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Spoligotyping of *Mycobacterium tuberculosis* Isolates from Multiple-Drug-Resistant Tuberculosis Patients from Bombay, India

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Spoligotyping was undertaken in 65 multiple-drug-resistant *Mycobacterium tuberculosis* isolates from Bombay, India. The spoligotype patterns showed seven closely related clusters, a cluster with 2 Beijing-like isolates, and unique spoligotypes (43%). Of the clusters, one with 29% of all the isolates suggested transmission of a dominant resistant clone.

The increasing worldwide prevalence of multidrug-resistant (MDR) strains of *Mycobacterium tuberculosis* represents a major threat to tuberculosis (TB) control programs (8). Although data on drug-resistant TB are lacking in India due to the absence of a reliable surveillance network, a World Health Organization survey in 1997 in the state of Tamil Nadu (28) and a limited study in an urban tertiary care center in Bombay (25) reported figures of 7.1 and 58% prevalence of multidrug resistance, respectively, indicating that MDR TB may pose significant problems. The design of strategies for the management of MDR TB depends on an understanding of the development and spread of resistant isolates. Well-documented outbreaks in settings of low endemicity demonstrate the efficacy of MDR TB isolates in generating new incident cases (4), but less is known about the ability of resistant isolates to compete with other strains of *M. tuberculosis* in areas of high endemicity such as Bombay.

The objective of this study was therefore to obtain an initial assessment of the extent to which the transmission of dominant clones of *M. tuberculosis* contributes to MDR TB in Bombay. The few studies on the molecular epidemiology of Indian *M. tuberculosis* isolates show low copy numbers of the IS*6110* insertion element, making them refractory to typing by the standard restriction fragment length polymorphism (RFLP) system (19, 20). The present study used spoligotyping, a PCR technique based on DNA polymorphism at the direct repeat locus of the genome of the *M. tuberculosis* complex (11, 13), as an alternative to IS*6110* RFLP. Although the overall discriminatory power of spoligotyping is lower than that of IS*6110* typing (11), it has the specific advantage of higher discrimination of strains with low copy numbers of IS*6110* (3).

We describe a cross-sectional analysis of a panel of 65 MDR TB isolates from Bombay. The patients were referred to the Foundation for Medical Research by local public and private providers for refractoriness of their infections to standard antituberculous therapy. Demographic details of the patients are presented in Table 1.

Patient specimens, including early morning sputum samples ($n = 53$) or blood, in the absence of productive cough (in 12 human immunodeficiency virus [HIV]-positive patients), were collected. The sputum and blood samples were processed by the modified Petroff's method (2) and the lysis-centrifugation method (15), respectively, cultured in Dubos' liquid medium and Löwenstein-Jensen slants and tested for niacin and pyrazinamidase production to confirm their identity as *M. tuberculosis* (14).

Drug sensitivity testing was performed in 53 of the 65 isolates by the radiorespirometric Buddemeyer technique (5). A growth index of ≥20% of the positive control for any drug was considered to indicate resistance to that drug. Multiple-drug resistance, as opposed to the classical definition, was defined as resistance to any two or more anti-TB drugs.

The genomic DNA from the isolates was extracted by the cetyltrimethylammonium bromide-phenol-chloroform method (22) and spoligotyped according to the method described previously (13). The spoligotype patterns were analyzed and corroborated manually by two independent observers. A cluster was defined as two or more isolates from different patients with identical spoligotype patterns, whereas nonclustered patterns were referred to as unique.

A total of 37 (57%) strains could be grouped into 8 different clusters (Fig. 1). The largest cluster (cluster 1) comprised 19 isolates, whereas the remaining seven clusters consisted of 2 to 4 isolates. Unique (nonclustered) spoligotype patterns were seen in 28 (43%) isolates. Of the 14 isolates from HIV-seropositive individuals, 9 belonged to the single largest cluster of 19 isolates (cluster 1).

The spoligotype pattern of the 2 isolates in cluster 8 (Fig. 1), with hybridization only to the 3′-terminal spacers 35 to 43, is characteristic of the Beijing genotype, a dominant strain in

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TABLE 1. Study population

Characteristic	Result for characteristic	No. (%) of clustered isolates	No. (%) of unique isolates	Total (n = 65) no. (%) of isolates	P value
Age (yr)	15–30	16 (57)	12 (43)	28 (43)	<0.9[a]
	31–45	16 (55)	13 (45)	29 (45)	<0.8[b]
	46–60	3 (75)	1 (25)	4 (6)	<0.8[c]
	>60		1 (100)	1 (1)	
	NA[d]	2 (67)	1 (33)	3 (5)	
Gender	Male	28 (57)	21 (43)	49 (75)	<0.8
	Female	9 (56)	7 (44)	16 (25)	
HIV status	Seropositive	16 (73)	6 (27)	22 (34)	<0.1
	Seronegative	21 (49)	22 (51)	43 (66)	
History of treatment	Previous anti-TB therapy	21 (51)	20 (49)	41 (63)	<0.3
	No previous treatment	14 (64)	8 (36)	22 (34)	
	Previous details NA	2 (100)		2 (3)	

[a] This P value reflects a comparison of the age ranges 15 to 30 years and 31 to 45 years.
[b] This P value reflects a comparison of the age ranges 31 to 45 years and 46 to 60 years.
[c] This P value reflects a comparison of the age ranges 15 to 30 years and 46 to 60 years.
[d] NA, not available.

many Asian countries (1, 26). IS*6110* RFLP of the isolates showed identical patterns of 15 bands (data not shown), again consistent with the Beijing genotype. There was no obvious epidemiological link between the two patients infected with Beijing strains with respect to the locations of their residences, their occupations, or the commonality of referring health centers. Both isolates were resistant to all individual first- and second-line drugs. A similar frequency of the Beijing genotype (3%) was previously reported among a panel of strains from India (26) and is in marked contrast to the dominance of this genotype in other parts of Asia (1, 26). The detection of the Beijing genotype is important, however, because of its reported association with drug resistance (1, 7) and with outbreaks of MDR TB (4, 7).

Two nonclustered isolates (Fig. 2, E7 and E8) had spoligotype patterns lacking terminal spacers 39 to 43, a profile generally associated with *Mycobacterium bovis* (6, 13, 16). Although both isolates were positive for niacin production, like *M. bovis*, they showed a negative pyrazinamidase test. However, further PCR-based genotyping of these isolates with previously described methods for RD5 and RD7 deletions (21), and commonly associated with *M. bovis* strains (10, 17, 21, 27), showed an absence of these deletion events, as did a novel method employing flanking primers for the RD8 region. The sequences of the RD8 primers were 5′-GAGTCTATATAGT GTGCTCATGGGGCTAGC-3′ (forward) and 5′-GCTTGCT GGCGATCATTGGTCT-3′ (reverse). These amplify a 178-bp product in strains which have undergone this deletion event. PCR conditions for the RD8 PCR were identical to those for the RD7 PCR. Moreover, amplification and sequencing of the *oxyR285* polymorphic site, as described previously (21, 24), showed nucleotide G, which is typical of *M. tuberculosis* isolates (23). The balance of evidence thus suggests the amplification failure of one or more terminal direct repeat spacers in *M. tuberculosis*, possibly due to the rearrangement or deletion of this region. Such intermediate profiles (12) may represent an ongoing evolution among these strains for broadening host tropism.

The chi-square test with Yates' correction was used to compare the association of the clustering of spoligotypes with HIV serostatus, age, and gender of patients. Overall, there was no significant difference in the clustering of spoligotypes between HIV-seropositive and -seronegative groups (Table 1). Similarly, there was no association of clustering with age, gender, history of previous treatment (Table 1), or specific drug susceptibility patterns (Table 2).

The detection of 36 different spoligotypes among the panel of drug-resistant isolates is consistent with a high rate of sec-

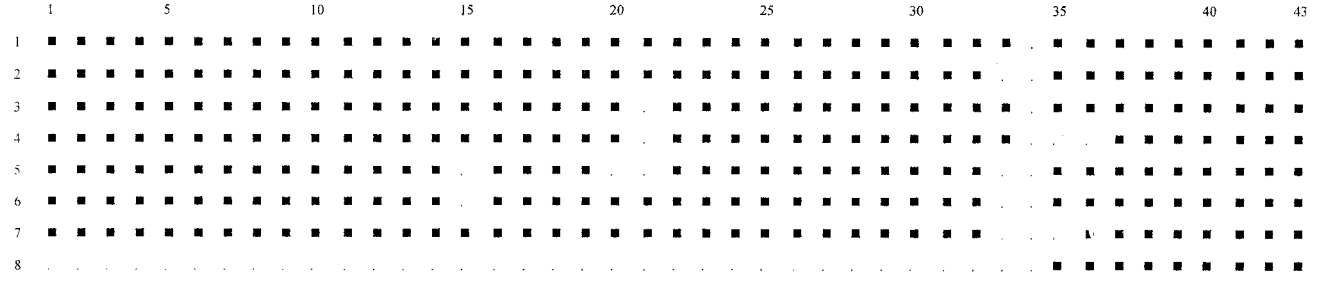

FIG. 1. Spoligotype patterns of clustered isolates. The filled boxes represent the presence of spacers, and the periods represent the absence of spacers. The numbers along the left of the figure are cluster numbers.

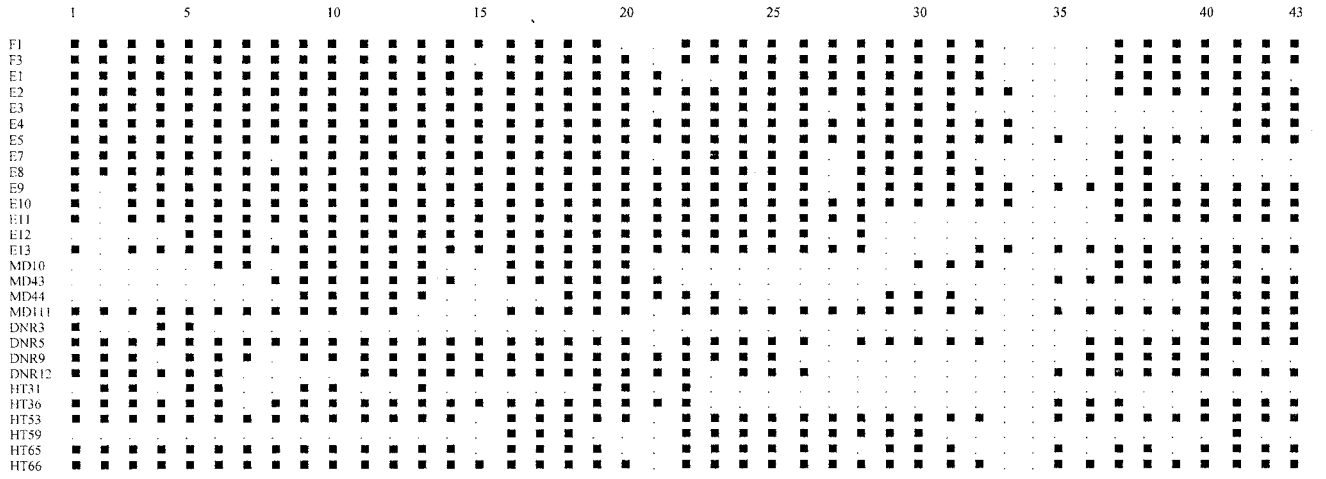

FIG. 2. Spoligotype patterns of unique isolates. The filled boxes represent the presence of spacers, and the periods represent the absence of spacers. The codes along the left of the figure are patient codes.

ondary, or acquired, resistance in this population, reflecting the problems of efficient TB control in a metropolis with overburdened health facilities (D. D'souza, N. F. Mistry, B. A. Rajgor, and N. H. Antia, submitted for publication). However, the sharing of a single spoligotype by 29% (19 of 65) of the isolates (cluster 1) suggests an important role for the transmission of a dominant resistant clone. While alternative genotypic techniques (9, 16, 18) are required to determine whether cluster 1 indeed represents a clonal population, this study may provide the basis for a systematic and extended study of MDR TB strains in India.

TABLE 2. Drug sensitivity testing of the isolates by using the radiorespirometric Buddemeyer assay

Test or characteristic	No. (%) of clustered spoligotypes	No. (%) of unique spoligotypes	Total (n = 65) no. (%) of spoligotypes
Tests			
Drug sensitivity[d]	36	17	53
Sensitivity to both first and second-line drugs[e]	33	15	48
Characteristics			
First and second-line drug resistance[g]	13 (39)	6 (40)	19 (40)
Rifampin resistance[g]	27 (75)[a]	15 (88)	42 (79)
Isoniazid resistance[g]	35 (97)[b]	17 (100)	52 (98)
Classical MDR[f,g]	27 (75)[a]	15 (88)	42 (79)
Resistance to any two drugs[g]	36 (100)[c]	17 (100)	53 (100)

[a] $P < 0.3$.
[b] $P < 0.5$.
[c] $P < 0.1$.
[d] Only first-line drug sensitivity was tested in three clustered and two unique isolates. Values are the number of isolates subjected to testing.
[e] First-line anti-TB drugs: isoniazid, rifampin, ethambutol, streptomycin, and pyrazinamide. Second-line anti-TB drugs: cycloserine, amikacin, ethionamide, kanamycin, and paraaminosalicylate. Values are the numbers of isolates subjected to testing.
[f] Classical MDR, resistance to at least isoniazid and rifampin.
[g] Values are the numbers of isolates displaying the characteristic listed.

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