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Evaluation of the Epidemiologic Utility of Secondary Typing Methods for Differentiation of *Mycobacterium tuberculosis* Isolates

Awewura Kwara,[1]* Ronald Schiro,[2] Lauren S. Cowan,[3] Newton E. Hyslop,[1] Mark F. Wiser,[4] Stephanie Roahen Harrison,[5] Patricia Kissinger,[5] Lois Diem,[3] and Jack T. Crawford[3]

Department of Tropical Medicine[4] and Department of Epidemiology,[5] Tulane University School of Public Health and Tropical Medicine, Section of Adult Infectious Diseases, Tulane University Health Sciences Center,[1] and Office of Public Health, Louisiana Department of Health and Hospitals,[2] New Orleans, Louisiana 70112, and Division of AIDS, STD, and TB Laboratory Research, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia 30333[3]

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Spoligotyping and mycobacterial interspersed repetitive unit–variable-number tandem repeat analysis (MIRU-VNTR) were evaluated for the ability to differentiate 64 *Mycobacterium tuberculosis* isolates from 10 IS*6110*-defined clusters. MIRU-VNTR performed slightly better than spoligotyping in reducing the number of clustered isolates and the sizes of the clusters. All epidemiologically related isolates remained clustered by MIRU-VNTR but not by spoligotyping.

DNA fingerprinting of *Mycobacterium tuberculosis* isolates is a powerful tool for studying the molecular epidemiology of tuberculosis (TB). IS*6110* fingerprinting has proven useful for detecting outbreaks (5) and for conducting population-based studies of recent transmission (1, 9). However, for low-copy-number strains (fewer than six copies of IS*6110*), it is a less reliable indicator of clonality and therefore a poor predictor of epidemiologic relationships (6, 12, 13). Secondary typing is often recommended for these isolates to decrease the number of falsely clustered isolates. Spoligotyping is the most widely used method for secondary typing; however, recent reports indicate that it does not appreciably improve strain differentiation and suggest that more discriminating methods should be used (3). Mycobacterial interspersed repetitive unit–variable-number tandem repeat analysis (MIRU-VNTR) is a relatively new typing method that determines the number of repeated mycobacterial interspersed repetitive units at 12 independent loci (7). In an initial study, 12 sets of epidemiologically related isolates remained clustered by MIRU-VNTR (7). In a study of low-copy-number isolates, MIRU-VNTR analysis was shown to further resolve some IS*6110* clusters, but the epidemiological significance was not investigated (2). In this study, we compared spoligotyping and MIRU-VNTR for the secondary typing of isolates that were previously clustered by IS*6110* and correlated the genotyping results with the epidemiologic data.

The study included 64 isolates from patients with TB diagnosed between 1999 and 2001. These isolates represent 74% of 87 IS*6110*-clustered isolates identified during an investigation of TB transmission in metropolitan New Orleans, La. The institutional review boards of Tulane University and Louisiana State University approved the study, and the consent form waiver was granted in accordance with federal regulatory code (45 CFR 46.116(d) [available online at http://ohrp.osophs.dhhs.gov/humansubjects/guidance/45cfr46.htm#46-116]). IS*6110* analysis was performed by the standard method (11), with some modifications. The PCR DIG probe synthesis kit and the DIG nucleic acid detection kit (Roche Diagnostics) were used to detect IS*6110* fragments. Images were analyzed with Molecular Analyst v. 1.6 (Bio-Rad, Hercules, Calif.). Spoligotyping (Isogen Bioscience BV, Maarssen, The Netherlands) was performed in accordance with previously published methods (8). Results were recorded as an octal code (4). MIRU loci (10) were amplified with primers specific for sequences flanking each locus (Table 1). The reaction mixture (20 μl) contained a 1-μl DNA sample, 1× *Taq* PCR buffer, 1× Q solution, 3 mM magnesium chloride, deoxynucleoside triphosphates (each at 0.2 mM), 1 U of AmpliTaq DNA polymerase (Qiagen, Valencia, Calif.), and the primer pair at 0.6 μM (each primer at 0.3 μM). The amplification profile consisted of 1 min at 94°C, followed by 40 cycles of 30 s at 94°C, 10 s at 60°C, and 1 min at 72°C in a GeneAmp 9700 PCR System (Perkin-Elmer Applied Biosystems, Foster City, Calif.). The size of each amplicon was determined with a CEQ2000 capillary sequencer (Beckman Coulter, Fullerton, Calif.). The number of MIRUs was determined in accordance with the convention described in Table 1. The results were recorded as a 12-digit number as previously described (2). Patient data were collected retrospectively from hospital and TB clinic records and from contact-tracing data obtained by TB control disease intervention specialists or by inpatient coordinators. A transmission link was said to exist if two patients lived or worked in the same facility at a time when one of the individuals was likely to have been infectious or if two patients independently identified contact with a prior common case.

Sixty-four isolates from 10 IS*6110* clusters were available for secondary typing; 30 were low-copy-number isolates, and 34 were high-copy-number isolates. The IS*6110* patterns are shown in Fig. 1. Spoligotyping identified eight types that defined seven clusters (63 isolates) and one unique isolate. Only one IS*6110*-defined cluster was further spilt by spoligotyping (Table 2). Of the 13 isolates in this cluster, 1 had a spoligotype

* Corresponding author. Mailing address: Infectious Diseases Division, Miriam Hospital, 164 Summit Ave., Providence, RI 02906-0609. Phone: (401) 793-2463. Fax: (401) 793-4704. E-mail: Awewura_Kwara@brown.edu.

TABLE 1. Sequences of oligonucleotides used in MIRU-VNTR

Oligonucleotide[a]	Sequence	Size (bp)[b]
2c(D3)	5′ CAGGTGCCCTATCTGCTGACG	236 + 47
2d	5′ GTTGCGTCCGGCATACCAAC	
4a(D2)	5′ GTCAAACAGGTCACAACGAGAGGAA	182 + 77
4b	5′ CCTCCACAATCAACACACTGGTCAT	
10a(D4)	5′ ACCGTCTTATCGGACTGCACTATCAA	272 + 53
10c	5′ CACCTTGGTGATCAGCTACCTCGAT	
16a(D2)	5′ CGGGTCCAGTCCAACTACCTCAAT	419 + 52
16b	5′ GATCCTCCTGATTGCCCTGACCTA	
20a(D2)	5′ CCCCTTCGAGTTAGTATCGTCGGTT	292 + 72
20b	5′ CAATCACCGTTACATCGACGTCATC	
23a(D4)	5′ CGAATTCTTCGGTGGTCTCGAGT	131 + 52
23b	5′ ACCGTCTGACTCATGGTGTCCAA	
24a(D3)	5′ GAAGGCTATCCGTCGATCGGTT	365 + 53
24b	5′ GGGCGAGTTGAGCTCACAGAAC	
26a(D4)	5′ GCGGATAGGTCTACCGTCGAAATC	291 + 48
26b	5′ TCCGGGTCATACAGCATGATCA	
27a(D4)	5′ TCTGCTTGCCAGTAAGAGCCA	321 + 52
27b	5′ GTGATGGTGACTTCGGTGCCTT	
31a(D3)	5′ CGTCGAAGAGAGCCTCATCAATCAT	160 + 52
31b	5′ AACCTGCTGACCGATGGCAATATC	
39a(D2)	5′ CGGTCAAGTTCAGCACCTTCTACATC	238 + 47
39c	5′ GCGTCCGTACTTCCGGTTCAG	
40a(D3)	5′ GATTCCAACAAGACGCAGATCAAGA	276 + 50
40b	5′ TCAGGTCTTTCTCTCACGCTCTCG	

[a] One primer in each pair was 5′ end labeled with Beckman dye D2, D3, or D4.
[b] Predicted size of amplicons containing one MIRU copy plus size of additional copies.

TABLE 2. Genotyping results

IS6110 RFLP (no. of bands)	No. of patients	Spoligotype	No. of patients	MIRU-VNTR	No. of patients
LTB2 (2)	20	777776777760601	20	224325153323	16
				223325153323	2
				224315153323	1
				224325163323	1
LTB3 (3)	5	777776777760771	5	225325123421	4
				245225123421	1
LTB13 (3)	3	700036777760771	3	222325153324	3
LTB14 (3)	2	777776777760601	2	224325153323	2
LTB6 (6)	13	777776777760771	12	224325153324	13
		777776777760731	1		
LTB8 (7)	3	777777777720771	3	225325153323	3
LTB9 (9)	12	000000000003771[a]	12	223425173563	10[b]
				223425173564	2
LTB38 (11)	2	776037777760771	2	223125163324	2
LTB34 (12)	2	677777607760771	2	223226153321	2
LTB31 (18)	2	000000000003771[a]	2	223325173533	2

[a] Cluster with the Beijing genotype.
[b] Two of the isolates in this cluster were a result of laboratory cross-contamination.

that differed from that of the others by a single spacer. MIRU-VNTR identified 14 types that defined 11 clusters (61 isolates) and three unique isolates (Table 2). Three of the four largest IS*6110* clusters were subdivided into four subclusters and four unique isolates. Two of these clusters contained low-copy-number isolates, and the third contained high-copy-number isolates. The combination of all three methods identified 16 genotypes that defined 12 clusters (60 isolates) and four unique isolates (Table 2). The isolate that was unique by spoligotyping remained clustered by MIRU-VNTR.

Epidemiologic data were available for 61 patients; 16 (25%) patients had definite transmission links with another patient. With one exception, the genotypes of the isolates from these patients were identical (Table 3). Isolates from three family members were identical by IS*6110* and MIRU-VNTR, but one isolate had a slightly different spoligotype in that it was missing spacer 40. Such minor variations have been previously reported among outbreak isolates (6). Two of the patients in this study were diagnosed with TB as a result of laboratory cross-contamination. The genotypes of these three isolates were identical.

Genotyping of *M. tuberculosis* isolates from a given community is useful to the TB controller by identifying unsuspected cases of recently transmitted disease and areas in which traditional contact tracing has failed. However, large clusters of unrelated strains can confound these efforts, and therefore, a combination of genotyping techniques that provides the greatest level of discrimination is necessary for molecular typing to be useful to TB control programs. In this study, spoligotyping of IS*6110*-defined clusters did not improve strain differentiation. The only IS*6110*-clustered isolates that were subdivided by spoligotyping were epidemiologically related. In contrast, MIRU-VNTR had greater resolving power than spoligotyping

TABLE 3. Genotypes of isolates with documented transmission links

Type of transmission link	No. of patients	IS6110 RFLP	Spoligotype (no. of patients)	MIRU-VNTR
Family contact	2	LTB2	777776777760601	224325153323
Family contact	2	LTB3	777776777760771	225325123421
Family contact	3	LTB6	777776777760771 (2) 777776777760731 (1)	224325153324
Family contact	2	LTB13	700036777760771	222325153324
Work contact	2	LTB6	777776777760771	224325153324
Contact with common case	3	LTB9	000000000003771	223425173563
Social contact	2	LTB2	777776777760601	224325153323

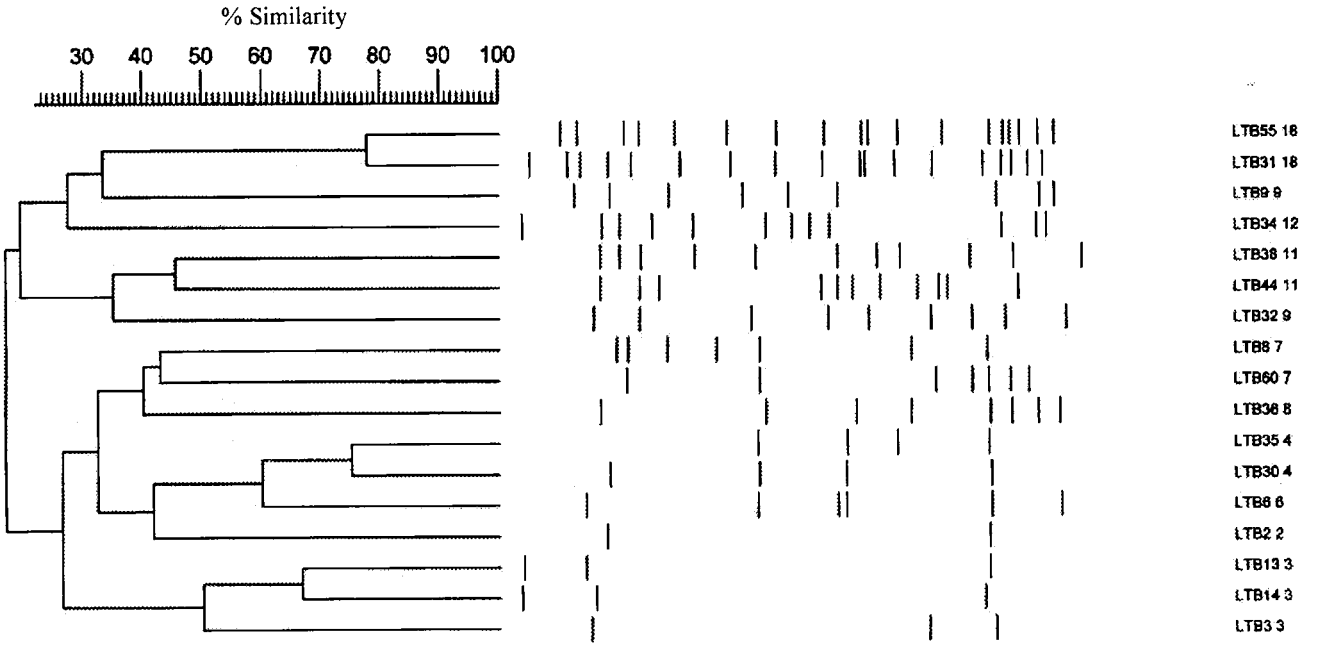

FIG. 1. Representative patterns of clustered isolates identified by IS*6110* RFLP. Numbers indicate pattern designations (LTB*x*) and numbers of IS*6110* bands. Clusters of the LTB55, LTB44, LTB32, LTB60, LTB36, LTB35, and LTB30 genotypes were excluded from this study because the isolates were not available for MIRU-VNTR typing.

and defined four additional genotypes; all epidemiologically related isolates remained clustered. We found that MIRU-VNTR is useful for secondary typing of IS*6110*-clustered isolates with both low and high numbers of IS*6110* copies. MIRU-VNTR further resolved two low-copy-number IS*6110* clusters and a high-copy-number cluster containing isolates with the Beijing spoligotype. The reduction in the number of falsely clustered isolates should allow more effective focused contact investigations. The ability of MIRU-VNTR to differentiate some IS*6110*-spoligotype-defined clusters suggests that the widely used genotyping combination of IS*6110* and spoligotyping may result in overestimation of recently transmitted disease.

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