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Transmission Dynamics and Molecular Characterization of *Mycobacterium tuberculosis* Isolates with Low Copy Numbers of IS*6110*

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Population-based analysis of *Mycobacterium tuberculosis* transmission in Houston, Tex., over 5 years identified 377 patients infected with an isolate containing one to four copies of IS*6110*. The isolates were analyzed by spoligotyping and assigned to one of three major genetic groups based on nucleotide polymorphisms in codons *katG* 463 and *gyrA* 95. Prospectively obtained patient interviews were reviewed to assess epidemiologic links between apparently clustered patients. A total of 13 groups of isolates with the same IS*6110* profile were identified, representing 326 of the 377 patients (86.5%; range 2 to 113 patients). In contrast, 28 groups of isolates containing 334 patients (88.6%) had the same spoligotype (range, 2 to 143 patients). Combination of IS*6110* profile and spoligotype data identified 31 clusters with 300 patients (79.6%; range, 2 to 82 patients). All 377 isolates belonged to major genetic group 1 (77 patients) or genetic group 2 (300 patients); no major genetic group 3 isolates were identified. Among the 228 patients interviewed, 33 patients (14.5%) were directly linked to another patient in the same cluster. Possible epidemiologic links were also found among 11 patients. Moreover, many clusters consisted of individuals with the same ethnicity. In conclusion, we confirmed that IS*6110* profiling and spoligotyping together provide enhanced molecular discrimination of *M. tuberculosis* isolates with low copy numbers of IS*6110*. Identification of epidemiologic links among some of the patients verified that the combination of these two methods reliably indexes tuberculosis transmission.

Characterization of *Mycobacterium tuberculosis* complex isolates by restriction fragment length polymorphism (RFLP) with the IS*6110* element as a probe is the standard tool used in tuberculosis epidemiology studies. *M. tuberculosis* organisms usually contain several copies of IS*6110*, and the RFLP results are stable and reproducible, providing reliable differentiation of epidemiologically unrelated isolates. Moreover, large population-based studies have shown that identification of tuberculosis case clusters is significantly enhanced by IS*6110* profiling compared to conventional contact tracing (1, 16, 22).

However, *M. tuberculosis* isolates containing five or fewer IS*6110* copies cannot be reliably differentiated by the RFLP method (3), and in rare cases, the IS*6110* element is missing from the genome altogether (21). *M. tuberculosis* strains with low copy numbers of IS*6110* (hereafter in this work referred to as low-copy-number strains) have been more frequently isolated from Asian patients than from patients of European origin. For example, 56% of the strains collected from India and 33% of strains collected from Vietnam, Thailand, and Malaysia contained five or fewer IS*6110* elements (5, 15), whereas the frequencies of low-copy-number isolates in Denmark and France were 11 and 8%, respectively (2, 10). In the United States, analysis of isolates from Texas, California, and Colorado found that 25% contained fewer than six IS*6110* copies (23).

Other molecular characterization techniques, such as RFLP analysis of the polymorphic GC-rich repetitive sequence (PGRS) (13, 21) or spoligotyping, can to be used to differentiate the IS*6110* low-copy-number isolates. Spoligotyping discriminates among *M. tuberculosis* isolates on the basis of genetic polymorphisms in the chromosomal direct repeat (DR) region consisting of identical DRs and unique spacer sequences (9, 11). Spoligotyping has been reported to be more sensitive than standard IS*6110* profiling for subtyping *M. tuberculosis* isolates with five or fewer IS*6110* copies (2, 7, 8, 13, 17). However, epidemiologic relationships among patients infected with IS*6110* low-copy-number isolates and clustered by spoligotyping have not been systematically investigated in detail.

In order to address this issue, we characterized low-copy-number *M. tuberculosis* isolates obtained from patients in Houston by IS*6110* profiling, spoligotyping, and major genetic group determination and investigated the epidemiologic links between patients in case clusters identified by these molecular methods.

MATERIALS AND METHODS

Bacterial isolates. The analysis was performed as part of an ongoing, population-based tuberculosis epidemiology study in Houston, Tex. During the 5-year study period, from October 1994 to September 1999, 502 patients were identified that were infected with an *M. tuberculosis* isolate having zero to four IS*6110* copies. This number represented 20.3% (502 of 2,478) of the total number of patients studied. During this time period, 85% of culture-positive tuberculosis (TB) patients reported in Houston were enrolled in our study, and the average yearly TB case rate was 19.7 per 100,000 members of the population. Isolates from 383 (76.3%) patients were available for this analysis. Only one isolate per patient was included. A total of 377 patients were infected with an isolate with

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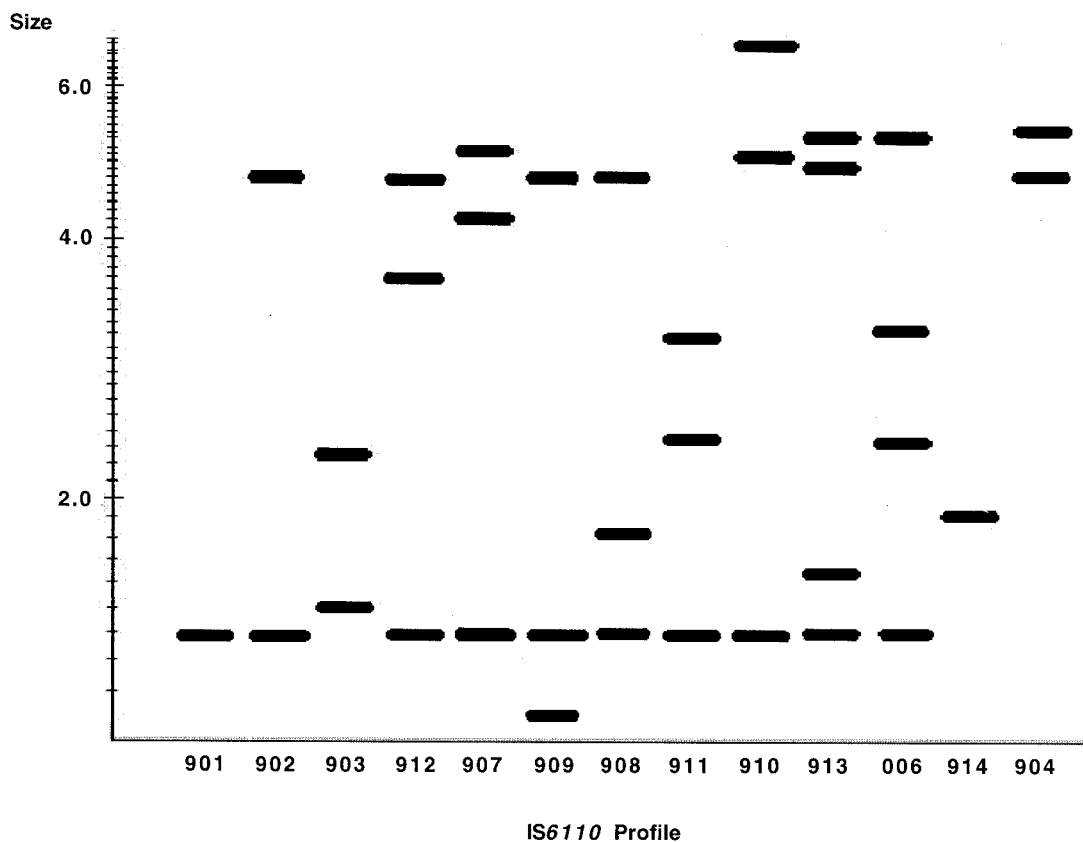
FIG. 1. Schematic representation of the IS*6110* profiles of the 13 IS*6110* groups obtained from the clustered low-copy-number *M. tuberculosis* isolates.

one to four IS*6110* copies (low-copy-number isolates), and six patients were infected with an isolate having no copies of IS*6110* (zero-copy-number isolates). The specimen source was pulmonary in 296 (77.3%) cases, extrapulmonary in 50 (13.1%) cases, and unknown in 37 (9.7%) cases. If the patient had both pulmonary and extrapulmonary isolates, the extrapulmonary isolate was used in this study.

DNA methods. DNA extraction and IS*6110* RFLP analysis were performed by an internationally standardized protocol (19). The IS*6110* profiles were analyzed with the BioImage (Ann Arbor, Mich.) Whole Band Analysis program, version 3.2. Spoligotyping was performed with a commercially available kit (Isogen Bioscience BV, Maarssen, The Netherlands) according to the instructions of the manufacturer. The isolates were assigned to one of three principal genetic groups on the basis of nucleotide polymorphism at codon 463 and 95 of the genes encoding the catalase-peroxidase and A subunit of DNA gyrase, respectively (18). Isolates lacking IS*6110* elements were also characterized by PCR-based sequencing of a 360-bp segment of the *hsp65* gene as described previously (12). A subset of the low-copy-number isolates was also analyzed by the double-repetitive-element PCR (DRE-PCR) according to a previously published protocol (6, 14). The DRE-PCR method is based on PCR amplification of genomic segments located between the IS*6110* and PGRS repetitive sequences. A cluster was defined as two or more patients infected with an *M. tuberculosis* isolate having the same IS*6110* profile and spoligotype.

Patient interviews. Prospectively obtained interviews were available from 228 of the 300 (76.0%) patients that were clustered by the combination of the IS*6110* profiling and spoligotyping results. The extensive questionnaire inquired about demographic information; living situation; use of tobacco, alcohol, and illicit drugs; sexual preferences and related habits; and patient medical history, including possible location of exposure to *M. tuberculosis*.

Statistical analysis. A chi-square analysis was performed on the effect of clustering and major genetic grouping by the country of origin. A *P* value of ≤0.05 was considered statistically significant.

RESULTS

IS*6110* profiling. Analysis of the 377 low-copy-number isolates by IS*6110* profiling alone revealed 64 distinct profiles. Fifty-one isolates had a unique profile, while 326 (86.5%) belonged to one of 13 groups (Fig. 1) consisting of 2 to 113 patients.

Spoligotyping. A total of 72 spoligotypes was identified among the 377 isolates. Forty-four of the 377 isolates had a unique spoligotype, while for 334 isolates (88.6%) 2 to 143 patients shared 1 of 28 spoligotypes. One *Mycobacterium bovis* isolate and four *M. bovis* BCG isolates were identified based on characteristic spoligotypes (11). The most common spoligotypes observed were S12 (*n* = 143) and S3 (*n* = 43) (17).

Combination of IS*6110* profiling and spoligotyping. Combination of the IS*6110* profile and spoligotyping data identified 31 clusters with 300 of the 377 patients (79.6%). The cluster size varied from 2 to 82 patients (mean, 9.7; median, 4) (Table 1). Isolates assigned to the two largest clusters (999.008 and 999.016) were also analyzed by the DRE-PCR method. All isolates in both of these clusters had the same DRE-PCR profile.

U.S.- and foreign-born patients. Of the 377 patients infected with low-copy-number isolates 241 (63.9%) were U.S. born, 129 (34.2%) were foreign born, and the birthplace was unknown for 7 (1.9%) individuals. The majority of foreign-born patients originated from Mexico (*n* = 57) and Vietnam (*n* =

TABLE 1. Characteristics of the clusters identified by combination of IS*6110* profile and spoligotype

Cluster[a]	IS*6110* copy no.	Genetic group[b]	IS*6110* profile[a]	Spoligotype[c]	No. of patients	No. of links[d]	Patient characteristic(s)[e]
999.001	1	1	901	S10	13	0	Vietnamese
999.002	1	1	901	S20	2	0	
999.003	1	1	901	S22	3	0	Vietnamese
999.004	1	1	901	S55	5	0	
999.005	1	1	901	S101	2	0	
999.006	1	1	901	S162	3	0	Vietnamese
999.007	2	2	902	S3	12	0	Hispanic
999.008	2	2	902	S12	82	6	Black, jail, homeless, drug use, 7 cross-contaminants
999.009	2	2	902	S52	7	0	Black, homosexual, HIV positive
999.010	2	2	902	S60	2	0	
999.011	2	2	902	S63	2	0	Hispanic
999.012	2	1	903	S11	6	4	White
999.013	2	2	904	S19	2	0	Vietnamese
999.014	3	2	909	S3	3	0	
999.015	3	2	907	S3	2	0	
999.016	3	2	908	S12	30	0	Black, jail
999.017	3	2	909	S15	7	2	Black, homosexual, HIV positive
999.018	3	2	909	S21	4	0	White
999.019	3	2	909	S53	3	0	Black, homeless, jail, prison
999.020	3	2	910	S18	3	0	
999.021	3	2	911	S54	7	0	Black
999.022	3	2	912	S242	3	0	Hispanic, jail, prison, homeless
999.023	4	2	913	S3	8	0	
999.024	4	2	006	S3	14	0	Hispanic
999.025	4	2	006	S25	26	14	Black
999.026	4	2	006	S26	4	0	
999.027	4	2	006	S27	8	2	Hispanic
999.028	4	2	006	S29	8	0	
999.029	1	1	914	BCG	4	0	
999.030	3	2	910	S12	2	0	
999.031	3	2	912	S12	23	5	Six cross-contaminants
Total					300	33	

[a] Arbitrary designation.
[b] Defined by Sreevatsan et al. (18).
[c] Defined by Soini et al. (17).
[d] Number of patients with a direct epidemiologic link to another patient in the same cluster.
[e] The ethnic groups refer to the ethnicity of the majority (>50%) of the patients in this cluster.

45). Comparison of clustering and place of birth is shown in Table 2.

Major genetic group designation. Seventy-seven isolates (20.4%) and eight clusters were assigned to major genetic group 1, and 300 isolates (79.6%) and 23 clusters belonged to major genetic group 2 (18). No group 3 isolates were identified among the 377 isolates. A comparison of major genetic group and place of birth is shown in Table 3. Of note, significantly more Vietnamese patients were infected with an isolate belonging to major genetic group 1 (37 of 45; 82.2%), whereas significantly more Mexican patients were infected with major genetic group 2 isolates (51 of 57; 89.5%) ($P < 0.01$).

Proven or potential epidemiologic links. Among the 228 patients interviewed, only 69 (30.3%) individuals had a history of contact with a known TB patient. A direct epidemiologic link, as defined by known person-to-person contact between two patients in the same cluster, was found between 33 (14.5%) patients in six clusters (Table 1). For example, in cluster 999.025, 14 of the 26 patients were epidemiologically

TABLE 2. Comparison of clustering and place of birth among patients infected with low-copy-number isolates

Birth	No. (%)			P
	All patients	Clustered	Nonclustered	
U.S. born	241 (63.9)	218 (72.7)	23 (29.8)	<0.01
Foreign born	129 (34.2)	75 (25.0)	54 (70.1)	<0.01
Unknown	7 (1.9)	7 (2.3)	0 (0.0)	0.35[a]
Total	377	300 (79.6)	77 (20.4)	

[a] Fisher's exact, two-tailed test.

TABLE 3. Comparison of major genetic group and place of birth among patients infected with low-copy-number isolates

Birth	No. (%)			P
	All patients	Group 1[a]	Group 2[a]	
U.S. born	241 (63.9)	13 (16.9)	228 (76.0)	<0.01
Foreign born	129 (34.2)	61 (79.2)	68 (22.7)	<0.01
Unknown	7 (1.9)	3 (3.9)	4 (1.3)	0.15[b]
Total	377	77 (20.4)	300 (79.6)	

[a] Defined by Sreevatsan et al. (18).
[b] Fisher's exact, two-tailed test.

TABLE 4. Spoligotypes of the IS*6110* zero-copy-number *M. tuberculosis* isolates

Spoligotype[a]	Spoligotype pattern[b]
S293	■■■■□□□□■■■■■■■□□□□□□□□□□□□□□□□□□□□■■
S294	■■■■■■■■□□□□■■□■□□□□□□□□□□□□□□□□□□□■■
S295	■■■■■■■■□■□□■■□■□□□□□□□□□□□□□□□□□□□■■
S296	■■■■■■■■■■■■■□□□□□□□□□□□□□□□□□□□□□□■■

[a] Arbitrary designation.
[b] Black squares denote hybridization with the spacer probe; open squares denote lack of hybridization.

linked. This cluster consisted mainly of black patients living in two neighborhoods on the north and the south side of Houston that were linked by a public bus route. In addition, 11 patients in each of three clusters (999.009, 999.019, and 999.022) shared similar TB risk factors even though direct links between the patients could not be identified. For one of the three clusters, patients were human immunodeficiency virus (HIV)-positive homosexual men, and for two clusters the patients had a history of homelessness and incarceration. Seven patients in cluster 999.008 and six patients in cluster 999.031 were identified as laboratory cross-contaminants. Overall, for 18 of the 31 clusters, the majority (51 to 100%) of the patients belonged to the same ethnic group. Four clusters consisted mainly of Vietnamese patients, five of Hispanic patients, two of white patients, and seven of black patients. No epidemiologic links were identified between patients whose isolates were not clustered.

Zero-copy-number isolates. Six patients were infected with *M. tuberculosis* isolates that did not hybridize with the standard, right-side IS*6110* probe, suggesting that the isolates did not contain IS*6110*. PCR-based sequencing of the *hsp65* gene confirmed that the isolates belonged to the *M. tuberculosis* complex. Four different spoligotypes were obtained from the six isolates. The four spoligotypes had similar patterns (lacking spacers 19 to 41) but have not been previously identified (Table 4). Two U.S.-born patients were infected with *M. tuberculosis* isolates with the spoligotype arbitrarily designated S295, whereas two of the four Vietnamese patients were infected with isolates with the spoligotype arbitrarily designated S296. However, no direct epidemiologic links were found between the patients sharing the same spoligotype. All zero-copy-number isolates belonged to major genetic group 1.

DISCUSSION

The aim of this study was to evaluate spoligotyping as a technique for subtyping IS*6110* low-copy-number isolates cultured in Houston, and to investigate the epidemiologic links between patients in case clusters identified by the molecular methods.

Clustering and epidemiologic links. The number of low-copy-number *M. tuberculosis* clusters increased from 13 when examined by IS*6110* profiling alone to 31 after using a combination of IS*6110* profiling and spoligotyping results. However, the clustering percentage only decreased from 86.5 to 79.6%. This clustering percentage is much higher than that observed in a similar study performed in Denmark, where the clustering percentage decreased from 83 to 55% (2), and higher than the 59% clustering observed in Houston overall (data not shown).

A review of patient interviews and contact investigation reports revealed that only 33 patients (14.5%) were directly linked to another patient in the same cluster. However, possible epidemiologic links were also found among 11 patients in three clusters, and 13 patients were found to be laboratory cross-contaminants. Moreover, many clusters consisted mainly of individuals with the same ethnicity. We thus believe that the clustering results obtained by combining IS*6110* profile and spoligotype represent true clustering. However, clustering of *M. tuberculosis* isolates measured by DNA clonality does not always indicate recent transmission but may represent transmission of strains endemic to the area. Since some clusters consisted of foreign-born patients only, it is possible that the clustering results reflect reactivation of a latent infection with a common organism endemic in the country of origin of the patients. An analysis of a large sample of common *M. tuberculosis* isolates from these countries could verify this hypothesis.

The combination of the methods used may have overestimated clustering of the low-copy-number isolates in some cases. Two of the largest clusters (999.008 and 999.016) had related IS*6110* profiles (902 and 908 [Fig. 1]) that shared two IS*6110* elements and had the same spoligotype, S12, but no epidemiologic links were identified between members of the two clusters. We have previously shown that this spoligotype is the most common spoligotype among low-copy-number *M. tuberculosis* isolates cultured in Houston (17). However, when the large clusters were analyzed by an additional typing method, DRE-PCR, they were not further divided, suggesting that these two clusters are distinct. Nevertheless, it is possible that in some cases clustering results could be further improved by using the PGRS RFLP method or by analyzing the isolates with the new spoligotyping test that contains additional spacer probes (20).

U.S.- and foreign-born patients. Studies performed in Denmark and France show that the majority of the patients infected with low-copy-number isolates were foreign born (2, 10). In this study, 63.9% of the isolates originated from U.S.-born patients. However, the proportion of U.S.-born patients infected with low-copy-number isolates was slightly lower than the 70.3% that was observed in Houston overall (data not shown). Low-copy-number isolates from foreign-born patients were significantly less likely to be clustered than isolates from U.S.-born patients ($P < 0.01$).

Major genetic groups. Sreevatsan et al. (18) have shown that *M. tuberculosis* complex isolates can be differentiated into three major genetic groups based on DNA polymorphism in codons

katG 463 and *gyrA* 95. They proposed that group 1 isolates are evolutionarily older and have subsequently evolved to group 2 and 3 organisms (18). Our results show that major genetic group 1 isolates were significantly more prevalent among patients of Asian origin, whereas the majority of Mexican and U.S.-born patients were infected with isolates belonging to major genetic group 2. These data add to the concept that *M. tuberculosis* isolates of different genetic groups are prevalent in different areas of the world. No major genetic group 3 isolates were identified in our study, confirming the previous observation that low-copy-number *M. tuberculosis* isolates belong to evolutionarily older genetic groups 1 and 2 (18).

Zero-copy-number isolates. The first isolate lacking IS*6110* was identified in 1993 from an Indian patient (21), and thus far, about 15 such isolates have been reported in the literature, originating mainly from Asian patients (4, 5, 7, 13, 23). These isolates can be identified by other molecular typing methods such as spoligotyping (13). We identified six zero-copy-number isolates from four Vietnamese patients and two U.S.-born patients. The six isolates were divided into four spoligotypes that had similar patterns, suggesting that they may have evolved from a common ancestor. Our data also confirmed that the DR region appears to be present in genomes of all *M. tuberculosis* isolates, even in those that lack IS*6110* (7, 13). All zero-copy-number isolates belonged to major genetic group 1, suggesting that they represent evolutionarily older *M. tuberculosis* isolates (18).

Conclusions. Our study confirmed that IS*6110* profiling and spoligotyping together provide an enhanced molecular discrimination method of low-copy-number *M. tuberculosis* isolates relative to IS*6110* profiling or spoligotyping alone. Identification of epidemiologic links among some of the patients verified that the combination of the two methods reliably indexes tuberculosis transmission. In addition, all low-copy-number *M. tuberculosis* isolates were found to belong to the evolutionarily older major genetic groups 1 and 2.

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