detection of the presence of different spacers between the repeats by reverse hybridization on membrane.[4] Another strategy for TB control is the rapid detection of drug resistance to implement an adequate treatment.[1] Because of the slow growth rate of *M. tuberculosis*, phenotypic drug susceptibility testing (DST) takes several weeks, and different molecular methods have been developed for rapid detection of mutations associated with drug resistance.[5] The objective of this study was to evaluate a molecular method based on multiplex PCR and hybridization on microbeads for simultaneous spoligotyping and detection of mutations associated with rifampicin (RIF) and isoniazid (INH) resistance, termed Tuberculosis-Spoligo-Rifampicin-Isoniazid Typing (TB-SPRINT, Beamedex SAS, Orsay, France).[6]

A total of 67 *M. tuberculosis* complex strains isolated in Spain were retrospectively selected from the collection in Instituto de Investigación Sanitaria de Aragón, Zaragoza, Spain. The strains were selected including different lineages with diverse IS*6110*-RFLP patterns. DST was performed with VersaTREK Myco Susceptibility Kit (Trek Diagnostics, Cleveland, OH) or Bactec MGIT960 (Becton Dickinson, Sparks, MD). Critical concentrations used were 1 μg/mL for RIF, and 0.4 μg/mL and 0.1 μg/mL for INH.[7] Genomic DNA was extracted from strains cultured on solid medium following the cetyltrimethylammonium bromide protocol. Conventional spoligotyping on membrane and IS*6110*-RFLP were performed as described previously.[3,8] The spoligotyping method allowed analysis of 22 DNA samples in approximately 5 hours, whereas with IS*6110*-RFLP 16 samples could be analyzed in 3 days. DNA sequencing for detecting mutations in *rpoB*, and pyrosequencing for detecting mutations in *katG* codon 315 and *inhA* promoter were performed as previously described.[9,10]

Strains were blindly analyzed with the TB-SPRINT assay (Beamedex SAS, Orsay, France; www.beamedex.com).[6] Briefly, the CRISPR region, *rpoB*, *katG*, and the promoter region of *inhA* were simultaneously amplified by PCR using dual-priming oligonucleotide primers. Subsequently, PCR product was hybridized to oligonucleotide-precoupled microbeads, and detection was performed either with the flow cytometry-based Luminex 200 system (Luminex Corp, Austin, TX) and XPONENT software for LX100/LX200 (version 3.1.871.0), or BioPlex200 (Biorad, Hercules, CA) running under Bio-Plex Manager 5.0. Raw TB-SPRINT results regarding relative fluorescence intensity values for each probe were interpreted as previously described.[6,11] Numerical data were uploaded to BioNumerics version 6.1 software (Applied Maths, Sint-Martens-Latem, Belgium). The TB-SPRINT method allowed analysis of 96 DNA samples in approximately 2.5 hours. Spoligotyping patterns obtained with either membrane-spoligotyping or TB-SPRINT were compared with those in the International Spoligotyping Database (SITVITWEB) of the Pasteur Institute of Guadeloupe (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/), and Spoligotyping International Types (SITs) were assigned.

Strains were also subjected to typing of seven lineage-specific SNPs (SNP typing) with multiplex PCR, microbead-based hybridization, and detection with the Luminex 200 system as previously described.[12]

Genotyping results obtained for the 67 *M. tuberculosis* complex strains included in this study are shown in Figure 1.

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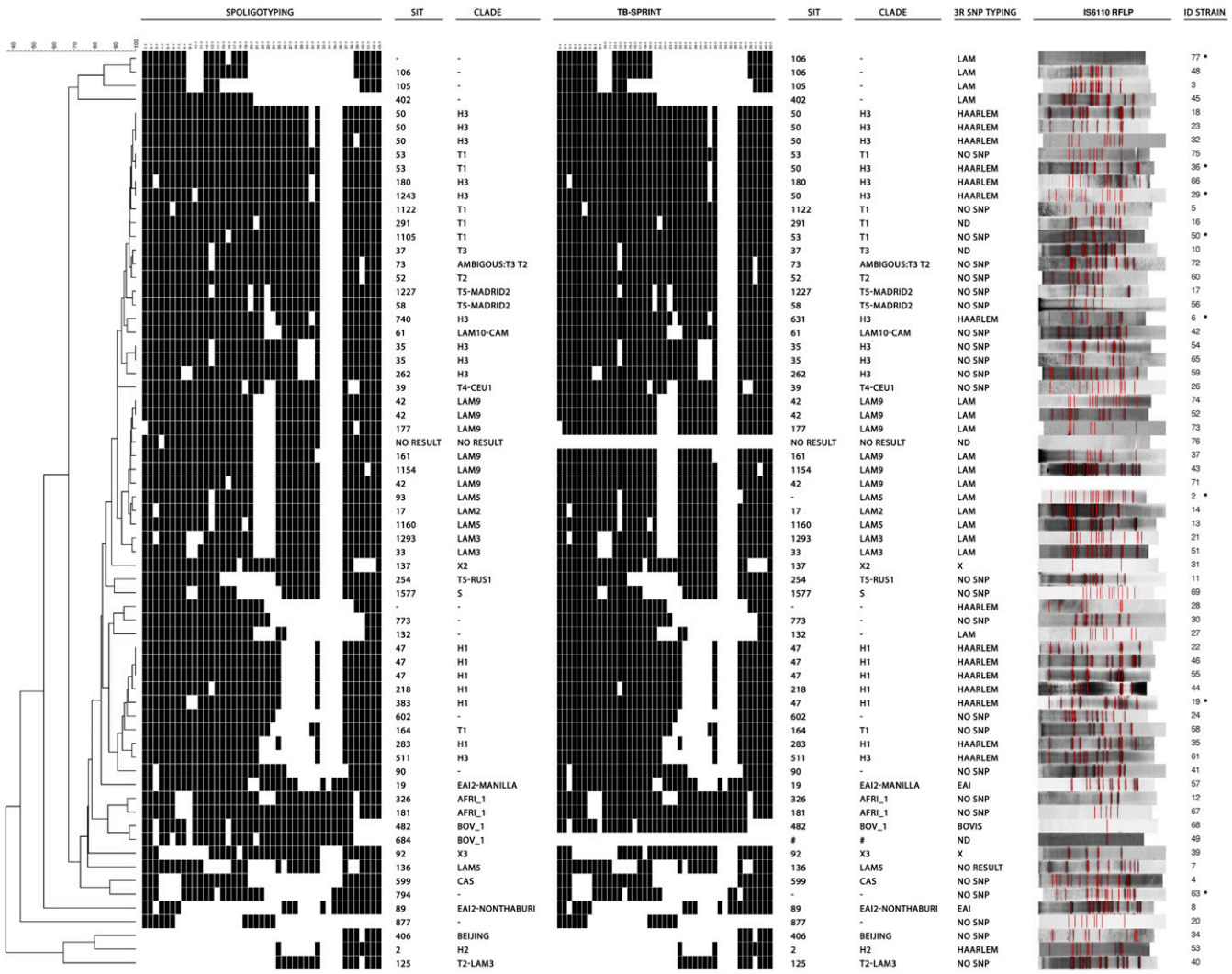

FIGURE 1. Genotyping results obtained for the 67 *Mycobacterium tuberculosis* complex strains included in this study. From left to right are shown the dendrogram, spoligotypes, SIT, and clade obtained with membrane-based spoligotyping; spoligotypes, SIT, and clade obtained with TB-SPRINT; clade identified with SNP typing, IS*6110*- RFLP pattern, and strain ID. SIT and clades were identified according to SITVITWEB (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/index.jsp). Strains with any discordance between membrane-based spoligotyping and TB-SPRINT are marked with an asterisk. TB-SPRINT spoligotyping result for strain 49 (marked with #) suggested the presence of two different populations and it is not shown. TB-SPRINT = Tuberculosis-Spoligo-Rifampicin-Isoniazid typing; SNP typing = typing of lineage-specific single nucleotide polymorphisms; RFLP = restriction fragment length polymorphism; ND = not done. Dendrogram built with BioNumerics version 6.1.

Among all the strains tested, TB-SPRINT result could not be obtained for one strain (strain 76) likely because of DNA degradation. For an additional strain (strain 49), TB-SPRINT spoligotyping result suggested the presence of two different populations, which was confirmed with analysis of mycobacterial interspersed repetitive units—variable number tandem repeats (MIRU-VNTR). Therefore, TB-SPRINT may be useful for detecting mixed infections, a relevant aspect in the management of TB patients. MIRU-VNTR typing has been also useful for this purpose, and has become another widespread used method for epidemiological studies.[13]

According to TB-SPRINT results, distribution of families among the 65 strains with a result was as follows: H, $N = 18$ (27.7%); LAM, $N = 13$ (20.0%); T super-family (T), $N = 13$ (20.0%); AFRI, $N = 2$ (3.1%); EAI, $N = 2$ (3.1%); X, $N = 2$ (3.1%); Beijing, $N = 1$ (1.5%); Bovis (BOV-1), $N = 1$ (1.5%); Central Asian (CAS), $N = 1$ (1.5%); S, $N = 1$ (1.5%); for nine strains (13.8%) a SIT number could be assigned but the family to which they belonged was unknown; finally, the remaining two strains (3.1%) did not match any pattern of the SITVITWEB (Figure 1). Considering the 65 strains, the concordance between membrane-based spoligotyping and TB-SPRINT was 99.6% (2,785/2,795 spoligotype data points). For eight of the 65 strains, discordant results between membrane-based spoligotyping and TB-SPRINT were obtained for some spacers (Table 1).

TB-SPRINT detected six clusters that were resolved by IS*6110*-RFLP (Figure 1). On the other hand, IS*6110*-RFLP detected only one cluster of two strains (strains 49 and 68) that harbored a single IS*6110* copy, which was resolved by membrane-based spoligotyping (Figure 1). The higher discriminatory power of spoligotyping for strains with less than five IS*6110* copies has been previously reported.[14] It is of note that despite strain 49 was excluded from the TB-SPRINT

TABLE 1
Membrane-based spoligotyping, TB-SPRINT, and SNP typing results of the strains with discordant results between spoligotyping methods

		Membrane-based spoligotyping			TB-SPRINT			
Strain	Spacer	Spacer	SIT	Lineage	Spacer	SIT	Lineage	SNP typing
2	37	Present	93	LAM5	Possibly absent*	Orphan	LAM5	LAM
6	23	Absent	740	H3	Present†	631	H3	Haarlem
19	9, 10	Absent	383	H1	Present†	47	H1	Haarlem
29	10	Absent	1,243	H3	Present†	50	H3	Haarlem
36	31	Present	53	T1	Absent‡	50	H3	Haarlem
50	16	Absent	1,105	T1	Present†	53	T1	NR§
63	22, 37	Present	794	CAS1-Delhi	Possibly absent*	Orphan	Orphan	NR¶
77	16	Absent	Orphan	Orphan	Present†	106	Orphan	LAM

NR = no result; SIT = Spoligo-International type; SNP typing = typing assay based on detection of lineage-specific single nucleotide polymorphisms; TB-SPRINT = Tuberculosis-Spoligo-Rifampicin-Isoniazid typing.
* Relative fluorescence intensity (RFI) values for the discordant spacers were near the cutoff.
† RFI values for the discordant spacers were significantly high.
‡ RFI values for the discordant spacers were significantly low.
§ No T1-specific SNP was included in this analysis.
¶ No CAS-specific SNP was included in this analysis.

analysis, the different microbead-based spoligotyping results allowed discriminating these strains.

Table 2 shows the drug susceptibility results for RIF and INH for the strains with any resistance or mutation detected by either phenotypic methods, DNA sequencing/pyrosequencing, or TB-SPRINT. TB-SPRINT showed good concordance with both phenotypic DST and DNA sequencing/pyrosequencing results (Table 3), although the number of resistant strains was low. In a previous work, Gomgnimbou and others obtained complete concordance with DNA sequencing for detecting resistance to both RIF and INH, and with phenotypic DST for RIF.[6]

Current technologies to diagnose, treat, and prevent dissemination of drug-resistant TB have limitations, and there is an increased need for more rapid, simple, sensitive, and affordable methods. In this study, we present additional evidence of the utility of TB-SPRINT, a microbead-based assay for simultaneous spoligotyping and detection of mutations in *rpoB*, *katG*, and *inhA*, associated with resistance to RIF and INH. Either TB-SPRINT or microbead-based spoligotyping has been successfully used in previous works.[15–18] Compared with conventional membrane-based spoligotyping, this method improves the throughput and flexibility for first-line screening of potential epidemiological links. In addition, due to the multiplexing capacity, this method has the potential to simultaneously target a well-defined set of mutations associated with drug resistance, increasing the sensitivity of molecular resistance detection. Although molecular testing cannot replace phenotypic DST yet, it may be valuable as a complementary tool, especially to rule out the considered drug for treatment in case that drug resistance is detected.[19] In the medium term, and subject to availability of a Luminex device, the combined approach of genotyping and detection of drug resistance may be attractive for low/middle-income countries with a high burden of drug-resistant TB where spoligotyping is routinely performed.[6,15]

In conclusion, spoligotyping results obtained with TB-SPRINT are in agreement with those obtained with conventional spoligotyping. In addition, TB-SPRINT is a more rapid and high-throughput assay that allows simultaneous detection of molecular resistance to RIF and INH in the same tube. Implementation of this method would be useful to improve epidemiological surveillance, and to obtain a preliminary drug susceptibility profile before phenotypic results are available, thus improving the management of TB patients and preventing further spread of drug-resistant *M. tuberculosis* strains.

TABLE 2
Phenotypic and molecular drug susceptibility result for rifampicin and isoniazid for the strains with any resistance or mutation detected by either phenotypic drug susceptibility testing, DNA sequencing/pyrosequencing, or TB-SPRINT

	Phenotypic DST		DNA sequencing/pyrosequencing			TB-SPRINT		
Strain	RIF	INH	*rpoB*	*katG*	*inhA*	*rpoB*	*katG*	*inhA*
5	S	S	wt	wt	C-15T	wt	wt	C-15T
7	S	S	wt	wt	wt	No 531 wt*	wt	wt
16	S	S	NR	wt	wt	wt	wt	No –15 wt†
21	S	R	wt	wt	C-15T	wt	wt	C-15T
39	S	R	wt	wt	wt	wt	wt	wt
73	R	R	S531L	wt	C-15T	S531L	wt	C-15T
74	R	R	H526D	wt	wt	H526D	wt	wt
75	R	R	H526Y	S315T	C-15T	No 516 wt*	S315T	wt
76	R	R	S531L	S315T	C-15T	NR‡	NR‡	NR‡
77	R	R	S531L	wt	C-15T	S531L	wt	C-15T

DST = drug susceptibility testing; INH = isoniazid; NR = no result; RIF = rifampicin; R = resistant; TB-SPRINT = Tuberculosis-Spoligo-Rifampicin-Isoniazid typing; S = susceptible; wt = wild-type.
* The result of the specified probe was considered negative since the RFI value was low, and the strain was regarded as RIF resistant by TB-SPRINT.
† The result of the specified probe was considered negative since the RFI value was low, and the strain was regarded as INH resistant by TB-SPRINT.
‡ TB-SPRINT result could not be obtained for strain 76, likely because of DNA degradation.

TABLE 3

Sensitivity, specificity, and concordance of TB-SPRINT for detecting drug resistance using phenotypic drug susceptibility testing or DNA sequencing/pyrosequencing as reference methods

	Sensitivity (no. detected/total no. [%], [95% CI])	Specificity (no. detected/total no. [%] [95% CI])	Concordance (no. detected/total no. [%])
RIF*	4/4 (100) (39.6–100)	59/60 (98.3) (89.9–99.9)	63/64 (98.4)
INH*	4/6 (66.7) (24.1–94.0)	56/58 (96.6) (87.0–99.4)	60/64 (93.8)
rpoB†	4/4 (100) (39.6–100)	56/57 (98.2) (89.4–99.9)	60/61 (98.4)
katG†	1/1 (100) (0.05–100)	63/63 (100) (92.8–100)	64/64 (100)
inhA†	4/5 (80.0) (29.9–98.9)	58/59 (98.3) (89.7–99.9)	62/64 (96.9)

CI = confidence interval; INH = isoniazid; RIF = rifampicin.

* Phenotypic drug susceptibility testing was used as reference method for sensitivity, specificity, and concordance calculations.

† DNA sequencing/pyrosequencing was used as reference methods for sensitivity, specificity, and concordance calculations.

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