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Amplified-Fragment Length Polymorphism as a Complement to IS*6110*-Based Restriction Fragment Length Polymorphism Analysis for Molecular Typing of *Mycobacterium tuberculosis*

Monserrat Ruiz,[1]* Juan Carlos Rodríguez,[1] Francisco Rodríguez-Valera,[2] and Gloria Royo[1,2]

Section of Microbiology, Hospital General Universitario de Elche,[1] and Department of Microbiology, Universidad Miguel Hernández,[2] Elche (Alicante), Spain

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The amplified-fragment length polymorphism (AFLP) technique was applied to clusters of *Mycobacterium tuberculosis* clinical isolates obtained by using IS*6110*-based restriction fragment length polymorphism (RFLP). Ten of the RFLP clusters showed identical AFLP patterns also, but the other 13 could be resolved into subclusters by AFLP. Our results suggest that some RFLP clusters may not be due to recent transmission and that AFLP may be a useful complementary technique.

The standardized protocol of restriction fragment length polymorphism (RFLP) of the insertion sequence IS*6110* is a very important tool that has been used in many instances (7, 9). However, the complexity of the epidemiology of *Mycobacterium tuberculosis* makes it necessary to use other methodologies with higher resolution in order to resolve the closest relationships (5, 8).

In this paper we studied the epidemiological relationships among *Mycobacterium tuberculosis* isolates by using IS*6110*-based RFLP (IS*6110*-RFLP) and amplified-fragment length polymorphism (AFLP).

Demography. The study included 147 patients, who account for 59.34% of the patients diagnosed microbiologically as having tuberculosis between 1993 and 1999 in the Elche Health District (population, 250,000), situated on the Mediterranean coast in southeast Spain. The samples from all the patients in the Elche Health District for whom there was a clinical suspicion of tuberculosis were processed in our laboratory. All the samples were cultivated in Lowenstein's medium, and all samples, except those of urine and sputum, were also inoculated in the liquid medium ESP-II (Difco). The mycobacteria were maintained frozen at −70°C until the study commenced.

For each patient, classical epidemiological study data were collected: name, age, sex, nationality, profession, attendance at a community center, employment status, telephone ownership, place of residence, district and street address, date disease was diagnosed, human immunodeficiency virus serology, parenteral drug user status, alcoholism, smoker status, homelessness, other causes of immunodepression, pulmonary cavitation, previous tuberculosis, current treatment, site of the disease, hospital admission, type of sample, quantification of the staining, quantification of the culture, and susceptibility to anti-tuberculostatic drugs. We also included classical contact study data: percentage of involvement in the first circle (people living under the same roof, couples, coworkers who spend many hours together in a closed room), second circle (contacts on nonconsecutive days in closed places), or third circle (occasional contacts), involvement or lack of involvement up to the last circle, involvement outside the family, and number of families studied. All the data were obtained prospectively.

IS*6110*-RFLP. The IS*6110*-RFLP technique was used by following a standardized protocol (6). An analysis of the patterns obtained was made by using the Gel Compar system, comparative analysis of electrophoresis patterns version 4.1 (Applied Maths, Kortijk, Belgium), and the patterns were also examined visually.

An RFLP cluster of *M. tuberculosis* isolates was defined as two or more isolates for which RFLP revealed an identical number and location of IS*6110* sequences when five or more copies of IS*6110* were present.

AFLP. The AFLP technique was applied to all the strains found clustered together according to IS*6110*-RFLP (71 strains).

DNA extraction. DNA extraction was carried out as described previously (9).

Enzymatic digestion. Four hundred to 600 ng of genomic DNA was digested simultaneously with *Eco*RI (Life Technologies, Paisley, Scotland) and *Mse*I (Life Technologies), using *Mse*I buffer and incubating it overnight at 37°C. Endonucleases were inactivated at 70°C for 10 min before the ligation reaction.

Adaptor ligation. Restriction-halfsite-specific adaptors (Applied Biosystems) were ligated to the double-digested DNA by a T4 DNA ligase (Life Technologies) and were incubated for 2 h at 37°C, generating template DNA for subsequent PCR amplification.

Preselective amplification. The sequences of the adaptors and the restriction site serve as primer binding sites for a subsequent low-level selection or preselective amplification of the restriction fragments. Only those genomic fragments that have an adaptor on each end amplify exponentially during PCR amplification.

Selective amplification. Selective amplification was carried out by using *Eco*RI and *Mse*I primers that amplified primarily *Eco*RI-*Mse*I-ended fragments.

* S. Microbiología, Hospital General Universitario de Elche, Camí de L'Almazara, no. 11, 03203 Elche (Alicante), Spain. Phone: 34-966679048. Fax: 34-966679108. E-mail: micro_elx@gva.es.

Primers used for the selective amplification. Two combinations, *Eco*RI 0-*Mse*I 0 and *Eco*RI 0-*Mse*I C, were selected on the basis of the degree of discrimination between epidemiologically different strains.

Detection and fragment analysis. An ABI PRISM 377 DNA (Perkin Elmer) sequencer was used to produce an electropherogram of fluorescent dye-labeled AFLP products.

Statistical analysis. All the variables considered in the study were qualitative except for age, which was classified in groups. Contingence tables and the Chi-squared statistical test were used.

In a previous study using IS*6110*-RFLP, 52.4% of 147 strains was found to be clustered in 23 clusters (6). Classical epidemiological studies confirmed only five (13 isolates) of these. When AFLP was used, 10 of the clusters showed identical AFLP patterns also, but the other 13 could be resolved into subclusters according to the AFLP patterns.

The two primer combinations used generated a total of 81 differently sized fragments, ranging in size from 80 to 380 bp. With primers *Eco*RI 0 and *Mse*I 0, 36 (41.8%) different fragments were generated. The AFLP patterns generated a number of bands ranging from 8 to 23.

The *Eco*RI 0-*Mse*I C combination (53.5%) generated 45 bands (between 5 and 25 per strain). The average number of fragments per strain with the two combinations used was 14. Most of these fragments ranged in size from 100 to 300 nucleotides.

With the *Eco*RI 0-*Mse*I 0 combination, two fragments were common to all the mycobacteria analyzed, three were common to 90% of mycobacteria analyzed, three to 80%, and two to 75%. With the *Eco*RI 0-*Mse*I C combination, two fragments were common to 100% of the strains, two to 90%, one to 80%, and two to 75%. Comparing the two primers used showed that the use of a primer with a selective base (*Eco* R1 0-*Mse*I C) produces a more discriminative pattern, although the number of fragments common to most strains were similar for both primers.

RFLP clusters including more than four strains always presented differences when analyzed by AFLP. Even smaller RFLP clusters were resolved into different AFLP patterns in 47% of the cases. The time span between isolations was also an important factor to be considered. Isolates obtained from the same RFLP cluster over a time span of more than 250 days showed differences in the AFLP patterns in 58.3% of the cases.

However, strains from the same RFLP cluster were more similar according to AFLP, with there being only 1 to 6 different bands (an average of 2.6). This indicates that IS*6110*-RFLP does indeed reflect the relationships at the genomic level. The strains with different RFLP patterns show much larger differences when AFLP is used (24.7 bands on average).

When we used classical epidemiological studies to investigate the epidemiological relationships, we were able to confirm only five clusters (13 patients) (Table 1). Furthermore, when these five clusters were analyzed by AFLP, we found that in only two of them (three and two patients, respectively) did the strains of each cluster show the same band patterns. We found small differences among the strains of each of the other three clusters (Table 1).

The statistical analysis showed a significant association between discrepancy in the RFLP-AFLP results and criteria such as residence outside the city, number of strains in the cluster, and prior tuberculosis.

TABLE 1. Characteristics of the clusters obtained by using IS*6110*-RFLP

Cluster no.	No. of isolates	Epidemiological relationship	No. of different bands by AFLP	Interval between isolations (mo)
1	3	Relatives	0	24
2	2	Friends	2	12
3	4	Relatives	1	12
4	2	Relatives	0	1
5	2	Relatives	3	1
6	7	No relation	3	41
7	8	No relation	6	11
8	5	No relation	6	14
9	4	No relation	1	3
10	3	No relation	0	47
11	3	No relation	4	37
12	3	No relation	1	11
13	3	No relation	0	15
14	3	No relation	0	0
15	3	No relation	0	0
16	2	No relation	0	0.5
17	2	No relation	0	1
18	2	No relation	3	0
19	2	No relation	2	23
20	2	No relation	0	3
21	2	No relation	1	0.3
22	2	No relation	0	60
23	2	No relation	1	2

Although it is well known that there is little genomic variability in *M. tuberculosis*, AFLP makes it possible to detect differences among the strains studied. AFLP enables the complete genome to be studied and can provide interesting results concerning the microevolution of this microorganism (2, 3, 4).

The high proportion of heterogeneities found by AFLP among the clusters of strains with identical IS*611*0-RFLP patterns leads us to question the efficiency of this methodology in our setting, particularly considering that there are times when epidemiological and clinical studies do not reveal any association among patients that cluster together according to RFLP (1).

It has been shown that AFLP can differentiate between clusters identified by RFLP, particularly in the case of large clusters, including strains obtained after lengthy disease periods, i.e., strains in which there are small but relevant changes in the genome that are not shown by IS*6110*-RFLP.

Strains with identical RFLP but different AFLP patterns may have a common ancestor; however, they would not correspond to recent transmission but rather to previous tuberculosis that allowed the strains to diverge genomically. The statistical study and classical epidemiological studies support this and suggest that not all the strains associated by IS*6110*-RFLP are associated with recent transmission. On the basis of these results, we consider AFLP to be a complementary technique in the molecular typing of *M. tuberculosis*.

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