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Spoligotype Signatures in the *Mycobacterium tuberculosis* Complex▽

E. M. Streicher,[1] T. C. Victor,[1] G. van der Spuy,[1] C. Sola,[2] N. Rastogi,[2] P. D. van Helden,[1] and R. M. Warren[1]*

DST/NRF Centre of Excellence in Biomedical TB Research, Division of Molecular Biology and Human Genetics, Faculty of Health Sciences, Stellenbosch University, Tygerberg, South Africa,[1] and Institut Pasteur de Guadeloupe, Pointe à Pitre, Guadeloupe[2]

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Evolution of the direct repeat region in *Mycobacterium tuberculosis* has created unique spoligotype signatures specifically associated with IS*6110*-defined strain families. Spoligotyping signatures may enable the analysis of the strain population structure in different settings and will enable the rapid identification of strain families that acquire drug resistance or escape protective immunity in drug and vaccine trials.

Numerous repeat sequences have been identified in the genome of the *Mycobacterium tuberculosis* complex, including transposable elements (3), trinucleotide repeats (36), variable number tandem repeats (10), mycobacterial interspersed repetitive units (27), and the direct repeat (DR) region (29). The DR region is one of the most extensively studied loci and consists of direct repeat sequences (36 bp) interspersed with unique spacer sequences (34 to 41 bp), which together are termed direct variable repeat (DVR) sequences (15, 29). The DR region has evolved through the deletion of DVR sequences by homologous recombination, single nucleotide mutations, and the integration of IS*6110* elements (1, 29, 34). These events are believed to be unidirectional and to occur over time, making the DR region an informative locus for studying the evolution and epidemiology of the *M. tuberculosis* complex (9, 13, 35).

Spoligotyping was developed as a genotyping tool to provide information on the structure of the DR region in individual *M. tuberculosis* strains and in different members of the *M. tuberculosis* complex (16). The simplicity of this method has allowed for the establishment of an international spoligotype database which describes 39,295 entries from 122 countries (4). Alignment of the spoligotype patterns has allowed authors to group isolates according to similarity to create clades or strain families (8). In addition, distinctive spoligotype patterns have been linked to defined species of the *M. tuberculosis* complex (8, 18). However, these evolutionary relationships have not been extensively tested with other genotyping methods (9). A positive association between spoligotype and single nucleotide polymorphism cluster groups (SCGs) could not be demonstrated in all instances (9). We hypothesize that the evolution of the DR region has created unique patterns of DVR deletion within the spoligotype and that these patterns are specific to the different IS*6110* DNA fingerprinting-defined strain families within the described SCGs. These unique patterns of DVR deletion have been termed spoligotype signatures.

To determine whether evolutionary relationships exist between the IS*6110*-defined families and spoligotype patterns, *M. tuberculosis* isolates from patients residing in the epidemiological field site near Cape Town, South Africa, were genotyped according to the internationally standardized IS*6110* DNA fingerprinting method (28). DNA fingerprints were analyzed with GelCompar software, using the unweighted-pair group method using average linkages and Dice coefficients (14). Isolates with an IS*6110* similarity index of ≥65% were grouped into strain families (22). In this study, DNA fingerprints from isolates of *M. tuberculosis* collected between 1993 and 1998 were available from 834 tuberculosis (TB) patients, and these fingerprints were grouped into 33 strain families. Representative strains from each strain family were classified into principal genetic groups (PGGs) by DNA sequencing of the *katG* and *gyrA* genes (Table 1) (25).

At least one strain representing each IS*6110* banding pattern within a strain family was subjected to spoligotyping using an internationally standardized method (16). In this study, a spoligotype signature was defined as the deletion of either a single DVR or multiple DVRs unique to all members of a specific strain family. Random deletions of DVRs were ignored, as they probably represent recent evolutionary events which occurred after the evolutionary event that generated the signature and were not inherited by all progeny. Spoligotype signatures were compared to previously published spoligotype data (2, 4, 6–8, 11, 12, 17, 19–21, 24, 26, 31, 35, 37).

Table 1 shows that the deletion of DVRs 33 to 36 was common to all members of PGGs 2 and 3. Twenty-seven of 33 (82%) strain families had spoligotype signatures. Eighteen of these spoligotype signatures were unique to a specific strain family, suggesting that these strain families had evolved independently. In contrast, five strain families (F9, F11, F13, F15, and F26) shared a distinct spoligotype signature (with DVRs 21 to 24 deleted) and had evolved their own defining signature, suggesting that these families are closely related and have evolved from a common progenitor. This evolutionary relationship was supported by IS*6110* insertion site mapping (33). Comparison of these spoligotypes with the SpolDB4 database shows that these families form part of the previously described Latino-American and Mediterranean (LAM) family (8). Family F11 has the characteristic LAM3 signature, F13 corre-

* Corresponding author. Mailing address: DST/NRF Centre of Excellence in Biomedical TB Research, Department of Medical Biochemistry, Stellenbosch University, P.O. Box 19063, Tygerberg, South Africa 7505. Phone: 27-21-9389482. Fax: 27-21-9389476. E-mail: rw1@sun.ac.za.

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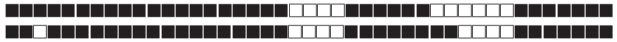
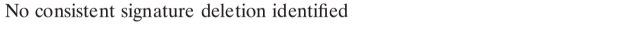
TABLE 1. Spoligotyping signatures of the *Mycobacterium tuberculosis* complex

PGG[a]	Species (strain family[b])	ST no.[c]	Core spoligotype pattern	International family name [reference(s)][d]
1	*M. africanum*	181		AFRI1 (4, 8)*
	M. africanum	331		AFRI2 (4, 8)*
	M. africanum	438		AFRI3 (4, 8)*
	M. bovis	482		BOV1/*M. bovis*-BCG (4, 8)*
	M. caprae	647		BOV4 (4)
	M. pinnipedii	593		PINI1 (4)
	M. pinnipedii	637		PINI2 (4)
	M. canettii	592		canettii (4)
	M. microti	539		microti (4)
	M. tuberculosis (F27; *n* = 6)	1		Beijing (12)*
	M. tuberculosis (F29; *n* = 318)	1		Beijing (12)*
	M. tuberculosis (F31; *n* = 31)	1		Beijing (12)*
	M. tuberculosis (F25; *n* = 12)	21		CAS1/Kili (2, 4, 8, 24)
	M. tuberculosis (F20; *n* = 9)	26		CAS1/Delhi (2, 4, 8, 24)
	M. tuberculosis	288		CAS2 (2, 4, 24)*
	M. tuberculosis	19		Manila/EAI2 (4, 6)*
	M. tuberculosis	236		Ancestral Mtb/EAI (26)
2	*M. tuberculosis* (F26; *n* = 11)	1241		
	M. tuberculosis (F13; *n* = 26)	20		LAM1 (4, 8)*
	M. tuberculosis (F11; *n* = 304)	33		LAM3 (8, 31)*
	M. tuberculosis (F9; *n* = 28)	59		LAM11-ZWE(4, 7)
	M. tuberculosis (F15; *n* = 17)	60		LAM4 (4, 8)
	M. tuberculosis	209		LAM12/Madrid 1 (4, 11)
	M. tuberculosis	41		LAM7-TUR (4, 37)
	M. tuberculosis	61		LAM10-Cameroon (4, 20, 21)
	M. tuberculosis (F14; *n* = 52)	765		
	M. tuberculosis (F30; *n* = 1)	2309		
	M. tuberculosis (F28; *n* = 122)	34		S/PZA^R-Quebec (4, 8, 19)*
	M. tuberculosis (F7; *n* = 25)	136		
	M. tuberculosis (F3; *n* = 4)	102		
	M. tuberculosis (F19; *n* = 6)	37		T3 (8)
	M. tuberculosis (F6; *n* = 16)	39		T4-CE1(4)
	M. tuberculosis (LCC; *n* = 230)[e]	119		X1 family (4, 8, 35)
	M. tuberculosis (1banders; *n* = 21)[f,g]	53		T1 (8)*
	M. tuberculosis (F1; *n* = 4)	47		Haarlem 1 (4, 8, 17)*
	M. tuberculosis (F10; *n* = 6)	47		Haarlem 1 (4, 8, 17)*
	M. tuberculosis (F2; *n* = 57)	50		Haarlem 3 4, 8, 17)
	M. tuberculosis (F4; *n* = 47)	50		Haarlem 3 (4, 8, 17)
	M. tuberculosis (F24; *n* = 24)		No consistent signature deletion identified	
3	*M. tuberculosis* (F22; *n* = 4)[g]	53		T1 (8)*
	M. tuberculosis (F5; *n* = 2)	52		T2 (8)
	M. tuberculosis (F8; *n* = 4)	1067		
	M. tuberculosis (F12; *n* = 1)			
	M. tuberculosis (F16; *n* = 16)		No consistent signature deletion identified	
	M. tuberculosis (F17; *n* = 12)		No consistent signature deletion identified	
	M. tuberculosis (F18; *n* = 31)		No consistent signature deletion identified	
	M. tuberculosis (F21; *n* = 29)		No consistent signature deletion identified	
	M. tuberculosis (F23; *n* = 30)		No consistent signature deletion identified	
	M. tuberculosis	58		Madrid 2 (11)

[a] PGGs are based on *katG463* and *gyrA95* classification (25).
[b] Strain classification of local families for *Mycobacterium tuberculosis* according to a report by Richardson et al. (22). *n*, number of cases.
[c] Shared-type number as described in the SpolDB4 database (4).
[d] *, matches the SPOTCLUST algorithm using a randomly initialized model (32).
[e] LCC, low-copy-number clade (35). These strains have two to six bands on the IS*6110* fingerprint pattern.
[f] The strains in this strain family have one copy of IS*6110* (35).
[g] Although there is a uniform spoligotype pattern, no signature other than the deletion of spacers 33 to 36 is present.

sponds to LAM1, F15 corresponds to LAM4, and F9 corresponds to LAM11-ZWE (Table 1) (4). A review of the literature showed that spoligotype signatures were also identified for the other members of the *M. tuberculosis* complex (Table 1) (4, 16, 23, 29, 30).

Spoligotype signatures were absent from six strain families (Table 1). The absence of a spoligotype signature was associated with PGGs 2 and 3 (Table 1). These strains were previously described as belonging to the T-strain family, thereby confirming a previous report which demonstrated that the T-strain family spoligotypes were distributed in a number of different SCGs (9). Although the T-strain family remains ill-defined, certain clones within the T-strain family have been characterized by specific deletions of DVRs (Table 1) (4). The presence of other defining signatures within the different T-strain family members cannot be excluded, as still-recognized signatures may have evolved within the DR regions of these strains and may fall outside of the 43 DVR sequences routinely analyzed.

The visual method of defining a spoligotype signature compared well with the previously described SPOTCLUST algorithm (32).

We acknowledge that the definition of a spoligotype signature used in this study has certain limitations, as it is possible that, although such events are rare, extensive deletion of DVR sequences may lead to convergence (34). Similarly, deletion of a small number of DVRs may lead to convergence, resulting in misclassification. Although no cases of misclassification were detected in this study, the potential limitation of the use of spoligotypes in phylogenetic analysis should be highlighted.

Despite these limitations, spoligotyping remains a highly informative genotyping method, and the identification of strain family-specific signatures provides an important marker for clonality. The classification of most strains into distinct evolutionary groups will enable the rapid stratification of patients in studies aimed at identifying pathogenic characteristics associated with the disease-causing strain (5, 9). Furthermore, this method will enable the rapid identification of emerging strain families. We propose that the identification of spoligotype signatures will provide a means to determine the strain population structure in different geographical settings and on a global scale. Identification of spoligotype signatures will also be an important tool in the monitoring of drug and vaccine trials, as it will enable the detection of strain families which may have a greater propensity to acquire drug resistance or to escape protective immunity.

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