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Use of Restriction Fragment Length Polymorphism as a Genetic Marker for Typing *Mycobacterium avium* Strains

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Restriction fragment length polymorphism (RFLP) was used to study 75 clinical isolates identified as *Mycobacterium avium*. Two repetitive insertion sequences, IS*1311* and IS*900*, were used as DNA probes. Although less than 25% of isolates showed RFLP patterns with IS*900*, all strains gave banding patterns with IS*1311*. *M. avium* strains isolated from patients with AIDS exhibited marked polymorphism with both probes.

Mycobacteria of the *Mycobacterium avium* complex (MAC) behave as opportunistic pathogens and can be isolated from environmental, animal, and human sources. *M. avium* particularly causes disseminated bacterial infections in patients with AIDS (12, 13).

Human sources of infection and routes of transmission of *M. avium* are not completely clear. Nevertheless, study of these factors is important to clarify the epidemiology of infection of AIDS patients with this potential pathogen. Molecular biology techniques which allow differentiation of strains within the *M. avium* group are important approaches for epidemiological study and control of disease dissemination.

Pulsed-field gel electrophoresis (PFGE) (15) has been shown to be a useful method for determining strain relationships within the *M. avium* cluster (16, 18). Analysis with this technique clearly established that water is a reservoir for *M. avium* infection in AIDS patients (20). Although PFGE has been useful, it requires a greater amount of DNA than restriction fragment length polymorphism (RFLP) does for good visualization of the ethidium bromide-stained fragments.

RFLP techniques with strain-specific markers are also useful in epidemiological studies of bacteria. IS*6110* in particular has been an excellent tool for tracing the world-wide distribution of the pathogenic *Mycobacterium tuberculosis* (1, 3, 19).

Several insertion sequences have been reported for the MAC group: IS*900* in *Mycobacterium paratuberculosis* (7), IS*901* and IS*902* in *M. avium* which share 98% of homology at the DNA level (14, 17), and IS*1110* also in *M. avium* (11).

The utility of these insertion sequences as probes for RFLP studies to differentiate isolates of *M. avium* was shown to be limited in studies of *M. avium* strains isolated from AIDS patients (10, 11).

Stable integration by transposition has been demonstrated with IS*900* (5). However, IS*900* and IS*901* show a very low degree of mobility which restricts their use as probes for *M. avium* epidemiology (11). In contrast, IS*1110* is a highly mobile genetic element, but its acquisition by *M. avium* appears to be rather recent and therefore it is rarely reported within the *M. avium* group (11).

Recently a new insertion sequence, IS*1245*, has been described (8), which is present in high copy number in *M. avium* isolated from both human and animal sources. It belongs to the IS*256* family of insertion sequences, which include other elements identified in mycobacteria: IS*1081* in *Mycobacterium bovis* (2) and IS*6120* in *Mycobacterium smegmatis* (9).

RFLP studies with IS*1245* as a probe showed a discriminatory power similar to that of PFGE for strain differentiation (8), indicating the usefulness of IS*1245* in *M. avium* epidemiological studies.

Another element in *M. avium*, IS*1311*, also identified by the same researchers (Gen Bank accession number U16276), showed 85% homology at DNA level with IS*1245* and may result in faint signals as a consequence of cross-hybridization when the conditions employed are not stringent (unpublished results).

In this study, we checked and compare the results of IS*900* and the new element IS*1311* in the differentiation of *M. avium* strains isolated from AIDS patients and from non-human immunodeficiency virus (non-HIV)-infected patients over a period of several years.

A total of 75 *M. avium* strains isolated from AIDS patients (52 isolates from 40 patients) and non-HIV-infected patients (23 isolates from an equal number of patients) were studied by RFLP during the period from 1989 to 1992 in the 12 October Hospital (Madrid, Spain). Two DNA probes were used: IS*900*, an insertion sequence described in *M. paratuberculosis* (7) and IS*1311* from *M. avium*. The strains were identified by Gen-Probe tests (San Diego, Calif.); all strains were positive with the *M. avium* probe and negative with the *Mycobacterium intracellulare* probe. These isolates represented about 90% of the MAC isolated during this period. The strains were grown from the following clinical specimens: (i) for AIDS patients, Blood culture, 33; respiratory tract, 9; bone marrow, 8; and liver biopsy, 2; and (ii) for non-HIV-infected patients, respiratory tract, 13; lymph node, 3; gastrointestinal tract, 3; bone marrow, 1; liver biopsy, 1; peritoneal biopsy, 1; and lung biopsy, 1.

M. avium DNAs were obtained as described elsewhere (4). Mycobacterial DNAs (1 to 2 μg) were digested with the restriction enzyme *Pvu*II (Promega Corp., Madison, Wis.), and the fragments were separated by horizontal electrophoresis on 0.75% agarose gels and vacuum transferred to nylon membranes (Hybond-N; Amersham Iberica, Madrid, Spain). Lambda DNAs digested with *Eco*RI and *Hin*dIII (Sigma Diagnostics, Madrid, Spain) were used as molecular size markers.

The probes used in this study were labelled with [α-^{32}P] dCTP (Amersham Life Science) by using the Prime-α-Gene

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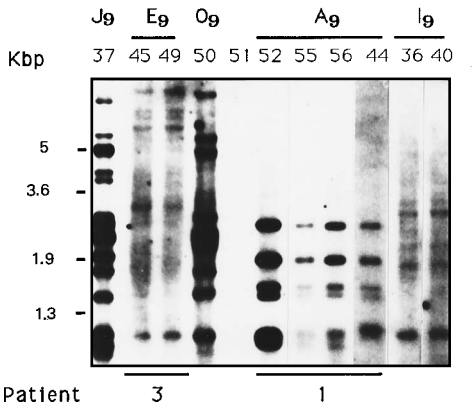

FIG. 1. RFLP patterns of *M. avium* strains isolated from AIDS patients with IS*900* as a probe. The strains (numbers) are given over the lanes. No signal was detected for strain 51. Patterns are identified with letters. The subscript 9 after each letter stands for the probe IS*900*. Strains sharing the same pattern are overlined. Strains isolated from the same patient are indicated in the lower part of the figure. Molecular marker sizes (in kilobase pairs) are indicated to the left of the gel.

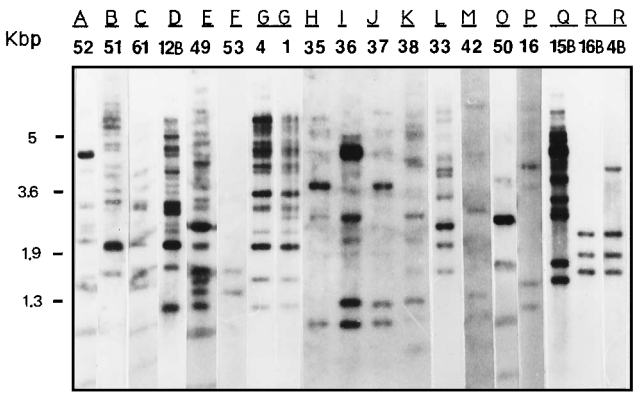

FIG. 2. RFLP patterns of *M. avium* strains with IS*1311* as a probe. Strains (numbered) and patterns (letters) are indicated over the lanes. *M. avium* strains isolated from AIDS patients are numbered, and *M. avium* strains isolated from non-HIV-infected patients are numbered and have the letter B. Strains 1 and 4B were assigned to patterns G and R, respectively, despite the presence of an extra band. Molecular marker sizes (in kilobase pairs) are indicated to the left of the gel.

System (Promega Corp.) according to the manufacturers' instructions.

RFLP analysis was carried out with the stringent conditions for hybridization and washing described elsewhere (4). The same filters were hybridized with both probes sequentially and washed for reprobing, following manufacturer's instructions.

IS*900* RFLP analysis. The insertion sequence IS*900* was obtained as a 1.5-kbp *Nru*I fragment from plasmid pMB22 (kindly provided by J. J. McFadden). With this probe, hybridization signals occurred in a minority of the *M. avium* strains studied (less than 25%). Patterns obtained could be distributed into seven different groups: A_9, E_9, I_9, J_9, O_9, P_9, and R_9 (Fig. 1).

M. avium strains with IS*900* multibanding patterns were checked for the presence of IS*901* by using PCR conditions and specific oligonucleotides as described by Kunze et al. (13). IS*901* was not detected in these strains (not shown).

Since IS*900* is not present in *M. avium*, strains showing RFLP banding patterns with this element must contain some IS*900*-related element. IS*1110* (a recently identified insertion element related to IS*900*) has been described as being uncommon in *M. avium* isolates. This result indicates that the bands obtained could correspond to another IS*900*-related element, still unidentified.

IS*1311* RFLP analysis. A 200-bp internal fragment of IS*1311* from the *M. avium* clinical strain 2B was amplified by PCR with the specific oligonucleotides: DD2 (5′ GTC GGG TTG GGC GAA GAT) and DD3 (5′ GTG CAG CTG GTG ATC TCT GA) (kindly provided by A. Telenti), with the following conditions: 30 cycles, with 1 cycle consisting of 1 min at 94°C, 1 min at 65°C, and 1 min at 72°C, followed by a 10-min extension at 72°C.

When the PCR-amplified product from IS*1311* was used as a probe for hybridization, banding patterns were identifiable in all strains studied. Up to 19 different RFLP patterns, labelled from A to S, were found (Fig. 2, 3, and 4). RFLP patterns in strains isolated from AIDS patients showed high variability. We deduced that our AIDS patients were not infected by a highly conserved *M. avium* strain, as has been described previously in other countries (6, 10).

In general, strains belonging to the same patient showed the same RFLP pattern (Fig. 3). However, sequential isolates from one AIDS patient showed different patterns along the clinical evolution of the disease (strains 13, 14, and 16 [Fig. 3]). This observation was likely attributable to infection with multiple strains of *M. avium*. Patterns Q, R, and S (Fig. 2 and 4) were detected only in non-HIV-infected patients.

There was no clear relationship between patterns and the source of specimens; however, pattern L (Fig. 2) was obtained only from respiratory tract specimens from AIDS patients.

Within *M. avium* strains isolated from AIDS and non-HIV-infected patients, we detected clustering of types during several time periods. Such clusters included strains with RFLP types D, G, I, and R (Fig. 4) as follows. (i) In 1989, seven of the eight strains obtained from AIDS patients had the pattern G (Fig. 4). (ii) From February to October 1991, 27 *M. avium* strains were isolated. Of these 27 strains, 12 (44.4%), all from non-HIV-infected patients, shared pattern R (Fig. 4). Moreover, in April of the same year, eight of nine strains isolated had pattern D (either from AIDS or non-HIV-infected patients; Fig. 4). (iii) From October 1991 to February 1992, 17 *M. avium*

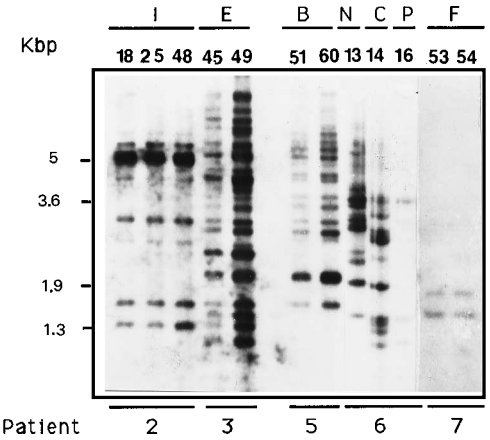

FIG. 3. RFLP patterns of *M. avium* strains isolated from AIDS patients with IS*1311* as a probe. Strains (numbers) and patterns (letters) are indicated over the lanes. Strains sharing the same pattern are overlined. Strains isolated from the same patient are indicated in the lower part of the figure. Molecular marker sizes (in kilobase pairs) are indicated to the left of the gel.

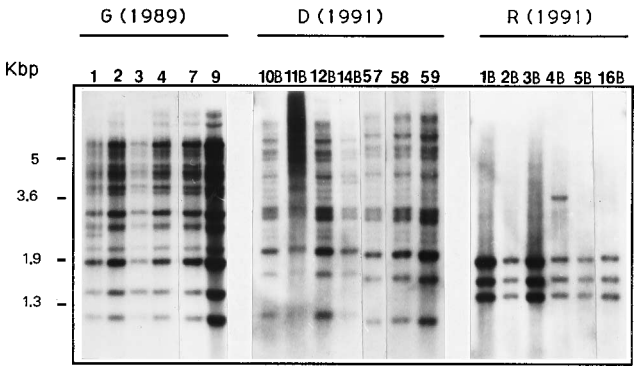

FIG. 4. RFLP patterns of *M. avium* strains corresponding to possible temporal clusters (see text) with IS*1311* as a probe. Strains sharing the same pattern are overlined. Patterns (letters) and the year of isolation (in parentheses) are given at the top. *M. avium* strains isolated from AIDS patients are numbered, and *M. avium* strains isolated from non-HIV-infected patients are numbered and have the letter B. Molecular marker sizes (in kilobase pairs) are indicated to the left of the gel.

strains were isolated from AIDS patients, 15 of which (88.2%) shared the same RFLP pattern (pattern I [Fig. 2]). Only one strain from a non-HIV-infected patient showed pattern I; this strain corresponds to an isolate of September 1992, which was probably unrelated to the others. Two strains from the 15 mentioned above with pattern I were isolated from the same patient (18 and 25 [Fig. 3]). A third specimen, isolated from this same patient in May 1992 (strain 48), also had the same RFLP pattern (Fig. 3).

In strains isolated from February 1991 to July 1992, some RFLP patterns were detected only from non-HIV-infected patients (patterns Q, R, and S [Fig. 2]). During this period, 21 strains were isolated from non-HIV-infected patients and 37 strains from AIDS patients. These results suggest that strains showing the patterns mentioned rarely produced infection in AIDS patients.

Results obtained show that IS*1311* is a useful tool in the differentiation of *M. avium* strains. Its use and that of related element IS*1245* as molecular probes could facilitate epidemiological studies of *M. avium* infection in AIDS patients.

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