

# Restriction Fragment Length Polymorphism (RFLP) Analysis and Tuberculosis Epidemiology<sup>1</sup>

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*In order to study polymorphisms of the DNA insertion sequence 6110 (IS6110) in Mycobacterium tuberculosis strains isolated from Colombian patients, together with resistance to antituberculous medications in the Department of Quindío, Colombia, a prospective study was conducted using a consecutive sample of 59 patients with symptomatic pulmonary tuberculosis whose cases had been confirmed by bacilloscopy, both with and without a history of treatment. The patients, who were participating in the Tuberculosis Control Program of the Regional Health Institute of Quindío in Armenia, included all individuals attending local health centers and hospitals between March and July 1993 who were referred to the regional institute.*

*Sputum specimens from each patient were cultured and subjected to drug sensitivity tests. Subsequently, restriction fragment length polymorphisms (RFLP) of IS6110 from 27 patients were analyzed. The patients' treatment histories were used to classify their cases according to WHO criteria.*

*Forty-five cultures were found positive, 44 for M. tuberculosis and 1 for M. africanum. Initial drug resistance was observed in 4 of 42 new cases, or 9.5% (95% CI: 0.6, 18), 2 showing resistance to isoniazid (INH) and 2 to isoniazid plus streptomycin (INH-SM). Acquired resistance was observed in 2 of the 3 chronic cases and relapses, the bacteria being resistant to isoniazid, rifampicin, and streptomycin (INH-RM-SM) in one case and to isoniazid, ethambutol, rifampicin, and streptomycin (INH-EMB-RM-SM) in the other. In those 27 strains subjected to RFLP analysis, the number of copies of IS6110 ranged from 6 to 17. Similarity coefficients revealed five distinct groups of strains.*

*Overall, the RFLP analysis permitted most of the strains to be distinguished from one another, implying that the polymorphisms involved are sufficient to permit effective employment of this technique, which appears to have considerable potential for use in epidemiologic studies and in work designed to provide a basis for tuberculosis control program decision-making.*

**T**he complex problems posed by the current status of tuberculosis (1-9) call for the design of new strategies and control methods. Restriction fragment length polymorphism (RFLP) analysis that

shows the insertion sequence 6110 (IS6110), an exclusive component of strains belonging to the *M. tuberculosis* complex, represents a new and promising tool for conducting epidemiologic studies of the

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disease (10). IS6110 is a DNA sequence with the ability to transpose itself to numerous sites on the chromosome. Although the exact function of insertion elements is unknown, they have sequences that encode for the production of proteins essential for genetic recombination. Such elements are present in varying numbers and positions, producing specific polymorphic patterns ("fingerprints") that vary from one bacterial strain to another and that can be observed through RFLP analysis (1).

To date, this method has been used primarily to determine sources of infection in outbreaks of drug-resistant nosocomial tuberculosis (11–13). Recent measures implemented in the United States to control these small outbreaks have been based on data obtained by this method. It is also possible, through isolation of strains from the same geographic region, to conduct studies of contacts in order to discover the origin of infections not detected by traditional methods (14).

The primary aim of the study reported here was to describe the polymorphic nature of IS6110 in strains of *M. tuberculosis* isolated from patients with pulmonary tuberculosis in the Department of Quindío, Colombia, and to assess the relationship of these strains to strains from African and European patients. A second objective was to determine the potential for using RFLP analysis to conduct epidemiologic studies and evaluate current resistance to antituberculous drugs in the area.

## MATERIALS AND METHODS

A consecutive sample was taken of 59 patients with pulmonary tuberculosis, both with and without a history of prior antituberculous treatment. The sample consisted of patients with respiratory symptoms and positive bacilloscopy results who were seen between March and

July 1993 in urban and rural hospitals and health centers and who were referred to the tuberculosis control program of the Regional Health Institute of Quindío. Like 80% of Quindío Department's 320 000 residents, these patients were not protected by social security or private insurance coverage; they therefore received their health coverage from the public health service. The tuberculosis control program is decentralized and employs a standardized, supervised (direct observation), and short-term (six-month) therapeutic scheme for treating new pulmonary and extrapulmonary cases in accordance with the scheme proposed by the tuberculosis control program of the Health Ministry of Colombia.

The patients were classified in accordance with their history of treatment, using the following WHO criteria (2):

- New case: The patient had been receiving antituberculous treatment for one month or less.
- Relapsed case: The patient was cured in the past and now exhibits a new case of active tuberculosis.
- Chronic case: The patient continues to yield positive bacilloscopy results even after repeated treatment.

The sputum specimens were transported to the laboratory in cetylpyridinium chloride medium (15). Decontamination of the sputum, a procedure needed to prevent growth of other bacteria, was done using the Petroff method (16). Cultures of each sample were grown in two test tubes with Löwenstein-Jensen medium, two tubes with Ogawa medium (for culturing all types of mycobacteria), and one tube with Stonebrink medium for detecting *M. bovis*. The test tubes were incubated at 37 °C and read every 15 days. Cultures exhibiting no mycobacterial growth were considered negative after 10 weeks. Identification of mycobacteria was accomplished using the method of Jen-

kins et al. (17). Drug sensitivity was determined by the proportion method using Löwenstein-Jensen medium that has been described by Cannetti et al. (18). The following drugs were used for sensitivity testing on Löwenstein-Jensen medium: isoniazid (INH, 2 µg/ml), streptomycin (SM, 4 µg/ml), ethambutol (EMB, 2 µg/ml), rifampicin (RMP, 40 µg/ml), and para-aminosalicylic acid (PAS, 0.5 µg/ml). The type of resistance was classified in accordance with the patient's chemotherapeutic history, as follows:

- Initial resistance: The strain is resistant but the patient denies having taken an antituberculous drug in the past.
- Acquired resistance: The strain is resistant and was obtained from a patient who had received prior antituberculous treatment.

RFLP analyses were performed with 27 Colombian *M. tuberculosis* isolates. In addition, 33 strains of *M. tuberculosis* and one of *M. africanum* that had been isolated from tuberculosis patients in Rwanda (n = 20), Burundi (n = 4), Guinea (n = 4), and Belgium (n = 6), as well as one *M. bovis* isolate, were examined together with the Colombian strains. The study used the protocols of the National Institute of Public Health in Bilthoven, the Netherlands, which are internationally accepted for purposes of standardization and comparability of results (19).

From each *M. tuberculosis* culture, bacteria were removed with a loop, were transferred to a microcentrifuge tube containing 500 µl of buffered "TE" solution (100 mM Tris HCl pH 8, 10 mM EDTA), and were killed by heat. Bacterial lysis was accomplished by adding 50 µl of lysozyme (10 mg/ml), 70 µl of sodium dodecyl sulfate (SDS), and 6 µl of proteinase K (10 mg/ml) to each tube. Then, to separate the proteins from the nucleic acids, 100 µl of 5M NaCl, and subse-

quently 80 µl of cetyl NNN-trimethyl ammonium bromide (CTAB)/NaCl, were added to each tube. Protein extraction was performed by adding an equal volume (700 µl) of chloroform/isoamyl alcohol (24:1), and the DNA was precipitated with 0.6 volume isopropanol. The restriction enzyme *Pvu* II (Boehringer Mannheim, Germany), which recognizes the palindromic sequence CAG|CTG, was used to digest the DNA. The fragments obtained were separated by length using slow electrophoresis (0.8 V/cm). Transfer of DNA from the agarose gel to the nitrocellulose membrane was done by the vacuum method (Milliblot-V), and detection of fragments containing *IS6110* was accomplished using a specific probe prepared by polymerase chain reaction. Within the *IS*, this specific probe detects a sequence located to the right side of the *Pvu* II restriction site.

The probe was labeled with peroxidase (ECL, Amersham). Hybridization took place overnight at 42 °C with a probe concentration of 10 ng/ml hybridization buffer in a revolving hybridization incubator (Robbins Scientific). Detection of the probe bound on the membrane was accomplished by chemiluminescence (Amersham, United Kingdom). Light emission was recorded by autoradiography (Hyperfilm; Amersham, United Kingdom).

The genetic polymorphisms thus detected were analyzed in order to construct dendrograms based on similarity coefficients (SAB), which were obtained using the modified formula of Godfrey and Stoker (20). This formula, in which two strains (A and B) are compared with regard to the numbers of bands revealed by RFLP analysis, is as follows:

$$SAB = \frac{A\&B}{A + B - A\&B}$$

where A = A bands, B = B bands, and A&B = bands shared by strains A and B. Isolated strains with identical bands

will have an SAB of 1, while strains with no bands in common will have an SAB of 0. The dendrograms were constructed manually (21). To form groups of strains that appeared associated, an SAB coefficient greater than 0.25 was selected. The confidence intervals for the proportions involved were calculated using the formula  $p \pm 1.96\sqrt{pq/n}$  (22).

## RESULTS

Of the 59 sputum specimens collected, 45 (76%) yielded positive culture results, 44 strains of *M. tuberculosis* and 1 strain of *M. africanum* being identified. This latter strain was found sensitive to thio-phenol 2-carboxyl hydrazide (TCH) and produced niacin (23). Sensitivity tests revealed that four of the 45 strains isolated (13%) were resistant to one or more drugs (95% CI: 3, 23). The distribution of these

6 strains in terms of initial and acquired resistance is indicated in Table 1.

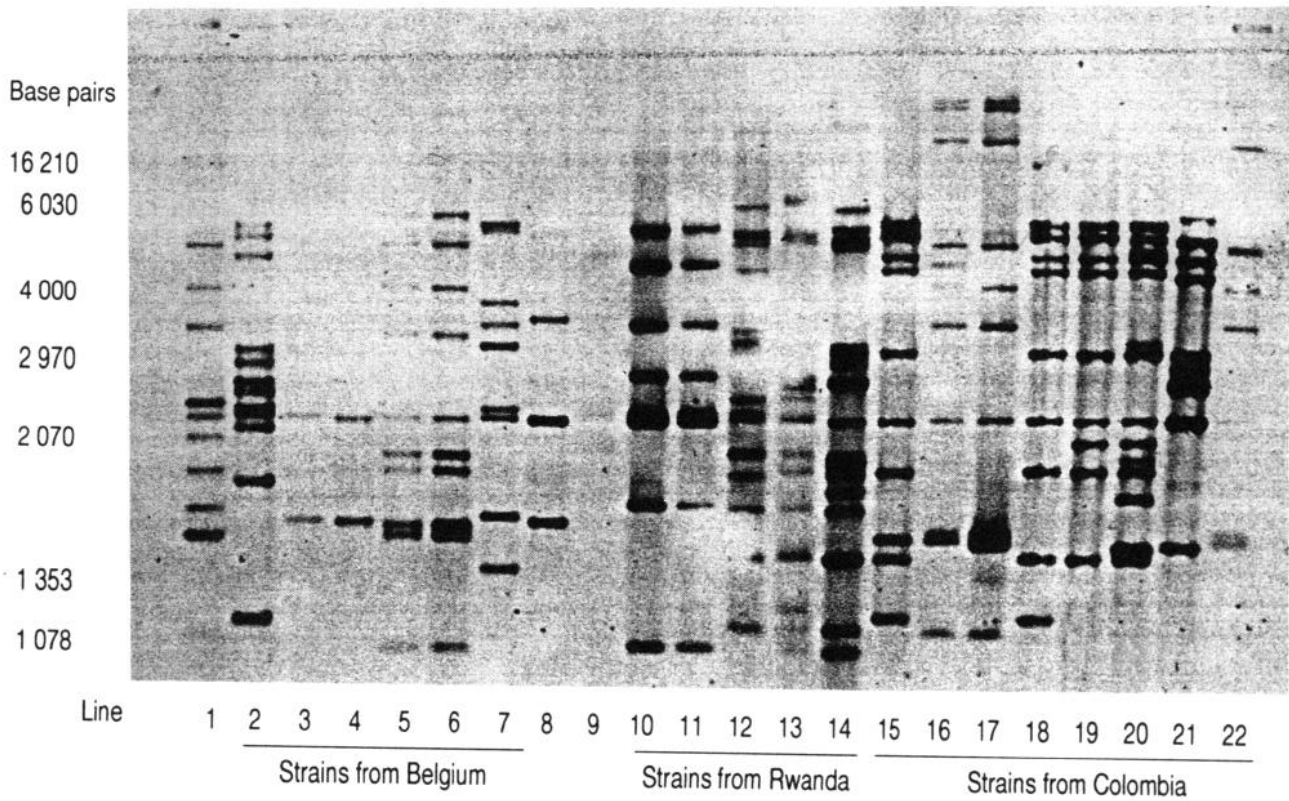
Two examples of autoradiographs obtained by analysis of the RFLPs of *IS6110* are shown in Figures 1 and 2. None of the strains from Africa or Europe exhibited profiles similar to those of the Colombian strains. In both non-Colombian and Colombian strains, however, the number of copies of *IS6110* found in the polymorphic profile ranged from 6 to 17. No strains without *IS6110* inserts were detected. The number of copies present in the 27 Colombian strains analyzed is shown in Figure 3.

In lines 15 and 16 of Figure 2 (pertaining to strains isolated from different patients) the profiles were identical. The two isolated strains also exhibited the same resistance phenotype (to INH and SM), leading to the conclusion that they were in fact the same. Line 10 in Figure 2 shows

**Table 1.** Initial and acquired resistance to antituberculous drugs found among 45 isolated strains of *Mycobacterium*. Armenia, Quindío, Colombia, 1993.

	Resistant strains (No.)/ total strains (No.)	%
<i>Initial resistance (strains from patients not previously treated):</i>		
Isoniazid	2/42	4.8
Isoniazid plus streptomycin	2/42	4.8
Total	4/42	9.5
		(95% CI: 0.6–18)
<i>Acquired resistance (strains from patients who had received prior antituberculous treatment):</i>		
Case of relapse (isoniazid, rifampicin, and streptomycin)	1/3	33.3
Chronic case (isoniazid, ethambutol, rifampicin, and streptomycin)	1/3	33.3
Total	2/3	66.6
		(95% CI: 13–100)

**Figure 1.** Autoradiograph of size polymorphisms (in base pairs) of insertion sequence 6110 in 19 strains of *Mycobacterium tuberculosis* from Belgium, Colombia, and Rwanda; 1 reference strain of *M. tuberculosis*, 1 strain of *M. africanum* isolated from a pig in South Africa, and 1 strain of *Mycobacterium bovis*. Line 1 corresponds to *M. tuberculosis* reference strain Mt 14323, which was used to show the size and location of bands on the nitrocellulose membrane; lines 2–7 are from the Belgian strains of *M. tuberculosis*; line 8 is from the *M. africanum* strain; line 9 (showing two barely visible bands) is from the *M. bovis* strain; lines 10–14 are from the Rwandan strains of *M. tuberculosis*; and lines 15–22 are from the Colombian strains of *M. tuberculosis*.



the profile of a strain resistant to multiple drugs (INH, RM, and SM). The remaining 24 Colombian strains analyzed by RFLP were sensitive to all of the drugs tested. The polymorphic profiles shown on lines 8 and 14, pertaining to sensitive strains obtained from different patients, were likewise found to be identical.

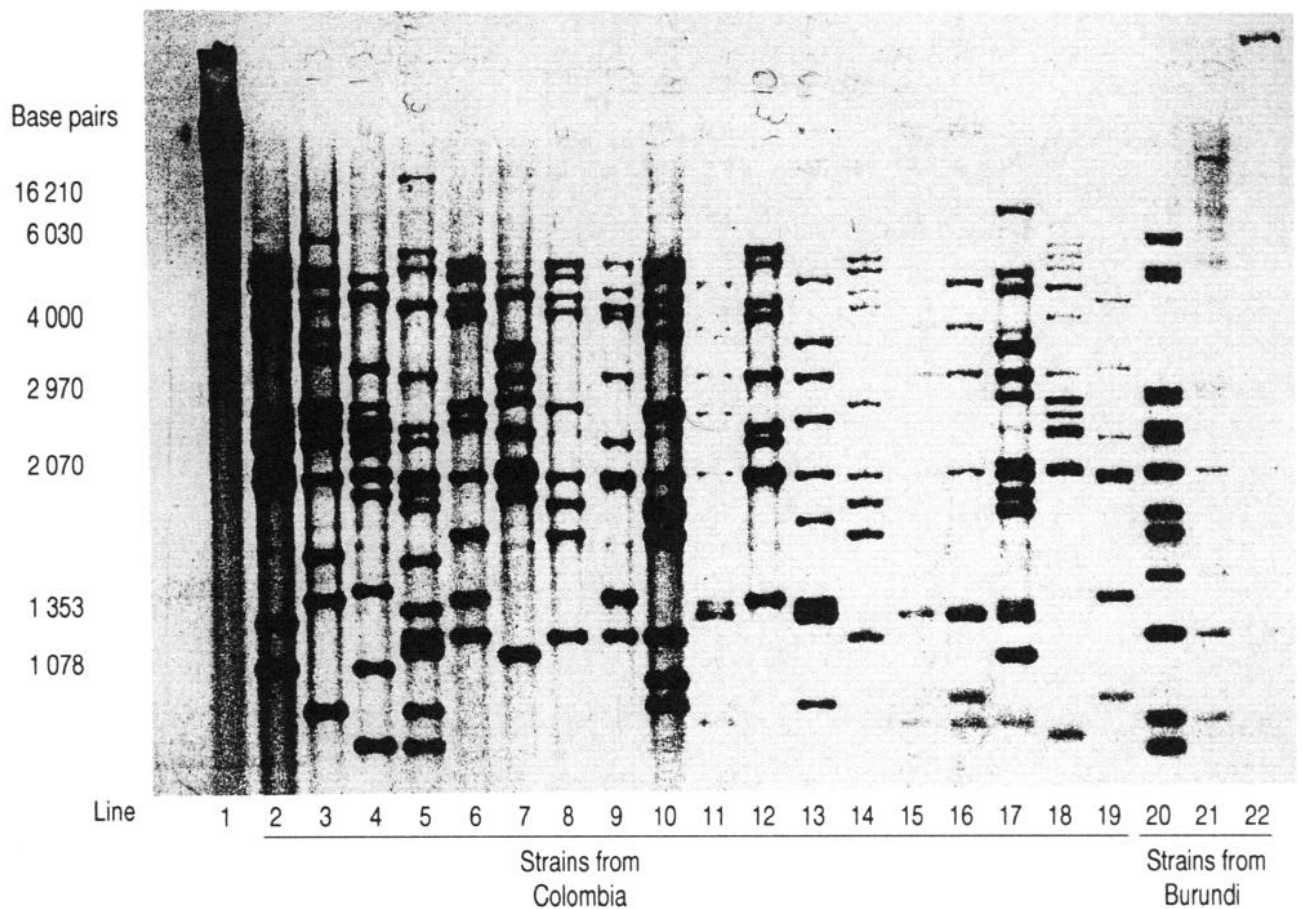
Twenty of the Colombian strains isolated revealed similar but not identical profiles. The dendrogram (Figure 4) derived from the SAB coefficients of the 27 strains assessed revealed five groupings, of which the largest was A (with 8 strains), followed by C (with 6 strains) and then by B, D, and E with 2 strains each.

## DISCUSSION

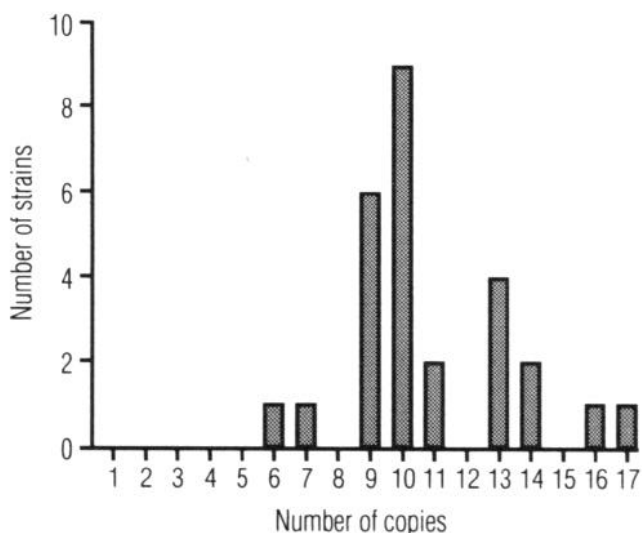
The sample studied illustrates the situation currently facing tuberculosis control programs with regard to bacilliferous patients. In terms of initial resistance to antibiotics, the results are consistent with those obtained in the regional survey conducted by PAHO that tested for resistance to INH and to INH plus SM (Kantor N, 1993, personal communication).

The general drug resistance situation in Colombia reveals a clear and urgent need for improvement of compliance with official treatment schemes, since this

**Figure 2.** Autoradiograph of size polymorphisms (in base pairs) of insertion sequence 6110 in 1 reference strain of *Mycobacterium tuberculosis* (strain Mt 14323, no bands visible), 18 strains from Colombia, and 3 from Burundi. This figure and Figure 1 provide examples of RFLP results but do not show all of the strains studied.



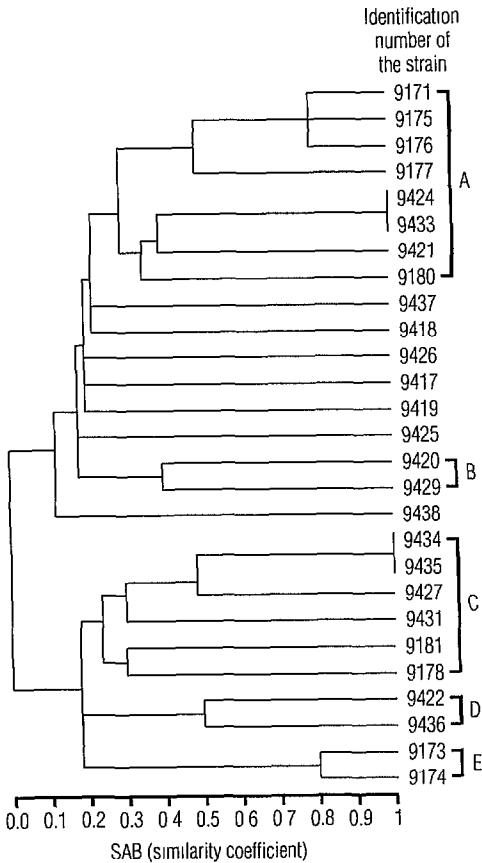
**Figure 3.** The 27 Colombian *M. tuberculosis* strains subjected to RFLP analysis contained anywhere from 6 to 17 copies of insertion sequence 6110. This figure shows the numbers of strains found to possess specific numbers of copies. Armenia, Quindío, Colombia, 1993.



measure offers the only effective means of reducing current levels of resistance. The six-month supervised therapeutic scheme continued to be effective in cases of initial resistance to SM and INH, in countries where the use of RMP and EMB was not widespread for a long period (24, 25). However, the situation is very different for cases resistant to RM and INH, because here the best available treatment is almost always ineffective and leads to high mortality (26). Strengthening tuberculosis control programs in Colombia today should be assigned the highest priority, as the country still finds itself in a situation that can be brought under control provided advantage is taken of currently available resources.

This study examines for the first time the polymorphism of *IS6110* in strains of

**Figure 4.** Dendrogram of the 27 *M. tuberculosis* strains subjected to RFLP analysis, showing the five groups of strains defined in terms of their individual members' similarity coefficients. Armenia, Quindío, Colombia, 1993.



*M. tuberculosis* isolated from Colombian patients. To the best of our knowledge it is also the first such report from Latin America. The initial important conclusion to be drawn is that the particular RFLP analysis selected for the study permitted most of the strains to be distinguished from one another. This finding implies that the polymorphism involved is sufficient to permit use of this technique as a tool within the framework of molecular epidemiology. It also raises questions about the usefulness of new molecular biology techniques in devel-

oping countries, where high rates of tuberculosis prevail among the general population. In particular, will such techniques serve to guide decision-making about control programs?

As the preceding indicates, it is evident that established priorities continue in effect; but tuberculosis is not following a static pattern of behavior in Latin America. To cite only one example, the number of new tuberculosis cases in the Department of Quindío has increased alarmingly, an increase that has clearly paralleled onset of the AIDS epidemic in the region. Studies are currently being conducted to examine the relationship between HIV-positive status and tuberculosis, and initial results have tended to confirm the suspicions raised by epidemiologic studies (unpublished results, Tuberculosis Control Program of the Regional Health Institute of Quindío).

In the face of this new epidemiologic situation, RFLP analysis will no doubt help in estimating the percentage of cases caused by reactivation of primoinfections versus recent transmission and in defining on this basis the appropriate duration of chemoprophylaxis for HIV-positive patients. In Latin America, it is to be expected that a fairly balanced distribution ascribable to both causal mechanisms will be found, given the complex epidemiologic situation prevailing in the region. Specifically, the existence of both old and new epidemics, together with relatively modern and sanitary living conditions, will tend to favor reactivation of primoinfections, while conditions of underdevelopment and poor sanitation affecting much of the population will tend to foster new cases through transmission.

Also important are the data on transmission of specific strains that RFLP analysis can provide. Of the 27 strains studied, 4 had identical profiles. This points to recent transmission in the patients involved rather than to reactivation of old infections (27). Thus, the technique made



it possible to discover two cases of transmission that had been unknown and undetected by traditional methods. In this regard, the most interesting result was that found for the two patients with resistance to isoniazid and streptomycin. The epidemiologic data normally used for identifying contacts had shown no relation between the two patients in question. The two were from the same city, but did not live in the same home and had no family connections, as a result of which there was no initial suspicion. Neither of the two had received prior antituberculous treatment, although one of them lived in contact with a patient who was receiving treatment. However, the drug resistance pattern and the fingerprint of the strain infecting this contact person are unknown.

The low percentage of specimens (76%) yielding a positive mycobacterial culture may have been ascribable to a decline in bacterial viability during transport and also to the occurrence of paucibacillary cases, as indicated by the low bacilloscopic indexes of many specimens.

The technique employed in this study could help to improve current knowledge of tuberculosis transmission mechanisms. Strain identification through RFLP analysis could be used by future studies to determine how often multiresistant strains appear and to explore factors favoring their spread in a given community. If the spread of multiresistant strains is common, it may be necessary to radically change control strategies, with a view not only toward detecting and treating cases but also toward eliminating circumstances favoring their spread. Recently, RFLPs have been used to conduct studies on community-based transmission in the United States and Switzerland (28–31). One such study, conducted in the canton of Bern, Switzerland, found that a single patient accounted for 13% of all tuberculosis cases identified in the canton (29); and another study, conducted in San

Francisco, California, found that a single patient was responsible for 6% of the cases surveyed during the study period (31). In our own study, we found that a single patient could probably account for two cases of initial resistance to the INH-SM drug combination. Findings of this sort could help in developing certain types of policies or actions, such as mandatory detention of patients representing a public health risk.

The present study found a close correlation between many of the Colombian strains, 20 of the 27 strains belonging to one of five groupings. This is what one would expect in a country where the frequency of transmission through contact with infected individuals is high. The correlation thus tends to confirm that there is intense circulation of *M. tuberculosis* in the surrounding area. This phenomenon is more evident when identical polymorphisms are detected in patients who have not been in contact with each other and who live in different cities (lines 8 and 14 of Figure 2), which means that the responsible strain has tended to spread more rapidly than the factors producing transposition of *IS6110*. On this basis, at least 4 of the 27 cases (14%) in this study appear attributable to recent transmission. The situation is similar to that found by one study in Africa, where 17 cases out of 118 (15%) could be attributed to recent transmission (20), but is very different from that found in specific settings in the United States, where recent transmission accounted for larger percentages of cases—191 cases out of 473 (40%) in San Francisco (31) and 39 out of 104 (37.5%) in New York (30)—and is also different from that found in the Menzel Bourguiba region of Tunisia, where recent transmission accounted for 53 of 128 cases (41%) (32). It should be noted that in Tunisia significant regional variations were found within the country itself, showing high polymorphism rates in some regions (32). It should also be noted that



the U.S. data reflect the impact of the AIDS epidemic, which has increased the total number of new tuberculosis cases and accounts for the high percentages of cases attributable to recent transmission. In this same vein, it is possible that additional studies in Latin America could detect local variations and identify recent microepidemic foci.

When the profiles of the 27 Colombian strains of mycobacteria were compared to those of the African and European strains, no similarities were observed, although all strains were found to have between 6 and 17 bands (1) regardless of their geographic origin. Also, our study detected no strains lacking IS6110 inserts, unlike studies of patients in Vietnam (33) and India (34). Our study is also the first to report symptomatic infection with *M. africanum* in Colombia.

Preliminary results of other studies comparing *M. tuberculosis* strains from different geographic regions make it appear feasible to create groupings based on strains' geographic origins (1, 20). To explore this possibility, several projects based on collection of a large number of strains and subsequent computerized analysis of those strains are now underway.

It should also be noted that RFLP analysis can be used to conduct epidemiologic studies in central or national reference laboratories. The low cost of analysis (approximately US\$ 1.50 per specimen) makes this technique affordable in developing countries. If the potential benefits of its application are considered, the cost-benefit ratio is commonly quite favorable and easily justifies the investment. It is also to be expected that studies conducted with this technique will lead to more effective control measures. Among the possible aims of such studies are determination of the frequency of therapeutic failure in cases acquired by transmission versus reactivation; follow-up of sources of dissemination of multi-

resistant strains; quality control in central or regional laboratories specializing in mycobacteria (35); and detection and study of outbreaks of nosocomial infection. In conclusion, it seems clear that RFLP analysis, which makes it possible to distinguish at the molecular level between various strains of *M. tuberculosis*, is a valuable tool for epidemiologic study of tuberculosis, one with a strong potential ability to clear up fundamental questions that have long gone unanswered.

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## *Tuberculosis and HIV Infection*

Tuberculosis has become the leading killer of HIV-positive individuals on a global scale. By the end of the decade, around one-third of all deaths among HIV-positive people will result from TB, according to WHO's Global Tuberculosis Program. That Program, in cooperation with the Global Program on AIDS, is mobilizing medical experts to develop a new HIV/TB research strategy. The strategy will seek to improve TB control programs already disabled in the face of growing HIV prevalence and to prevent devastation of TB programs in countries with an emerging HIV problem. The new Joint UN Program on AIDS (UNAIDS), which will become operational in January 1996, intends to further cooperate with the Global TB Program.

TB germs are transmitted through the air, and persons who are HIV-positive are probably more likely to become infected when inhaling the airborne germs. Once infected, they are 30 times more likely to become sick than TB-infected people who are HIV-negative. An increase in HIV cases thus can result in an increased number of infectious TB cases, facilitating the disease's spread to previously unaffected populations. Experts believe that today's TB research agenda must be broadened to reflect the complications caused by HIV/TB co-infection. The Global TB Program is seeking new partnerships with leading scientists from the TB and AIDS research communities to help communicate the new research priorities to funding agencies.

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Source: World Health Organization. TB has become world's leading killer of HIV-positive people. Geneva: WHO; 2 June 1995. (Press release WHO/43).