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Typing of *Mycobacterium avium* Isolates by PCR

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A *Mycobacterium avium* typing method based on PCR amplification of genomic sequences located between the recently described repetitive elements IS*1245* and IS*1311* was developed. This method was applied to a set of epidemiologically related and unrelated strains and compared with restriction fragment length polymorphism analysis with IS*1245* as the probe. This PCR typing consists of a rapid and simple technique, providing a reproducible *M. avium* characterization as discriminant as restriction fragment length polymorphism analysis.

Mycobacterium avium represents one of the main agents of mycobacterial diseases other than tuberculosis and leprosy. In patients without AIDS, this potential pathogen causes pulmonary infections in adults and submandibular adenopathies in children, whereas disseminated infections are highly frequent in the late stages of AIDS (6). Moreover, *M. avium* represents an important pathogen for animals, causing tuberculosis in birds and lymph node infections in swine and cattle. The formal taxonomic designation for the mycobacteria involved in such a large variety of diseases in humans and animals is *M. avium* subsp. *avium*, as two other subspecies, namely, *M. avium* subsp. *paratuberculosis* and subsp. *silvaticum*, correspond to strict pathogenic agents of zoonoses (13). However, in usual practice, *M. avium* subsp. *avium* is referred to as *M. avium.*

M. avium bacilli are widely present in the environment and have been isolated from various sources, including water, soil, dust, and air. They may be recovered from bronchopulmonary specimens or stools of healthy individuals (7). Human-to-human transmission of *M. avium* diseases has not been demonstrated, and infection is usually considered to be acquired from the environment. However, the sources of infection as well as the routes of transmission have not been clearly established (6, 7). A better understanding of these mechanisms requires the availability of methods able to discriminate between *M. avium* strains and to detect the similarities of epidemiologically related strains.

Strain typing of mycobacteria may be achieved by pulsed-field gel electrophoresis (PFGE) or restriction fragment length polymorphism (RFLP) analysis. Since its first application to mycobacteria in 1989, PFGE has been shown to allow *M. avium* subsp. *paratuberculosis* and subsp. *silvaticum* characterization (14) as well as *M. avium* subsp. *avium* (1, 3, 11), *M. haemophilum* (17), and *M. fortuitum-M. chelonae* (5, 16) strain typing. Recently, this technique established water as a possible reservoir for *M. avium* infections in patients with AIDS (15) and revealed the occurrence of polyclonal infections (1, 11). However, RFLP has not been employed in epidemiological investigations of *M. avium* infections, although it was widely exploited in surveys of tuberculosis cases that used various repeated DNA elements, especially IS*6110* (8). Despite descriptions of the IS*900* and IS*901* repetitive DNA sequences of *M. avium* subsp. *paratuberculosis* and subsp. *silvaticum* strains, respectively, RFLP could not be applied to *M. avium* strain typing since no polymorphism was detected for these elements (4). Two recently described insertion sequences, IS*1245* and IS*1311*, sharing 83% identity at the DNA level were found to be consistently present in *M. avium* strains and to display a highly diverse RFLP in human isolates (4, 9).

In this study, we aimed to develop a simple and rapid PCR typing method for *M. avium*. Primers to the ends of IS*1245* and IS*1311* were designed in an attempt to amplify DNA fragments between copies of both of these insertion elements (IS) and to obtain PCR patterns useful for discriminating between clinical isolates.

MATERIALS AND METHODS

Mycobacterial isolates. Twenty-six *M. avium* isolates, including 22 strains isolated from 14 patients with AIDS and 4 strains from 4 patients not infected with human immunodeficiency virus, were studied. All strains from human immunodeficiency virus-infected patients consisted of blood cultures, including 7 independent isolates from seven patients (B, C, D, F, G, K, and L) and 15 sequential isolates from seven patients (Fig. 1 and Table 1). Strains 1, 5, 8, and 10 (patients A, E, H, and J) were recovered from gastric aspirate, bronchopulmonary, or lymph node specimens from human immunodeficiency virus-seronegative patients. In this study, strains isolated from different patients were considered to be unrelated and sequential strains isolated over weeks from a single patient were considered to be related.

Strains were identified as *M. avium* on the basis of conventional tests and by using a specific probe, either DT6 (12) or the *M. avium* Gen-Probe kit. Serotypes were determined by thin-layer chromatography and detection of specific peptidoglycolipids after mild saponification of lipid extracts, as recommended by Brennan et al. (2).

RFLP. *M. avium* strains were scraped from Löwenstein-Jensen slants, resuspended in 5 ml of Middlebrook 7H9 broth containing 1 mg of D-cycloserine per ml, and incubated overnight at 37°C. Cells were heated for 20 min at 80°C, centrifuged, and resuspended in 250 μl of 25% (wt/vol) sucrose–50 mM Tris (pH 8)–50 mM EDTA containing 500 μg of lysozyme per ml. Incubation was continued for an extra night. Then 250 μl of a solution of 100 mM Tris, 1% (wt/vol) sodium dodecyl sulfate (SDS), and 400 μg of proteinase K per ml was added and incubated for 4 h at 55°C. DNA was extracted with phenol-chloroform, precipitated with ethanol, and dissolved in TE (10 mM Tris, 1 mM EDTA [pH 8]).

Two micrograms of mycobacterial DNA was digested with 10 U of *Pvu*II in a 30-μl reaction mixture. DNA fragments were resolved by overnight electrophoresis on a 1% agarose gel at 1.5 V/cm and transferred to a nylon membrane (N^+-Hybond; Amersham International, Amersham, United Kingdom) by the method of Southern. The membrane was hybridized overnight at 42°C with the probe prepared as described below. Then the membrane was washed twice for 10 min at 55°C with 0.5× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate containing 0.4% SDS) and washed twice for 5 min at room temperature with 2× SSC. The presence of peroxidase-labelled DNA probe was determined with the ECL detection system (Amersham International).

By the method of Guerrero et al. (4), a 427-bp target sequence within the transposase gene of IS*1245* was amplified and used as a probe for RFLP. Amplification products were analyzed by electrophoresis on a 1.5% agarose gel and detected by ethidium bromide staining. The 427-bp amplified product was recovered from agarose according to the GeneClean II kit instructions (Bio 101, Inc., La Jolla, Calif.). The probe was labelled with horseradish peroxidase by using ECL (Amersham International).

PCR typing. For PCR tests, approximately 1 μg of mycobacteria was taken from Löwenstein-Jensen slants, suspended in 100 μl of TE containing 1% Triton

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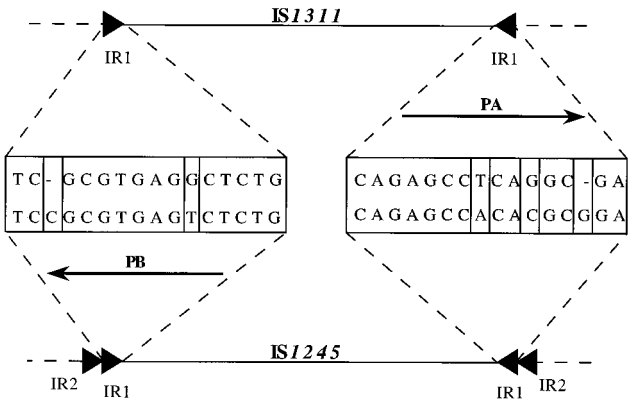

FIG. 1. Schematic representations of IS*1245* and IS*1311* and locations of primers PA and PB. IS*1245* is a 1,414-bp element flanked by two different inverted repeats, IR1 (a 16-bp unit) and IR2 (a 38-bp unit). IS*1311* is a 1,317-bp element delimited by IR1, a 15-bp unit. IR1 sequences from IS*1245* and IS*1311* show 85% identity at the DNA level.

X-100, and incubated for 30 min at 100°C. These lysates were used as DNA sources without further purification.

Amplification reactions were performed with 50-μl volumes containing 1× *Taq* polymerase buffer (50 mM Tris-HCl [pH 8.3], 50 mM KCl, 0.01% [wt/vol] gelatin), 1.5 mM $MgCl_2$, 200 μM (each) deoxynucleoside triphosphates, 1 μM (each) nucleotide primers (PA, 5′ CAGAGCCTCAGGCGA 3′, and PB, 5′ CAGAGCCTCACGCGGA 3′), and 2 U of *Taq* polymerase (Perkin-Elmer Corp., Norwalk, Conn.) covered with mineral oil. Two microliters of template DNA prepared as described above was added to the mix. The 35 amplification cycles consisted of 1 min at 95°C, 1 min at 55°C, and 2 min at 72°C and were followed by 10 min of extension at 72°C in a Perkin-Elmer thermocycler. These PCR conditions were selected because they provide clear and reproducible banding patterns of *M. avium* strains. Amplification products were analyzed by electrophoresis on a 2% agarose gel (MetaPhor agarose; FMC BioProducts, Rockland, Maine) and detected by ethidium bromide staining.

RESULTS

To evaluate the epidemiological value of PCR typing, related and unrelated strains were studied by both RFLP and PCR techniques.

First, isolates were analyzed by Southern blot hybridization with IS*1245* (Fig. 2A). The patterns obtained were polymorphic and complex, containing up to 27 copies (Fig. 2A). However, one strain presented a single-band profile corresponding to a high-molecular-weight restriction fragment (Fig. 2A, lane 3). To assess that the single band corresponded to a single IS copy, we performed RFLP with other restriction enzymes, namely, *Pst*I, *Eco*RI, and *Sma*I. The detection of a unique copy was confirmed with each digestion system (data not shown). The weaker bands observed in some patterns (Fig. 2A) may result from cross-hybridizations with the related element, IS*1311*, as suggested by Roiz et al. (9). It has to be stressed that IS*1311* contains a single *Pvu*II restriction site. Digestion with this enzyme releases two fragments, of which one 735-bp fragment contains the sequence used as the probe. The low-molecular-weight restriction fragments of less than 1.4 kb detected in RFLP patterns are likely to correspond to IS*1311.*

Unrelated strains isolated from 18 study patients produced different RFLP profiles (Fig. 2A). However, the RFLP patterns of sequential strains, isolated over 0.5 to 23 months (Table 1) from seven patients, were found to be identical for each of these patients (Fig. 2A, lanes 14 to 26 [patients F and M to R]), except for the first strain isolated from patient M (Fig. 2A, lane 13). The three strains isolated from patient M were also subjected to PFGE with two independent digestion enzymes, *Dra*I and *Xba*I. The PFGE patterns obtained with each digestion system confirmed the RFLP analysis (data not shown).

In the second part of this study, we selected primers consisting of consensus sequences of the imperfect inverted repeats of IS*1245* and IS*1311* (Fig. 1). We analyzed the same 26 isolates by PCR typing with primers PA and PB. Banding patterns consisted of less than 10 bands, with some corresponding to intense bands and others corresponding to weaker bands (Fig. 2B). Patterns were found to be reproducible for different PCR tests performed on the same bacterial extract (data not shown).

For all strains subjected to analysis, the typing results based on PCR profile interpretation were similar to those provided by RFLP (Fig. 2).

RFLP and PCR patterns were not altered by the acquisition of resistance to clarithromycin, as observed for sensitive strains 22 and 25 and for resistant strains 23 and 26 from patients Q and R, respectively (Fig. 2).

DISCUSSION

This report describes a reliable and rapid methodology for the characterization of *M. avium* strains on primary isolation that consists of PCR amplification of genomic sequences between IS. This strategy had been previously proposed by Ross et al. for *M. tuberculosis* typing, relying on the amplification of DNA fragments between IS*6110* copies (10). To increase the number of possible priming sites, we took advantage of the high-level homology of IS*1245* and IS*1311* and selected primers directed to both elements (Fig. 1).

The RFLP analysis performed in this study confirms the marked polymorphism provided by the repetitive elements IS*1245* and IS*1311* for *M. avium* strain typing (4, 9). Similar to the results for strains studied by Guerrero et al. (4), we found that the average IS copy number for the strains of this study was 20, ranging from 1 to 27 copies. Furthermore, we confirmed the occurrence of a human *M. avium* strain with a unique IS*1245* element (Fig. 2A, lane 3).

Similar to previous results observed with PFGE (1, 11), RFLP could discriminate between prolonged infections due to a single strain and infections involving different *M. avium* strains. The two distinct patterns found for patient M correspond to strains isolated over 23 months (Fig. 2, lanes 13 and

TABLE 1. Sequential isolates from patients with AIDS

Patient	Strain	Serotype(s)	Interval since previous isolate (mo)	Cluster[a]
M	13	4		I
	14	1	23	II
	15	1	14	II
N	16	No serotype		III
	17	No serotype	0.5	III
O	18	2 + 9		IV
	19	2 + 9	2	IV
P	20	21		V
	21	21	0.5	V
Q	22	21		VI
	23	21	2	VI
F	6	1		VII
	24	1	1	VII
R	25	8		VIII
	26	8	4	VIII

[a] Strains were assigned to clusters according to RFLP and PCR patterns (Fig. 2).

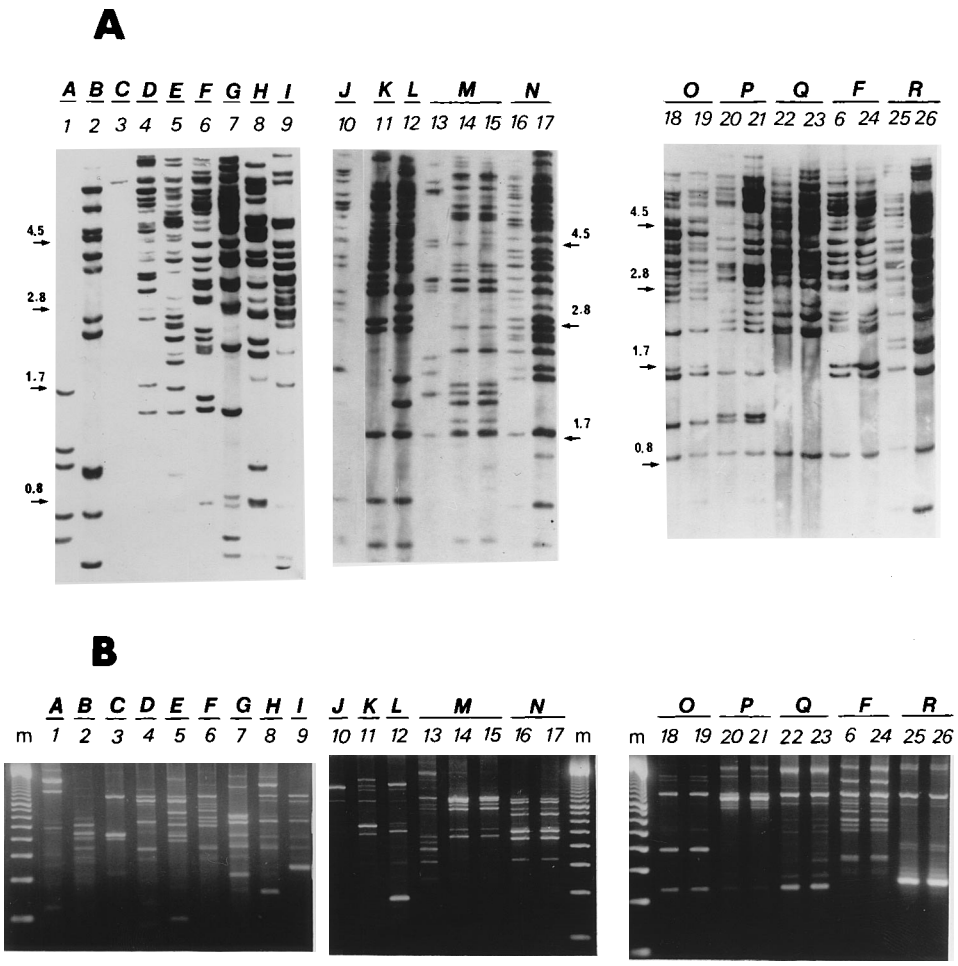

FIG. 2. (A) RFLP patterns of *M. avium* strains with IS*1245* as the probe; (B) PCR typing of the same strains. Patients are indicated by capital letters, and strains are indicated by numbers. The positions of molecular markers (in kilobase pairs) are indicated by arrows. Lanes m, DNA molecular weight markers consisting of 100-bp ladders.

14). This finding suggests the occurrence of subsequent or polyclonal infections. Identical profiles found for sequential isolates over 14 months (Fig. 2, lanes 14 and 15) underline the stability of IS distribution and the reliability of epidemiological techniques based on their detection. Moreover, the identical patterns of sequentially isolated strains emphasized the reproducibility of the PCR procedure developed in this study (Fig. 2B, lanes 14 to 26). One strain presented an RFLP pattern that consisted of a single band, whereas its PCR pattern consisted of several bands, two major and one minor (Fig. 2, lanes 3). These PCR products are likely due to the presence of nonspecific priming sites near the IS copy and likely are not related to the presence of undetected IS copies, as checked by RFLP analysis with different restriction enzymes.

The results of PCR typing with primers PA and PB were as discriminant as the results of RFLP. In both systems, unrelated strains produced different patterns and related strains displayed identical patterns.

This PCR technique may be more readily applied than PFGE or RFLP typing. In this study, template DNA consisted of a crude bacterial lysate, whereas for PFGE and RFLP methods, DNA purification is necessary. Moreover, this PCR method is quicker in terms of technical handling time. PFGE may be hampered by the necessity to release DNA in agarose plugs in order to prevent nonspecific physical breakage of the molecule, while RFLP is slowed down by the multiple steps required, including electrophoresis of digested DNA, Southern blot, and hybridization with labelled probes.

The availability of a rapid typing technique is of great interest for large epidemiological studies. It has recently been demonstrated that tap water could represent a source of contamination for patients with AIDS. This result justifies the search for *M. avium* in the environment of these patients and the comparison of environmental and human strains to determine the role of tap water in the acquisition of these frequent infections. Moreover, it has been shown that *M. avium* polyclonal infections are common (1, 11). PCR typing will be helpful in ascertaining the frequency of such polyclonal infections by rapid analysis of multiple colonies of mycobacterial cultures recovered from patients with AIDS. A simple typing method will also be useful in the follow-up of patients to differentiate recurrent infections from new infections and to substantiate decisions regarding patient management.

In conclusion, this study shows the significance of a PCR typing technique directed to the repetitive elements IS*1245* and IS*1311*, as this technique is simple, rapid, and reproducible and provides relevant characterizations of *M. avium* strains. This PCR method deserves to be tested on numerous isolates

in order to be validated for large epidemiological studies designed to identify the sources of human infection.

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