

Molecular Fingerprinting of *Mycobacterium tuberculosis* on a Caribbean Island with IS6110 and DRr Probes

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Received 6 November 1996/Returned for modification 19 December 1996/Accepted 10 January 1997

Because of a substantial increase in human immunodeficiency virus (HIV) infection and HIV-linked tuberculosis in the Caribbean, a molecular fingerprinting study of clinical isolates of *Mycobacterium tuberculosis* isolated at the Pasteur Institute of Guadeloupe from 1994 to 1995 was undertaken with the insertion sequence IS6110 and the direct repeat DRr probes. We present the results for 72 isolates from 51 patients. A major cluster (cluster A) representing isolates from 12 patients (24%) was detected upon PvuII-IS6110 fingerprinting, which revealed a pattern of four bands among these isolates. Homogeneity was retained when the isolates were further analyzed with the DRr probe or further characterized by *AluI* and *SmaI*-DRr restriction fragment length polymorphism analysis. The isolates of cluster A, from 10 men and 2 women, was present in people of all ages and of different ethnic and geographical backgrounds, and infection with these isolates was independent of the HIV status of the patients (except for 2 HIV-positive patients from the same ward from whom the tubercle bacilli were isolated at the same time). The percentage of reactivation versus active transmission events could not be precisely determined in this study. These results are discussed on the basis of the genetic advantage of predominant clusters and/or specific characteristics of the settings from which a similar cluster of isolates with four bands has so far been reported, which include South Africa, French Polynesia, and Guadeloupe.

Tuberculosis remains one of the major world health problems both in the developing world and in industrialized countries (11). The global incidence of tuberculosis is predicted to continue to increase mainly due to socioeconomic underdevelopment among people in poor rural areas or among certain parts of the population in large urban centers (1, 10). In such a context, molecular fingerprinting of *Mycobacterium tuberculosis* isolates has become a high priority of research for tracking the global circulation of this disease (8). The internationally agreed upon IS6110-restriction fragment length polymorphism (RFLP) methodology allows investigators to shed light on the relative prevalence of reinfection versus reactivation in a given population (10). However, despite its discriminatory potential, IS6110-RFLP analysis alone may not be sufficient for establishing a link between strains sharing less than five identical bands (2), and use of a second genetic marker, e.g., direct repeat (DRr) probes (7), the polymorphic GC-rich sequences (pTBN12) (4), and the (GTG)₅ oligonucleotide (17), may be needed to discriminate between such isolates. Finally, the IS6110 element may also be helpful as a tool for studying the phylogeny of the *M. tuberculosis* complex (12).

Guadeloupe is a relatively small (area, 1,780 km²) but dense island of the Lesser Antilles (417,000 inhabitants in 1996) with a resident population of African, European, Indian, and Syrian-Lebanese origin and with recent immigrants of Haitian and Dominican origin. A recent epidemiological survey of the evolution of tuberculosis in the French Caribbean island of Guadeloupe between 1982 and 1994 showed that the incidence of the disease decreased from 25/100,000 inhabitants in 1982 to 10/100,000 inhabitants in 1988 and has stabilized at about 11/

100,000 inhabitants since 1989 (9). Together with Martinique and French Guyana, this geographical area in the Lesser Antilles shows an elevated incidence of AIDS; e.g., 30% of all tuberculosis patients in 1994 were coinfecting with human immunodeficiency virus (HIV) in Guadeloupe (9). The present report is a first attempt to systematically type all the *M. tuberculosis* strains isolated from patients on a Caribbean island during a 2-year period (1994 to 1995) by the IS6110-RFLP methodology and describes the transmission of tuberculosis on the island of Guadeloupe.

MATERIALS AND METHODS

Preparation of bacterial DNA and IS6110-RFLP. All the strains used in this study were grown as fresh Löwenstein-Jensen slants at 37°C and were isolated from clinical specimens from local patients. DNA was prepared by the cetyltrimethylammonium bromide method (14). IS6110 fingerprinting was performed essentially by using the internationally agreed upon methodology (14). Southern hybridizations were also performed with the DRr probe (a recently described 36-mer oligonucleotide (7) with *AluI*- and *SmaI*-digested DNAs. Labelling and detection were performed by using indirect ECL kits (Amersham, Buckinghamshire, United Kingdom). The development of the autoradiographs was performed manually with standard Kodak photochemicals.

Computer-assisted analysis of results. RFLP patterns were scanned and digitized by using the Ofoto software and a Macintosh Power PC 7200/90 computer (Apple Computers, Cupertino, Calif.). The scanned images were analyzed by using the Taxotron software package (P. A. D. Grimont, Institut Pasteur). The probability of cross contamination for two strains presenting similar RFLP profiles and consecutive identification numbers was ruled out because in our study such samples were not processed for culturing on the same day.

RESULTS

Demographic and clinical data. Seventy-two clinical isolates from 51 patients were identified as *M. tuberculosis* by the classical procedures. The origins of the samples were diverse: sputum, fibroscopic aspirates, bronchoalveolar fluids, gastric tubing, pleural punctures, urine, and hemocultures. All except three of the strains originated from Guadeloupe; two strains were from Suriname and one was from Martinique. The 51

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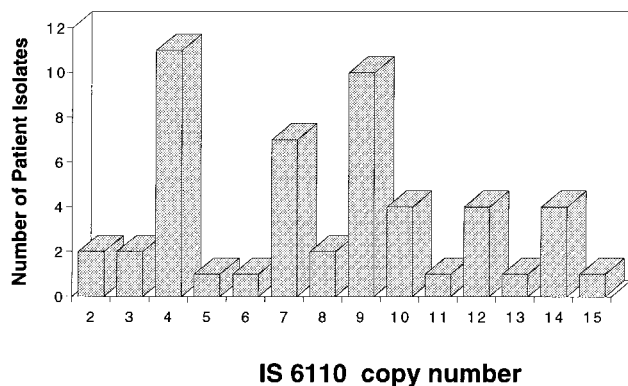


FIG. 1. Histogram showing the number of IS6110 copies in the chromosome of the *M. tuberculosis* clinical isolates studied ($n = 51$).

patients studied included 34 men and 17 women (sex ratio, 2). The number of HIV-seropositive patient was 18 (35%). Pulmonary tuberculosis prevailed in most patients (88%) (9).

The drug susceptibility patterns of the isolates showed that all except three isolates were sensitive to the common antimycobacterial agents; two isolates were resistant to a single drug (isoniazid), and another one was resistant to both isoniazid and rifampin. We did not detect any patient with successive infections with two genetically distinct strains.

IS6110-RFLP results. A total of 72 individual IS6110-RFLP patterns were obtained from the 72 isolates from the 51 patients. As we undertook a systematic genetic analysis of the strains isolated in our laboratory and since successive sputum or other samples from the same patient are usually available in order to check for the efficiency of antibiotic therapy, the redundancy of certain profiles was expected and was actually found for given patients, as demonstrated previously (3). Figure 1 shows the distribution patterns of the number of IS6110 copies for the 51 patient-derived isolates by eliminating the redundant profiles obtained. Contrary to certain regions of the world (6, 19), no isolates without or with a single copy of IS6110 was detected in our setting. The 51 patient-derived isolates in our case contained between 2 and 15 copies of IS6110. A computer-assisted analysis of our results was performed with 51 unique patient isolates, and the results are illustrated in Fig. 2. Three clusters were identified, a major four-band cluster (cluster A; bands of 4.8, 3, 2.3, and 1.4 kb) which included isolates with four bands from 12 patients and two minor clusters (clusters B and C), comprising 3 and 2 isolates, respectively. According to the specificity/sensitivity ratio chosen, 17 of 51 (33.4%) of the strains were clustered. When the pattern of isolates in cluster A was compared with previously published patterns of isolates from different regions of the world, this type of profile was found to be recently detected in at least two other regions, accounting for 15.6% of the profiles among isolates from French Polynesia (13) and an unspecified number among isolates from South Africa (17).

Subtyping with the DRr probe. The *PvuII*-IS6110 Southern blots were rehybridized with the DRr probe which may discriminate between isolates with identical IS6110-RFLP patterns with fewer than five copies of IS6110 (13). The representative patterns obtained with the IS6110 probe compared to the corresponding profiles obtained with the DRr probe are illustrated in Fig. 3. As shown in Fig. 3B and D, the patterns obtained with the DRr probe generally presented one to three bands whose positions could be superimposed with those of the existing IS6110 bands. Among 4 of 12 identical four-band pat-

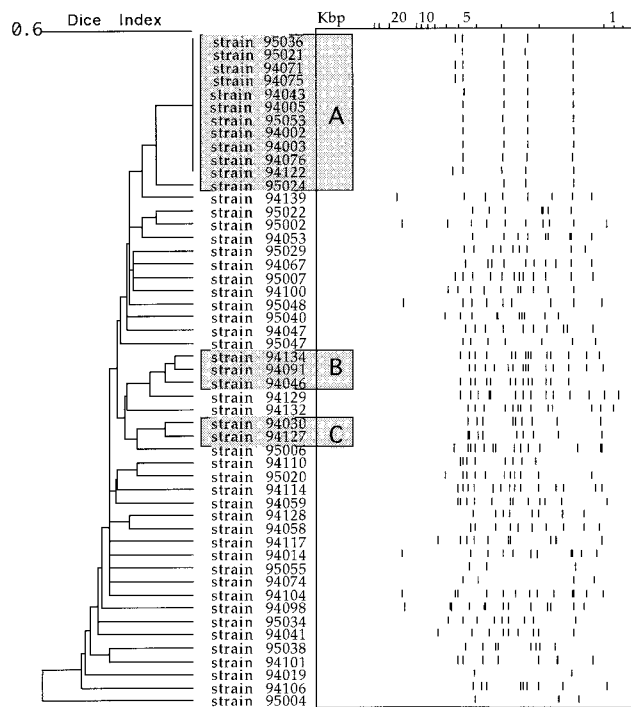


FIG. 2. Dendrogram and associated schematic view of the IS6110-RFLP patterns obtained for 51 clinical isolates of *M. tuberculosis* from Caribbean patients (the Dice index was used to compare the patterns, followed by the single-linkage method for clustering). The numbers designate various patient isolates. The clustered strains are shown within a grey frame and are termed clusters A, B, and C.

terns obtained with the IS6110 probe included in Fig. 3A, rehybridization with the DRr probe showed that this IS6110-defined cluster retained its homogeneity (Fig. 3B). When we correlated the hybridization patterns obtained with the DRr and IS6110 probes, our results demonstrated the link between the DRr locus and certain IS6110 copies. We also observed that no hybridization with the DRr probe was linked to the 1.4-kb IS6110 band of cluster A. However, an additional band by hybridization with the DRr probe in some clustered isolates was observed in a few cases and may have represented traces of nonspecific hybridization.

Confirmation of *PvuII*-DRr probe hybridization results by *SmaI*-DRr and *AluI*-DRr probe hybridizations. In order to establish the identities of cluster A isolates, DRr typing was repeated after *SmaI* and *AluI* digestions of cluster A isolates (Fig. 4A and B). All the strains presented the same pattern, confirming the homogeneity of the initial *PvuII*-IS6110-defined cluster A. All together, these results demonstrate unambiguously that between 1994 and 1995 24% of patients shared an identical isolate of *M. tuberculosis* in Guadeloupe and argued in favor of active transmission of disease.

Analysis of minor clusters. Minor cluster B (isolates 94091, 94134, and 94046) was also probed with the DRr probe as mentioned above and retained its homogeneity (data not shown). Minor cluster C, containing strains 94030 and 94127, was not investigated further because of an absence of any epidemiological link on the basis of demographic and clinical data.

DISCUSSION

Numerous studies have established the potential of IS6110 fingerprinting for studying the transmission of tuberculosis in

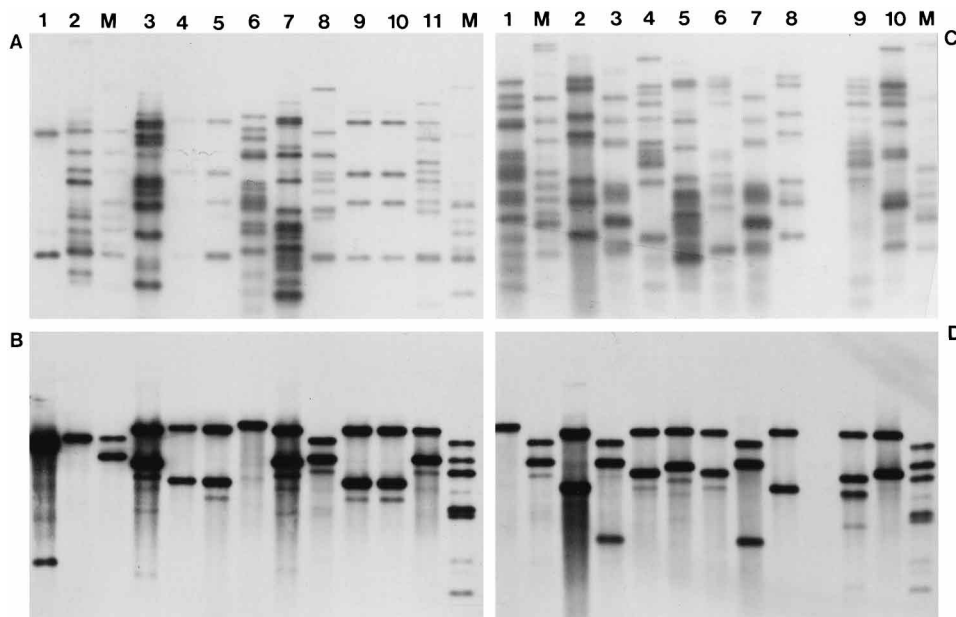


FIG. 3. *Pvu*II-IS6110 fingerprints of selected clinical isolates (A and C), followed by DRr probe rehybridization (B and D). (A and B) Lane 1, isolate 94019; lane 2, isolate 94106; lane 3, isolate 94030; lane 4, isolate 94005; lane 5, isolate 94043; lane 6, isolate 94046; lane 7, type strain H37Rv; lane 8, isolate 94041; lane 9, isolate 94075; lane 10, isolate 94071; lane 11, isolate 95034; lanes M, external marker, *M. tuberculosis* 14323. (C and D) Lane 1, isolate 94046; lane 2, isolate 94101; lane 3, isolate 95013; lane 4, isolate 95020; lane 5, type strain H37Rv; lane 6, isolate 94059; lane 7, isolate 95014; lane 8, isolate 94101; lane 9, isolate 94110; lane 10, isolate 94117; lanes M, external marker, *M. tuberculosis* 14323. It should be underlined that isolates 95013 (lane 3) and 95014 (lane 7) in panel C were isolated at a 12-day interval from the same patient and are identical to isolate 95022 shown in the dendrogram (Fig. 2), which was obtained 1 month later.

various geographical areas around the world (6, 8, 13, 17, 19). This technique also constitutes a powerful approach for the detection of outbreaks of multidrug-resistant strains (1, 10) and for population-based surveillance of the control of tuberculosis (18) because of the genetic stability of the IS6110 element (3, 15).

In order to better understand the mechanisms of tuberculosis transmission in Guadeloupe, we undertook a molecular fingerprinting study with the IS6110 and DRr probes as genetic markers and an internationally agreed upon RFLP procedure (15), and we were able to detect upon *Pvu* II-IS6110 fingerprinting a cluster of isolates with a major four-band pattern from among 24% of patients. A similar four-band pattern has recently been identified among isolates from French Polynesia (13) and South Africa (17). This four-band pattern could be further characterized on the basis of the patterns obtained with the DRr probe (13; this study), and it was recently hypothesized that it could represent an ancient common pool (ancestral clone) of mycobacteria in the general population responsible for both active transmission and reactivation of tuberculosis (13).

According to the currently prevailing opinion, IS6110 clusters of isolates from large rural geographic areas detected by IS6110 fingerprinting may not represent recent transmission events, and because fingerprints with five or fewer bands may lack specificity, these should be further typed with a secondary marker to define epidemiologically related strains (2, 4). Despite the fact that our setting covered a small geographic area, we decided to subtype the cluster A isolates using the DRr probe, which confirmed its genetic homogeneity.

It is a common practice to consider the Caribbean as a whole when discussing public health issues. However, one should also consider the socioeconomic differences which may exist between various Caribbean islands; e.g., data on the evolution and control of tuberculosis highlight the fact that islands with

both high and low incidences of tuberculosis do coexist; the incidence of new cases of pulmonary tuberculosis among children is as high as 80/100,000 in Haiti (5), as opposed to <1/100,000 in Guadeloupe (calculated on the basis of the total number of cases diagnosed during the last 13 years among children ages 0 to 14 years [9]). Essentially composed of a rural population with a low incidence of tuberculosis transmission (incidence of <12/100,000 inhabitants for a population of 417,000), but with as many European visitors per year, *M. tuberculosis* isolates from Guadeloupe are characterized by a dual IS6110 polymorphism: highly polymorphic patterns, due to extensive population mixing and independent recent transmission events through numerous visitors and many short events of close contacts in resorts, shopping centers, bars, restaurants, airports, etc., and low polymorphic patterns, representing the traces of an ancient pool of isolates among patients among the local population because the incidence was very high a few decades ago (9).

The percentage of reactivation versus active transmission events could not be precisely determined within this study, but it should be noted that cluster A was present in people of all ages and of different ethnic and geographical backgrounds and was independent of the HIV status of the patients. Cluster A was composed of isolates from 10 men and 2 women. The clinical diagnosis was pulmonary tuberculosis in 10 patients and extrapulmonary tuberculosis in 2 patients. No evident epidemiological link could be observed on the basis of geographical link, sex, age, family ties, or site of hospitalization (except for two HIV-positive patients from the same ward from whom the tubercle bacilli was isolated at the same time). Because the ages of the patients was from 25 to 76 years at the time of diagnosis, we conclude that the cluster A isolates caused both reactivation and active transmission events.

Contrary to previous studies which concentrated on either large cities in developed countries (1, 10) or samples of strains

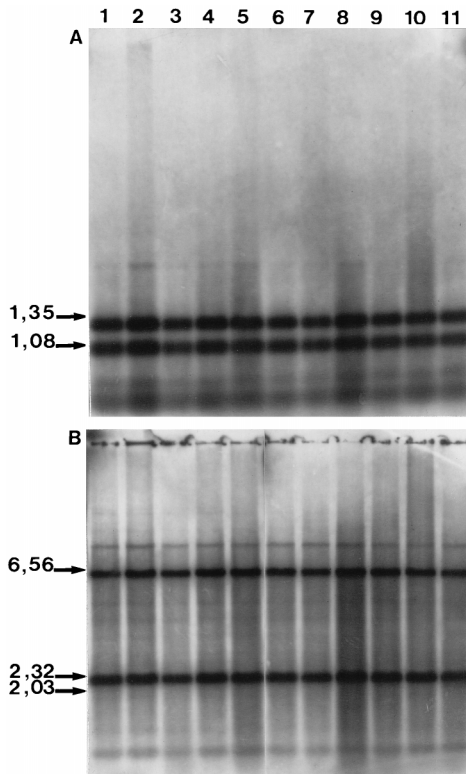


FIG. 4. *AluI*-DRr probe Southern blot hybridization (A) and *SmaI*-DRr probe Southern hybridization (B) of cluster A isolates (data not shown for isolate 94122).

from various areas of larger territories (8, 17, 19), the present study focused on all isolates from a Caribbean island where more than 30% of the patients are coinfecting with HIV. The epidemiological implication of these results will be the subject of an independent investigation by public health authorities in the near future and are instrumental in designing a large-scale, systematic IS6110-based fingerprinting study of clinical isolates from Guadeloupe, Martinique, and French Guyana over an extended time period. Nonetheless, the existence of a related IS6110 cluster of isolates from settings as diverse as French Polynesia (13), South Africa (17), and Guadeloupe (this study) stimulates more questions than answers. Although it may be purely coincidental, a possible explanation might reside in the fact that these long-isolated territories were left uninfluenced by migration for centuries. What we see today at the molecular level might be a remnant of a common past of IS6110 exchange between diverse migratory and/or selective forces and may be linked to a positive selection factor such as virulence. Evolutionary forces in our context may include, among others, people of diverse ethnic origins, interaction with *M. bovis*-infected cattle (12), and a long history of vaccination of these populations with live *M. bovis* BCG vaccines (16). Further studies of the genetic linkage between the IS6110 and the DR loci of the present isolates with four-band patterns may shed light on the mechanisms of genetic evolution of *M. tuberculosis* in settings like French Polynesia, South Africa, and the Caribbean.

ACKNOWLEDGMENTS

This project was supported through grants from Délégation Générale au Réseau International des Instituts Pasteur et Instituts Associés,

Institut Pasteur, Paris, France, and Fondation Française Raoul Follereau, Paris, France.

We are grateful to J. L. Guesdon and D. Thierry (Institut Pasteur, Paris) for IS6110-containing plasmid and to K. Kremer and J. D. A. Van Embden (RIVM, Bilthoven, The Netherlands) for providing the IS6110 fingerprinting methodology and *M. tuberculosis* 14323.

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