

A Combination of Two Genetic Markers Is Sufficient for Restriction Fragment Length Polymorphism Typing of *Mycobacterium tuberculosis* Complex in Areas with a High Incidence of Tuberculosis

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The incidence of tuberculosis (TB) in Madagascar is 150 cases per 100,000 people. Because of this endemicity, we studied the genetic diversity of *Mycobacterium tuberculosis* strains isolated in four big cities in 1994 to 1995 with the aim of monitoring TB transmission. Isolates from 316 cases of pulmonary TB (PTM⁺) were typed by Southern hybridization with genetic markers IS6110 and DR. Of the 316 PTM⁺ strains, 66 (20.8%) had a single IS6110 band and were differentiated by the DR marker into 33 profiles. Using both markers, 37.7% (119) of the patients were clustered, a proportion similar to that in countries with a high prevalence of TB. There was no significant difference between clustered and nonclustered patients in age, sex, *Mycobacterium bovis* BCG status, and drug susceptibility of strains. Clustering was significantly greater in the capital, Antananarivo, than in the other cities, suggesting a higher rate of transmission. However, most of the patients in clusters were living in different areas, and, within a distance of 0.7 km, we did not find epidemiologically unrelated strains with the same restriction fragment length polymorphism profile. Despite an apparently low polymorphism, genetic markers such as IS6110 are potentially valuable for monitoring TB transmission. However, the high proportion of Malagasy isolates with a single IS6110 copy makes this marker alone unsuitable for typing. Additional markers such as DR are necessary for the differentiation of the isolates and for epidemiological surveys.

Since the discovery of repetitive elements associated with polymorphism and their use as genetic markers for *Mycobacterium tuberculosis*, strain differentiation by DNA typing has greatly facilitated epidemiological studies (7, 8, 35). To type *M. tuberculosis* complex strains, the most extensively used method has been a standardized protocol of restriction fragment length polymorphism (RFLP) analysis with insertion sequence IS6110 as a marker (29, 31). This method has also been used successfully in confirming epidemics and nosocomial infections and in studies on transmission of tuberculosis (TB) in high-risk communities (5, 11, 13, 15, 17, 24). Another marker often used for discriminating strains is the direct repeat (DR) region, which contains multiple 36-bp DRs separated by variable spacers (31 to 45 bp in length), which are sources of diversity (21).

In Madagascar (about 13 million inhabitants), the incidence of pulmonary tuberculosis is very high (about 150 cases per 100,000 people) (9). In contrast, the prevalence of human immunodeficiency virus (HIV) is low, and between 1989 and 1994 only six of a total of 3,168 tuberculous patients tested were HIV seropositive (4). RFLP studies of clinical isolates in countries with a high incidence of TB describe limited polymorphism of TB genetic markers such as IS6110. To determine the usefulness of this marker to monitor transmission in these

countries, we studied the diversity of *M. tuberculosis* complex strains from pulmonary patients (PTM⁺) in four big cities in Madagascar. We have correlated strain polymorphism with geographic areas. Such data will allow the detection of epidemics and an understanding of TB transmission in this country.

MATERIALS AND METHODS

Study population. This study was carried out in the capital, Antananarivo, and in three other large cities, Antsirabe, Fianarantsoa, and Mahajanga, and was conducted jointly with a survey of *M. tuberculosis* primary resistance in 1994 to 1995 (10).

Between August 1994 and December 1995, of the 1,389 PTM⁺ patients identified in eight diagnostic and treatment centers, 1,108 new patients (81.5%) were included. For DNA fingerprinting, 316 patients were randomly sampled. They were representative of the eight centers: 153 patients from Antananarivo, 27 from Antsirabe, 64 from Fianarantsoa, and 72 from Mahajanga. The age of the patients was 11 to 74 years (mean age: 34 years). The male-to-female ratio was 1.53:1. *Mycobacterium bovis* BCG status was known for 306 patients, of whom 223 were vaccinated.

All patients included in this study were Malagasy and HIV seronegative.

Bacteriology. All clinical specimens were cultured on standard Lowenstein-Jensen (LJ) medium (Diagnostics Pasteur, Paris, France) and on LJ medium without glycerol but supplemented with 0.5% pyruvate. Mycobacterial isolates were identified according to growth on LJ medium, colony morphology, and biochemical tests for the following: niacin production, catalase, urease, and nitrate reductase (20). Drug susceptibility of the PTM⁺ isolates to streptomycin, isoniazid, rifampin, and ethambutol was tested using the proportion method (6), as recommended by the Global Tuberculosis Programme of the World Health Organization and the International Union against Tuberculosis and Lung Disease (19).

RFLP typing. Genomic DNA from only one strain from each PTM⁺ patient (316 isolates) was extracted according to the method described by van Soolingen et al. (36) and tested by RFLP analysis. DNA fingerprints were obtained using a standardized RFLP technique with the IS6110 insertion sequence (IS6110 pat-

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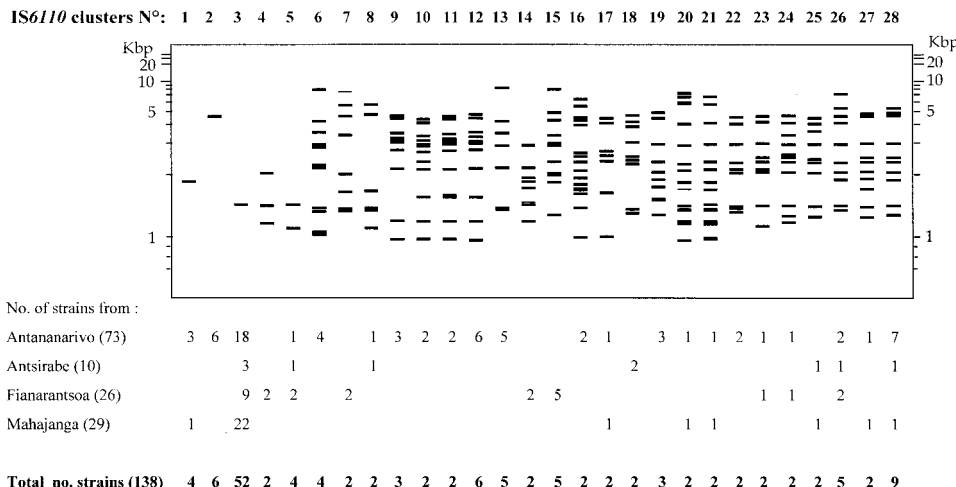


FIG. 1. IS6110 clusters of PTM+ isolates in Madagascar.

tern) (31). Briefly, chromosomal DNA was digested with restriction endonuclease *PvuII* (Pharmacia Biotech) and was hybridized with the 807-bp *PvuII-XhoI* fragment of IS6110 (29). For strains with one to four copies of IS6110, DNA was also digested with *AluI* (Pharmacia Biotech) and hybridized with the 36-bp DR sequence probe (5'GTCGTCAGACCCAAAACCCCGAGAGGGGACGGAAAC3') (21). All probes were labeled with horseradish peroxidase and detected with the enhanced chemiluminescence system (ECL; Amersham).

Analysis of RFLP patterns. The Taxotron software (P. A. D. Grimont, Institut Pasteur, Paris, France) was used for computer-assisted analysis of the RFLP patterns. Patterns were compared by the unweighted pair group clustering method of averages, and matching was further confirmed by visual examination. A cluster of *M. tuberculosis* strains was defined as two or more isolates with identical RFLP patterns. A cluster of patients was defined as two or more patients with identical strains. Epidemiological relationships between patients belonging to a cluster were also investigated. The geographical distribution of the clusters was assessed on the basis of the place of residence of the patients. The characteristics of clustered patients and nonclustered patients were compared. For assessing differences between percentages, we used the χ^2 test or Fischer's exact test for values ≤ 5 . Differences were considered significant if *P* values were < 0.05 .

RESULTS

DNA polymorphism of the *M. tuberculosis* complex strains isolated from PTM+ patients. The IS6110 RFLP types for 316 PTM+ strains, a representative sample corresponding to 28.5% of the patients included (and 23% of the patients identified in the four cities for the 1994 to 1995 period), were determined. Of these strains, 312 were identified as *M. tuberculosis* and 4 were identified as *M. bovis*.

One strain from Antananarivo had no hybridizing band, suggesting that it did not contain the IS6110 element. One hundred and seventy-seven isolates (56%) had unique IS6110 patterns, and 138 (43.6%) were assigned to 28 IS6110 clusters of 2 to 52 strains (Fig. 1). The number of IS6110 copies per strain was between 1 and 22 (Fig. 2). The number of bands in the 177 isolates with unique banding patterns also varied from 1 to 22, and in the clustered isolates the number varied from 1 to 17. Sixty-six strains (20.8%) had a pattern with only one hybridizing band, suggesting that they contained a single IS6110 copy. The proportion of strains with a single IS6110 copy was the highest in Mahajanga (36.1%); the proportions were 17.6, 15.6, and 11.1% in Antananarivo, Fianarantsoa, and Antsirabe, respectively. Among these 66 strains, seven frag-

ment sizes were observed: 1.35 (1 strain), 1.45 (52 strains), 1.56 (1 strain), 1.8 (4 strains identified as *M. bovis*), 4.1 (1 strain), 4.8 (6 strains), and 5.1 kb (1 strain). Moreover, 30 strains had a pattern with two to four bands containing IS6110. The strains with zero to four IS6110 copies were further genotyped with the DR marker (Fig. 3) (one strain of IS6110 cluster 4 was not viable and, therefore, could not be DR typed). Among those strains with zero to four IS6110 copies, from 4 to 14 restriction fragments were hybridized with the DR probe, producing 46 different DR patterns. Thirty-seven isolates had unique DR patterns, and 59 fell into nine clusters of 2 to 10 strains. There were isolates having the same DR pattern but having different IS6110 profiles (Table 1; Fig. 3). DR cluster A, for example, contained 19 isolates that could be differentiated into nine IS6110 profiles. However, for those strains with one IS6110 copy, strains with different IS6110 profiles also had different DR patterns (Table 2; Fig. 3A). Likewise, the same DR pattern was observed for all *M. bovis* strains, and it was different from the DR patterns of *M. tuberculosis* isolates. The strain without the IS6110 element had a DR profile different from that of all other strains.

On the basis of both IS6110 and DR patterns, 197 PTM+ patients (62.3%) gave isolates that were not clustered and 119

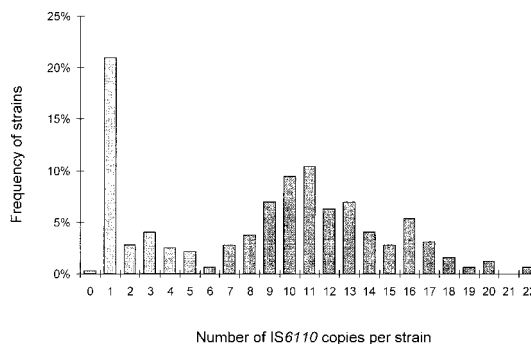


FIG. 2. Numbers of IS6110 copies in 316 *M. tuberculosis* complex strains isolated from PTM+ patients in Madagascar.

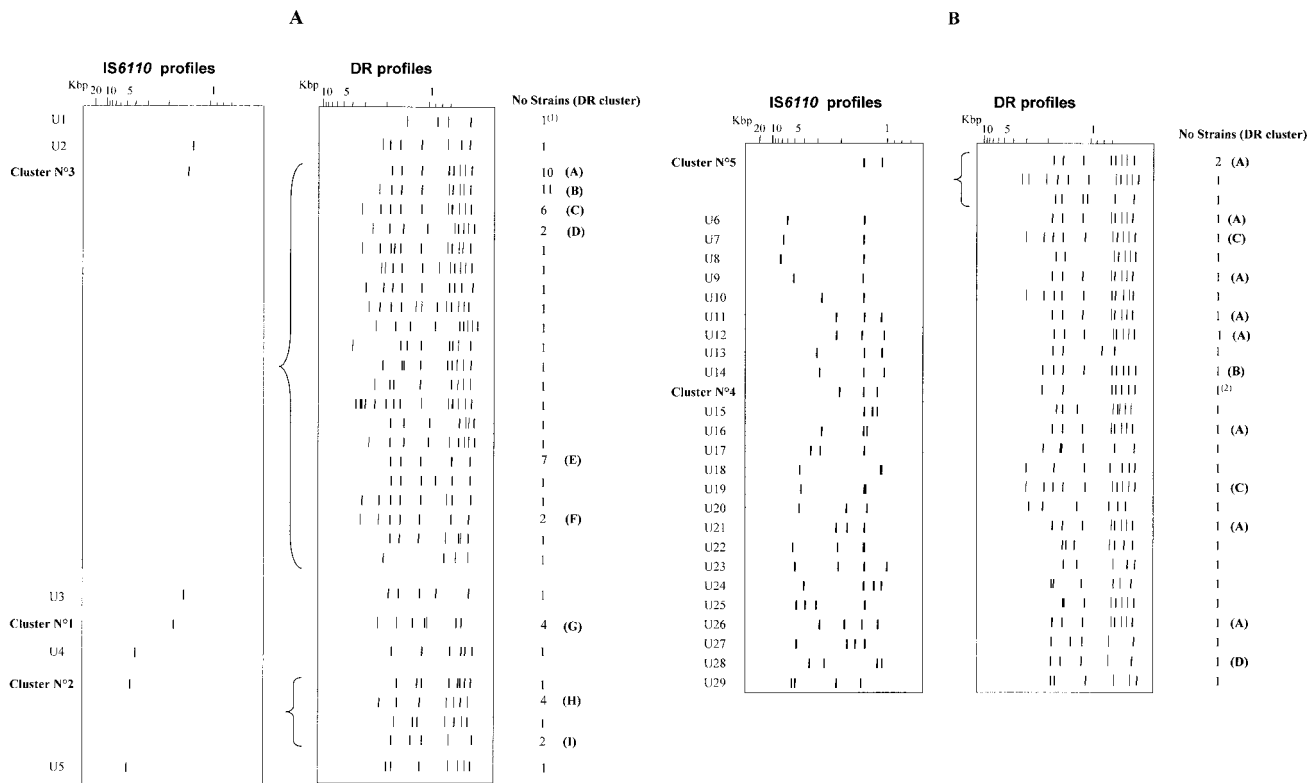


FIG. 3. (A) DR patterns of the isolates with zero or one *IS6110* copy. (B) DR patterns of the isolates with two to four *IS6110* copies. The numbers of *IS6110* clusters are given on the left. U1 to U29, strains with unique *IS6110* profiles; A through I, DR clusters; (1), *M. bovis* strains; (2), strain of *IS6110* cluster 4 not viable and not available for DR typing.

(37.7%) gave isolates that were contained within 34 clusters of 2 to 10 isolates (Table 3). The 19 additional strains demonstrating a unique profile with two markers were composed of two groups: (i) 17 strains with one *IS6110* band (Fig. 3A) and (ii) 2 strains with two *IS6110* bands (Fig. 3B). Therefore, it appears that DR typing is especially useful for discrimination between isolates with one *IS6110* copy. The small clusters were the most frequently observed group, accounting for 68% of the clusters but less than 50% of the isolates. These were further broken down as 21 clusters of two patients and 2 clusters of three patients. In comparing the characteristics of the clustered and the nonclustered patients (Table 4), we found that clusters of patients did not differ significantly in age, sex ratio, and BCG status and that the drug susceptibility patterns of the isolates also did not differ significantly. Similarly, no significant difference between the two groups within any of the individual cities was found (data not shown). Finally, a comparison of the RFLP profiles of drug-resistant and drug-susceptible strains revealed no correlation between drug resistance and RFLP pattern (36).

Geographical distribution of the clusters. One hundred and thirty-eight strains (43.6% of the studied strains) were assigned to 28 *IS6110* patterns. Thirteen of them (eight clusters of two patients and five with more than two patients) were recovered in at least two different cities (Fig. 1). One *IS6110* pattern (two patients) was found only in Antsirabe, 4 were found only in Fianarantsoa, and 10 were found only in Antananarivo. An RFLP pattern with an *IS6110* band at 1.45 kb (*IS6110* cluster

3) was the only profile found in all four cities. Of the 46 DR patterns of the strains with one *IS6110* copy, only 6 were found in two or more cities (data not shown).

When both the *IS6110* and DR markers were used together, the proportion of clustered patients differed significantly between the cities. It was higher in Antananarivo (38.5%; 59 of 153) than in the three other cities ($P = 0.003$): 26.5% (17 of 64) in Fianarantsoa, 25% (18 of 72) in Mahajanga, and 7.4% (2 of 27) in Antsirabe. In Antsirabe, Fianarantsoa, and Mahajanga, the patients belonging to any single cluster lived in different areas of the city. In Antananarivo, except for two patients living at the same address and eight patients in Antananarivo

TABLE 1. Comparison of DR clusters and *IS6110* patterns of the isolates with four or less *IS6110* bands

DR cluster	Total no. of strains	No. of <i>IS6110</i> patterns		
		Total	Unique	Clusters (no. of strains)
A	19	9	7	2 (12)
B	11	2	1	1 (10)
C	8	3	2	1 (6)
D	2	1	0	1 (2)
E	8	2	1	1 (7)
F	2	1	0	1 (2)
G	4	1	0	1 (4)
H	2	1	0	1 (2)
I	3	2	1	1 (2)

TABLE 2. Number of DR patterns in 66 strains with one IS6110 copy

Location (kb) of IS6110 band (cluster)	Total no. of strains	No. of DR patterns		
		Total	Unique	Clustered (no. of strains)
1.45 (3)	52	21	15	6 (37)
4.6 (2)	6	4	2	2 (4)
1.8 (1)	4	1	0	1 (4 ^a)
1.35	1	1	1	0
5.1	1	1	1	0
1.56	1	1	1	0
4.1	1	1	1	0
Total	66	33	21	9 (45)

^a *M. bovis* strains.

prison, all the patients within clusters lived in different districts of the city. The two closest patients within the same cluster lived in different districts, within a distance of about 0.7 km (IS6110 cluster 3; DR pattern A). Although the polymorphism of strains in these cities seems relatively low, RFLP typing may be a valuable epidemiological tool for identifying transmission.

DISCUSSION

The present study is the first on DNA polymorphism of the *M. tuberculosis* complex strains in Madagascar, a country in which TB is hyperendemic (3). Four of the largest cities (Antananarivo, Antsirabe, Fianarantsoa, and Mahajanga) were chosen for this study. Using a two-genetic-marker typing method (IS6110 and DR), we found three IS6110 clusters containing strains that were differentiated using the second marker. There were two IS6110 clusters with isolates having one IS6110 band pattern (clusters 2 and 3) and one cluster with two-band-pattern isolates (cluster 5). Moreover, for some isolates having an IS6110 profile with two to four bands, some DR clusters contained isolates with different IS6110 patterns (Table 1). These results indicate that the changes in the DR and the IS6110 profiles are independent events and that only isolates with the same IS6110 and DR patterns can be considered identical strains. Our results can be favorably compared with those of Gillespie et al., who used the IS6110 and the PGRS markers for typing isolates from Tanzania with six or more band profiles (18). They found that only 1 of 13 IS6110 clusters contained isolates with different PGRS patterns. In this study, genotyping with a second marker was done only on isolates with patterns of less than five IS6110 bands. Thus, both our results and those of Gillespie et al. showed the usefulness of two-marker genotyping. An additional advantage of typing with the DR sequence that we chose as a second marker is that it can be performed using the PCR technique, avoiding large-scale cultures of *M. tuberculosis* (23).

The overall polymorphism of the strains as assessed by the number of clustered strains for the two genetic markers, IS6110 and DR, was low (37.7% for PTM⁺ strains). This is similar to results reported from other African and Asian countries but differs from what is observed in Europe or North America (16, 22, 35, 36.). The majority of the clusters (21 of 34) contained only two strains, probably due to the limited sample size. Only 42% (13 of 28) of the IS6110 patterns and 13% (6 of 46) of the DR patterns were found in more than one

TABLE 3. Degree of discrimination of IS6110 versus IS6110 plus DR

Marker	Total no. of strains	No. of isolates from:	
		Single strains with unique patterns	Clustered strains (no. of clusters)
IS6110	315	177	138 (28)
IS6110 + DR	316	197 ^a	119 (34)

^a Includes one strain with no IS6110 band.

city, suggesting limited transmission between patients from different cities. Moreover, most patients contained within specific clusters lived in different areas in their city rather than in close proximity. The minimum distance between two clustered patients without any reported epidemiological link was 0.7 km. Despite the finding that the clustering of these patients was not related to risk, it is apparent that the polymorphism of markers used for *M. tuberculosis* strains is sufficient for detecting epidemics, for monitoring the transmission of resistant strains, and for tracking TB transmission in high-risk groups within particular areas (26).

Molecular epidemiology studies can lead to two hypotheses: first, that the degree of clustering can be used as a measure of recent and active transmission of TB (2, 28, 30), and second, that more young patients than old patients have recent infections and thus are more likely to belong to clusters. In our study, we found that the proportion of clustered patients was higher in Antananarivo than in the other cities, suggesting a more-active transmission of TB in the capital. However, ages of clustered and nonclustered patients were not significantly different. There are several possible explanations for this: (i) the population in the capital is highly mobile, (ii) the incuba-

TABLE 4. Characteristics of clustered and nonclustered PTM⁺ patients

Characteristic	Value for:		
	Total (n = 316)	Clustered (n = 119)	Nonclustered (n = 197)
Mean age (age range) (yr)	34.01 (11–74)	33.94 (13–70)	34.08 (11–74)
Sex (no. of patients)			
Female	125	52	73
Male	191	67	124
BCG status (no. of patients)			
Vaccinated	223	85	138
Unvaccinated	83	31	52
Unknown	10	3	7
Susceptibility to S, H, R, and E ^a (no. of patients)			
Susceptible to all	250	98	152
Resistant to			
One drug	48	13	35
Two drugs	4	1	3
Three drugs	1	1	0
H and R	1	1	0
ND	13	6	7

^a S, streptomycin; H, isoniazid; R, rifampin; E, ethambutol; ND, not determined.

tion time after an infection is highly variable, (iii) the prevalence of TB and the circulation of strains are so high that the risk of reinfection is the same throughout the population, and (iv) the reactivation of old infections is less frequent than expected (32). Another possibility is based on a recent study in Malaysia (12), where the authors reported an apparent increase in clustering with the age of the patients and suggested that strains with stable RFLP patterns may be in circulation for longer periods and thus may contribute to clustering. Differences in virulence among the strains could also explain the presence of some clusters. For example, a virulent strain would be isolated more frequently than a nonvirulent strain and thus would produce larger clusters.

Only one isolate did not contain the IS6110 genetic element. Zero-copy strains have previously been described, with slightly higher frequencies noted in patients from Vietnam, China, Thailand, and India (1, 14, 25, 36). One interesting characteristic of the Malagasy strains was the high proportion of single-IS6110-copy strains (21.6% of the PTM⁺ sampled strains) including seven different positions of the single-hybridizing band. These strains could be easily differentiated by using the DR marker. The strains having an RFLP pattern with a single-hybridizing band at 1.8 kb were all identified as *M. bovis*. This pattern is the same as that described for the majority of *M. bovis* strains elsewhere (27, 34). Several of the one-IS6110-band patterns have previously been found with similar frequencies in: Vietnam (12%) (37), India (30 to 40%) (14, 34), Thailand (20%) (25), and Malaysia (17.5%) (16). Thus, there appears to be a similarity between Asiatic and Malagasy strains (zero- or single-IS6110-band strains). This is not surprising because the Malagasy population is descended from African, Asian, and Arabic peoples who migrated to Madagascar several centuries ago, and there have been extensive economic and tourist contacts between countries of the Indian Ocean and Madagascar for centuries. It would be interesting to use other genetic markers or the spoligotyping method (23) to compare the Malagasy and Asiatic single-IS6110-copy strains and to look for the presence of other typical Asiatic patterns such as the Beijing and Nonthaburi groups (25, 33).

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