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IS*1245* Restriction Fragment Length Polymorphism Typing of *Mycobacterium avium* Isolates: Proposal for Standardization

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Mycobacterium avium **has become a major human pathogen, primarily due to the emergence of the AIDS epidemic. Restriction fragment length polymorphism (RFLP) typing, using insertion sequence IS*1245* as a probe, provides a powerful tool in the molecular epidemiology of *M. avium*-related infections and will facilitate well-founded studies into the sources of *M. avium* infections in animal and environmental reservoirs. The standardization of this technique allows computerization of IS*1245* RFLP patterns for comparison on a local level and the establishment of *M. avium* DNA fingerprint databases for interlaboratory comparison. Moreover, by combining international DNA typing results of *M. avium* complex isolates from a broad spectrum of sources, long-lasting questions on the epidemiology of this major agent of mycobacterial infections will be answered.**

The *Mycobacterium avium* complex (MAC) comprises opportunistic and obligate pathogens of animals and humans as well as less-defined (sub)species (12, 22, 29). Previously, on the basis of the production of similar polar glycolipid surface antigens which could be used in agglutination tests of bacterial cells, *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium scrophulaceum* were assigned to the MAC (29). Later, Thorel et al. proposed dividing the MAC into the species *M. avium*, *Mycobacterium silvaticum*, and *Mycobacterium paratuberculosis* because of differences in genotypic and growth characteristics, pathogenicity, and host range (22).

Several classical and novel techniques are available to identify and type MAC isolates for taxonomic or epidemiological purposes. Until a few years ago, most laboratories favored serotyping (1, 10), and extensive interlaboratory studies have been conducted to standardize this technique (28). More recently, other techniques have become available; these techniques include multilocus enzyme electrophoresis (28) and DNA-based methodologies, such as pulsed-field gel electrophoresis (14, 20), PCR-based typing (17, 21), and restriction fragment length polymorphism (RFLP) typing. For the latter technique, insertion sequences such as IS*900* (6), IS*901* (13), IS*902* (15), IS*1110* (9), IS*1141* (26), IS*1245* (7), and IS*1311* (19) have been proposed as possible epidemiological tools to type and distinguish isolates of the different groupings within the MAC. On the basis of RFLP typing, Guerrero et al. (7) and Bono et al. (2) determined the host range of IS*1245* to be limited to *M. avium*, while *M. intracellulare* appeared to be devoid of this genomic element. Devallois and Rastogi (3) showed that the highly similar IS*1245* and IS*1311* possess a similar discriminatory potential for *M. avium* isolates.

Highly polymorphic multibanded IS*1245* RFLP patterns were almost invariably found among *M. avium* isolates from humans (2, 7, 16, 18, 19). A significant part of the IS*1245* DNA fingerprints of *M. avium* isolates from pigs shared a high degree of similarity with the human isolates (2, 18). In contrast, isolates from a wide variety of bird species were found to possess identical three-band patterns (2, 18). The three-band pattern found in birds was also found in a small fraction of the pig isolates. As this pattern was only rarely encountered among human isolates, birds were found not to be an important source of *M. avium* infections in humans (18).

Other possible reservoirs for *M. avium* infection in humans have been reported to be tap water (27), hard cheese (11), and cigarettes (4). Extensive RFLP typing studies of *M. avium* isolates from these and other reservoirs are needed to investigate the epidemiological relatedness with human infections. This will also provide more insight into the taxonomy and evolutionary divergence within the MAC. To fully explore the possibilities of RFLP typing, international standardization of this method is required. This would facilitate the establishment of databases of *M. avium* DNA fingerprints and help to trace true sources of infection of this emerging potential pathogen. A previous international standardization of IS*6110* RFLP typing of *Mycobacterium tuberculosis* has resulted in an international database of fingerprints.

Proposal for standardization. Standardization of IS*1245* RFLP typing involves the following issues: the choice of the restriction enzyme, the electrophoresis conditions, the preparation of the probe (primers and target), the hybridization stringency, and the use of molecular size marker DNA.

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M. avium is a slow-growing microorganism, and the amount of bacterial culture obtained from a Löwenstein slant is often limited. Furthermore, the quantity of DNA extracted from *M. avium* bacteria is often less than that from *M. tuberculosis* complex cells. When insufficient growth is obtained on Löwenstein medium, an excellent way to obtain high yields of *M. avium* cells in the log phase can be achieved by inoculating bacteria from a viable culture in 5 ml of Middlebrook 7H9 liquid medium containing Tween 80 and albumin-glucose (20, 24). After 7 days, the culture is transferred into a volume of 50 ml and incubated (while being agitated) for an additional 10 days (optical density at 600 nm of 0.8 to 1.2). The cells are concentrated by centrifugation and resuspended in a total volume of 400 μl of Tris EDTA buffer for the DNA extraction. Cell lysis and DNA extraction should be performed as described previously (24).

The choice of the restriction enzyme is strongly dependent on the range of sizes of DNA fragments obtained after cleavage of genomic DNA from *M. avium* strains. Several restriction enzymes provide a wide range of DNA fragments and are capable of defining distinct banding patterns and clusters of identical or highly related isolates, and at least one enzyme, *Nru*I, has been proposed as appropriate for IS*1245*-based RFLP analysis (5). However, in most previous *M. avium* RFLP studies (2, 3, 7, 18, 19), the restriction enzyme *Pvu*II was used and *Pvu*II-based RFLP pattern databases have been established. We therefore recommend using *Pvu*II as the restriction enzyme. The use of this restriction enzyme yields restriction fragments ranging from 0.5 to 20 kb. The disadvantage of the use of this enzyme is the appearance of faint bands in the RFLP patterns (5, 18). This can largely be overcome by using a probe for hybridization prepared by PCR amplification on an IS*1245* DNA-containing plasmid and higher-stringency washing conditions after hybridization.

Except for the strains with the three-band pattern of birds, IS*1245* RFLP patterns of *M. avium* isolates consist of a high average number of bands, approximately 20 (18). In order to facilitate accurate computer-assisted analysis of these multibanded DNA fingerprints, it is necessary to have a high electrophoresis resolution. The use of relatively long agarose gels (minimum of 24 cm) and electrophoresis at a low voltage (0.5 V/cm) for 20 h can achieve this. The electrophoresis should be continued until the 872-bp fragment of an external DNA size marker, for example, *Hae*III-digested ϕX174 DNA, has reached a distance of 19 cm from the slots of the gel.

The probe used for the detection of IS*1245*-containing *Pvu*II restriction fragments in the hybridization procedure can be prepared by PCR with the primer set described by Guerrero et al. (7). The two primers P1 (5′-GCCGCCGAAACGATCT AC) and P2 (5′-AGGTGGCGTCGAGGAAGAC) amplify the region of IS*1245* sequence from positions 197 to 623 (accession no. L33879), resulting in a PCR product of 427 bp. The required PCR treatment consists of 30 cycles, with 1 cycle being 1 min at 94°C, 1 min at 65°C, and 1 min at 72°C, followed by one final extension step of 10 min at 72°C (7).

The high degree of similarity between the DNA sequences of IS*1245* and IS*1311* (19) may result in variable PCR products if DNAs from different strains are used as targets for PCR probe amplification. Furthermore, there may be more, yet unknown, IS elements in *M. avium* strains representing the same type of insertion sequence family. Therefore, to obtain a standardized and pure IS*1245* probe, the use of plasmid pMA12 (Fig. 1), containing the IS*1245* DNA sequence as an insert, is highly recommended as a target for probe amplification. This pUC-derived plasmid contains the *Nru*I/*Sph*I restriction fragment of IS*1245* between the *Sph*I and *Sma*I sites. Since both

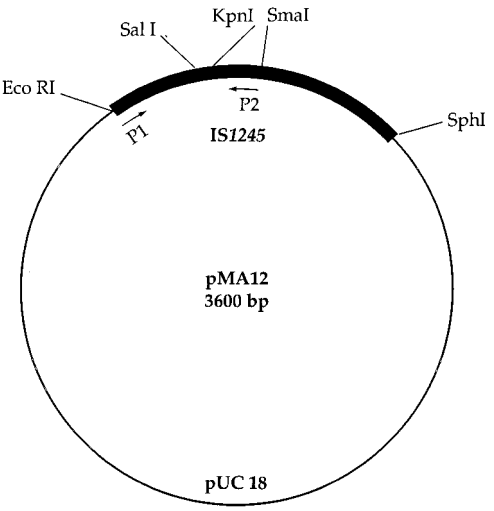

FIG. 1. Physical map of plasmid pMA12 containing an IS*1245* insert, which can be used as a target in PCR amplification resulting in a standardized IS*1245* probe.

*Nru*I and *Sma*I are blunt-end cleavers, after ligation both restriction sites are lost. However, the IS*1245* insert can be removed by using restriction enzymes *Sph*I and *Eco*RI. In PCR, 10 to 20 ng of undigested plasmid DNA is sufficient to ensure an optimal DNA target concentration.

The accurate determination of the sizes of IS*1245*-containing *Pvu*II restriction fragments requires the use of reference DNA size markers. Either internal or external size markers could be used. However, the use of internal size markers provides much more accurate band position determinations. We recommend using the same internal size markers as those used in the standardized method for RFLP typing of *M. tuberculosis* isolates (23). In short, each digested *M. avium* DNA sample is mixed with a reference DNA mix consisting of *Pvu*II-digested supercoiled DNA ladder and *Hae*III-digested ϕX174 DNA (23). The use of a mix of two internal size markers is necessary to obtain reference DNA fragments of the right size range. After electrophoresis and Southern blotting, the first hybridization enables detection of IS*1245*-containing *Pvu*II restriction fragments. An additional hybridization on the same membrane is performed by using a mix of *Pvu*II-digested supercoiled DNA ladder and *Hae*III-digested ϕX174 marker DNA as a probe to visualize the marker bands of known sizes (Fig. 2). During the computer-assisted analyses, both hybridization patterns are superimposed and the sizes of IS*1245* bands can be accurately determined.

It is also possible to use an external size marker with the right range of DNA fragments on at least three different parts of the gel. To facilitate the best achievable intralaboratory comparison of IS*1245* RFLP patterns with external size markers, we propose to use reference strain IWGMT49. The computer-assisted analysis based on three external markers will be less accurate than analysis based on internal size markers but will be sufficiently accurate to compare DNA patterns within an accuracy of 1.5% band position deviation.

The final hybridization patterns are strongly dependent on the choice of the stringency conditions during the hybridization and posthybridization washes. The ECL direct system (Amersham International plc) for labeling and detection of probes can be applied with the following modifications. It is recommended that after hybridization more stringent conditions are

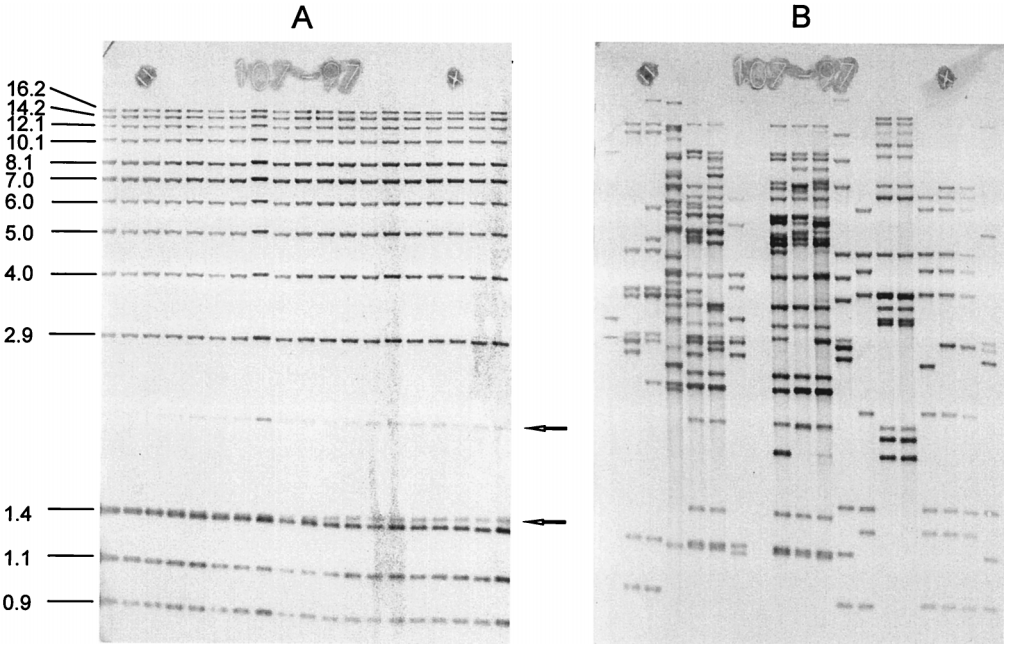

FIG. 2. IS*1245* RFLP patterns (B) and internal size marker patterns (A) prepared by the proposed standard method. The internal marker bands are *Pvu*II-digested supercoiled DNA ladder fragments with molecular sizes of 16.2, 14.2, 12.1, 10.1, 8.1, 7.0, 6.0, 5.0, 4.0, and 2.9 kb and *Hae*III-digested ϕX174 ladder fragments with molecular sizes of 1.4, 1.1, and 0.9 kb. Note that the bands indicated by the two arrows represent supercoiled DNA ladder fragments that were not well digested and that should be excluded from the computer analyses. The smallest fragment of the internal marker patterns is 0.9 kb. In standard DNA fingerprinting of *M. tuberculosis* isolates, the 0.6-kb band of the *Hae*II-digested ϕX176 DNA marker is also used for computer-assisted analysis (24). For typing of *M. avium*, this band is not required. The outermost IS*1245* RFLP patterns in panel B represent the external control strains R13 (leftmost lane) and IWGMT49 (rightmost lane).

used than those suggested by the manufacturer in order to obtain IS*1245*-specific hybridization patterns. This is achieved by washing the Southern blot twice for 10 min each time at 55°C with a 6 M urea primary wash buffer (supplemented with 0.1× SSC–0.4% SDS [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, and SDS is sodium dodecyl sulfate]), followed by a secondary wash for 5 min each time at 65°C with 2× SSC–0.1% SDS. Rinsing twice for 5 min at room temperature with 1× SSC completes the washing procedure.

Interlaboratory exchange of computerized IS*1245* RFLP patterns requires the standardization of the computer program and settings. The Gelcompar software (Applied Maths, Kortrijk, Belgium) has been successfully used before in both *M. avium* and *M. tuberculosis* epidemiology (8, 18, 25), but other DNA fingerprint analysis computer programs can be used (3). Due to the use of the 24-cm-long agarose gels and the on average high-copy-number of IS*1245*, a track resolution of 1,000 positions is recommended. The standard positions of the bands of the internal marker for normalization are 38, 48, 64, 88, 128, 157, 197, 253, 330, and 446 for the *Pvu*II-digested supercoiled DNA ladder and 784, 875, 957 for the three largest bands of *Hae*III-digested ϕX174 DNA. The external marker strains should be applied to each gel, one to the second slot and one to the penultimate slot. One of these control strains should provide a wide range of IS*1245*-containing *Pvu*II restriction fragments, and for this purpose, we recommend the use of strain IWGMT49 (band positions 62, 254, 447, 459, 481, 754, 840, and 934). For a second control strain, we recommend R13, representing the three-band IS*1245* RFLP pattern typical of birds (band positions 110, 416, and 452). The use of two external marker strains offers the possibility of controlling the superimposing of the IS*1245* and size marker patterns. The band position deviation between the DNA patterns of the control strains in different gels should not exceed 0.8%. The entire procedure for RFLP typing and computer-assisted analysis of mycobacteria has been described in detail in a laboratory manual (24).

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