Identification of *Mycobacterium avium* Genotypes with Distinctive Traits by Combination of IS*1245*-Based Restriction Fragment Length Polymorphism and Restriction Analysis of *hsp65*

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One-hundred eight *Mycobacterium avium* isolates from pigs, humans, birds, and bovines were typed by the IS*1245*-based restriction fragment length polymorphism (RFLP) method and PCR-restriction enzyme analysis (PRA) of *hsp65*. Nine clusters of isolates showing more than 80% similarity in their RFLP profiles were detected. The largest cluster (cluster B) included 32 of 79 pig isolates (40.5%), 3 of 25 human isolates (12%), and 1 of 2 bovine isolates, comprising 33% of all isolates. The second largest cluster (cluster A) included 18 pig isolates (22.8%) and 6 human isolates (24%). Six smaller clusters included six pig isolates (clusters C and D), four and two human isolates (clusters E and F, respectively), two pig isolates (cluster I), and two pig isolates plus one bovine isolate and the avian purified protein derivative strain (cluster H). Cluster G represented the "bird-type" profile and included the bird isolate in this series, one pig isolate, plus reference strain R13. PRA revealed four allelic variants. Seventy-seven isolates were identified as *M. avium* PRA variant I, 24 were identified as *M. avium* PRA variant II, 6 were identified as *M. avium* PRA variant III, and 1 was identified as *M. avium* PRA variant IV. Except for three isolates from cluster B, each of the RFLP clusters was associated with a single PRA pattern. Isolates with unique (nonclustered) RFLP profiles were distributed between PRA variants I and II, and there was one unique isolate of PRA variant IV. These observations are consistent with divergent evolution within *M. avium*, resulting in the emergence of distinct lineages with particular competence to infect animals and humans.

Disseminated infection due to isolates of the *Mycobacterium avium* complex (MAC) was rare prior to the human immunodeficiency virus (HIV) pandemic, but disseminated MAC infection emerged as the most prevalent disseminated bacterial infection in patients with advanced AIDS. MAC, which is widespread in the environment (22), includes isolates of the closely related species *M. avium* and *M. intracellulare* and probably other less well defined genospecies (7).

Members of MAC can be classified into serotypes on the basis of differences in the C-mycoside glycopeptidolipids. Saito et al. (16), using 16S rRNA probes, assigned serotypes 1 to 6, 8 to 11, and 21 to *M. avium* and serotypes 7, 12 to 20, and 25 to *M. intracellulare*. Certain *M. avium* serotypes (serotypes 1, 4, and 8) have been reported to predominate among isolates from AIDS patients (25). Although these same serotypes have been isolated from the environment, this technique does not have the discriminatory power needed to identify sources of transmission.

Although *M. avium* has been associated with a variety of diseases in both animals and humans, the specific routes of infection are not well defined. Person-to-person transmission

has not been demonstrated. Ingestion of environmental organisms followed by invasion through the gastrointestinal tract has been suggested as the main route of infection in AIDS patients because the organisms are frequently isolated from the stools of these patients (3). There is also an important positive correlation between the presence of MAC in respiratory samples and the subsequent development of disseminated disease (8). Immunocompetent individuals can have asymptomatic respiratory and intestinal colonization but rarely develop disseminated disease (7). No specific virulence factors have been identified to explain why, of the more than 100 species of nontuberculous mycobacteria, *M. avium* is the most common cause of infection in humans.

Investigation of these questions is complicated by the phenotypic and genotypic heterogeneity of *M. avium*. On the basis of its host range characteristics and pathogenicity, *M. avium* was recently subdivided into subspecies: *M. avium* subsp. *avium*; *M. avium* subsp. *silvaticum* or wood pigeon bacillus; and *M. avium* subsp. *paratuberculosis*, which is responsible for Johne's disease in cattle (23). It has been proposed that *M. avium* isolates from birds represent a separate taxon (14).

A considerable number of genetic tools for identification and typing of MAC isolates have become available in recent years. The most widely used system is a commercial hybridization assay (AccuProbe; Gen-Probe, San Diego, Calif.), in which species-specific labeled DNA probes bind to rRNA sequences. Specific probes are available for the identification of

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TABLE 1. Isolates included in this study[a]

No. of isolates	Host	Source	PRA type	RFLP type	AccuProbe reaction result
18	Pig	Lymph node	I	A	+
29	Pig	Lymph node	I	B	+
3	Pig	Lymph node	II	B	
6	Pig	Lymph node	II	C	+
6	Pig	Lymph node	III	D	+
1	Pig	Lymph node	I	G	
2	Pig	Lymph node	I	H	
2	Pig	Lymph node	II	I	
5	Pig	Lymph node	I	Unique	
7	Pig	Lymph node	II	Unique	
1	Human, HIV+	Sputum	I	A	
1	Human, HIV−	Sputum	I	A	
1	Human, HIV+	Bone marrow	I	A	
1	Human, ND	Bone marrow	I	A	
1	Human, HIV+	Blood	I	A	+
1	Human, ND	Blood	I	A	
1	Human, HIV+	Sputum	I	B	
1	Human, ND	Sputum	I	B	
1	Human, ND	Blood	I	B	
3	Human, ND	Sputum	II	E	
1	Human, ND	Bronchial fluid	II	E	
1	Human, HIV+	Gastric fluid	I	F	
1	Human, HIV+	Blood	I	F	
1	Human, HIV+	Lymph node	I	Unique	
1	Human, ND	Lymph node	I	Unique	
1	Human, ND	Sputum	I	Unique	
2	Human, ND	Sputum	II	Unique	
1	Human, HIV+	Bone marrow	IV	Unique	+
1	Human, ND	Abscess	I	Negative	+
1	Human, ND	Lymph node	I	Negative	+
1	Human, ND	Biopsy	I	Negative	+
1	Human, HIV+	Blood	I	Negative	+
1		Strain D4	I	H	
1	Bovine	Lung	I	H	
1	Bovine	Lymph node	I	B	
1	Crane	Intestine	I	G	

[a] Abbreviations and symbols: I, II, III, and IV, *M. avium* PRA variants (11, 12); A to I, groups of isolates showing similarities greater than 80%, as defined by IS*1245*-RFLP typing (Fig. 1); HIV+, HIV positive; HIV−, HIV negative; ND, nondetermined HIV infection status; +, result of AccuProbe analysis with *M. avium*-specific probe performed with one isolate in the group.

M. avium and *M. intracellulare*, but some MAC serovars do not hybridize with either species-specific probe and are designated "MAC-other" (19). Amplification of the DT1 and DT6 fragments was considered equally sensitive for species identification of *M. avium* and *M. intracellulare* (4). Insertion sequences IS*900* (5), IS*901* (10), IS*1245* (6), and IS*1311* (15) can be used as markers to make further subdivisions among *M. avium* isolates.

PCR-restriction enzyme analysis (PRA) is a method of identifying species of mycobacteria on the basis of analysis of restriction patterns in digests of a PCR-amplified fragment of *hsp65* (20). In the initial report of the method, all clinical *M. avium* isolates examined demonstrated a single PRA type. In our previous studies of isolates obtained from animals and humans (11, 12), we identified three additional allelic variants. In this report we describe a comprehensive genotypic analysis of 108 *M. avium* isolates from pigs, humans, birds, and bovines by IS*1245*- and IS*1311*-based restriction fragment length polymorphism (RFLP) analysis, PRA, and PCR-amplification of DT1 and DT6.

MATERIALS AND METHODS

Isolates. The study included 108 isolates from various species: 79 pig, 25 human, 2 bovine, and 2 bird isolates. The pig isolates, each of which was from a single infected animal, were cultured from animals slaughtered between November 1997 and July 1998 during an outbreak in an important center of swine meat production in the southern region of Brazil and were obtained from Empresa Brasileira de Pesquisa Agropeudria (Santa Catarina, Brazil).

Of the 25 human isolates, 23 were cultured from individual patients, of whom 7 were HIV positive, 1 was HIV negative, and the remainder were of unknown HIV status. Three of these patients had mixed infections with *M. avium* plus *M. kansasii*, *M. tuberculosis*, and *M. lentiflavum*, respectively (data not shown). The remaining two human isolates of *M. avium* were cultured from a single HIV-positive patient with a polyclonal infection (12). All of the clinical isolates were identified at Instituto Adolfo Lutz, São Paulo, Brazil, between July and September 1998, after the implementation of highly active antiretroviral therapy in Brazil.

Two bovine isolates and one bird isolate, cultured in 1996 and 1997, plus avian strain D4, which is used for the production of avian purified protein derivative, were obtained from the collection of the Instituto Biológico, São Paulo, Brazil. Each of the contributing institutions cited represent reference laboratories for the isolation and identification of mycobacteria from animals and humans in Brazil and are engaged in disease control.

Two *M. avium* reference strains, strain R13 (the "bird-type" strain) and strain IWGMT49 (originally isolated from a pig), were kindly provided by Dick van Soolingen (National Institute of Public Health and the Environment, Bilthoven, The Netherlands) and were included in all gels used for RFLP analysis.

Isolates were kept at −80°C in Middlebrook 7H9 liquid medium (Difco Laboratories, Detroit, Mich.) supplemented with OADC (oleic acid, albumin, dextrose, and catalase; Difco) plus 15% glycerol. Only isolated colonies of *M. avium* were included in the RFLP analysis.

Identification of mycobacteria. DNA from all isolates was obtained by three freeze-boil cycles of a loopful of mycobacteria grown on solid medium (Löwenstein-Jensen or Stonebrink) and suspended in 0.4 ml of TE (10 mM Tris, 1 mM EDTA [pH 8.0]) with 1% Triton X-100. Five to 10 μl was used for PCR.

Identification of *M. avium* was based on the results of PRA (20), amplification of *M. avium*-specific insertion sequences IS*1245* (6) and IS*1311* (15), and amplification of MAC-specific fragments DT1 and DT6 (21).

Biochemical identification and probe testing for the detection of rRNA specific for *M. avium* (AccuProbe; Gen Probe) were performed with selected isolates.

Strain typing. Strain typing was performed by RFLP analysis, as described by van Soolingen et al. (24). Briefly, isolates were grown in 5 ml of Middlebrook 7H9 liquid medium-OADC. After 7 to 15 days, the culture was transferred to a volume of 50 ml and incubated at 37°C in a shaker for an additional 10 to 15 days. The cells were centrifuged and resuspended in 0.4 ml of TE for DNA extraction, as described previously (24).

DNA was digested with *Pvu*II, and 2 μg of the DNA fragments generated was electrophoretically separated on a 24-cm 0.8% agarose gel until the 872-bp fragment of *Hae*III-digested φX174 DNA (external DNA size marker; Invitrogen, Carlsbad, Calif.) reached a distance of 19 cm from the slots of the gel. A mixture of a *Pvu*II-digested supercoiled DNA ladder (Invitrogen) and *Hae*III-digested φX174 DNA was used as an internal DNA size marker. DNA was blotted onto nylon membranes (Hybond N-plus; Amersham Biosciences do Brasil Ltda., São Paulo, Brazil) and probed in different PCR experiments with probes obtained by PCR from IS*1245* or IS*1311*. The internal marker was also labeled and used as a probe. The probes were labeled and detected with the ECL direct system (Amersham) or the RPN 3690 kit (Amersham) under stringent conditions. The membranes were exposed to X-ray film (X-OMAT; Kodak, Rochester, N.Y.).

Analysis of RFLP patterns. RFLP analysis was performed at least twice with each isolate. The best images were selected and analyzed with the GelCompar II (version 2.5) program (Applied Maths, Kortrijk, Belgium). Autoradiograms of the IS*1245* fingerprints were superimposed on the autoradiograms of internal markers for normalization. The Dice coefficient of similarity for all pairwise comparisons of patterns was calculated. A dendrogram of pattern relatedness among the strains was constructed by the unweighted pair group method with arithmetic averages clustering method.

RESULTS

The species identification and biochemical characterization of the 108 isolates tested in the present study were reported
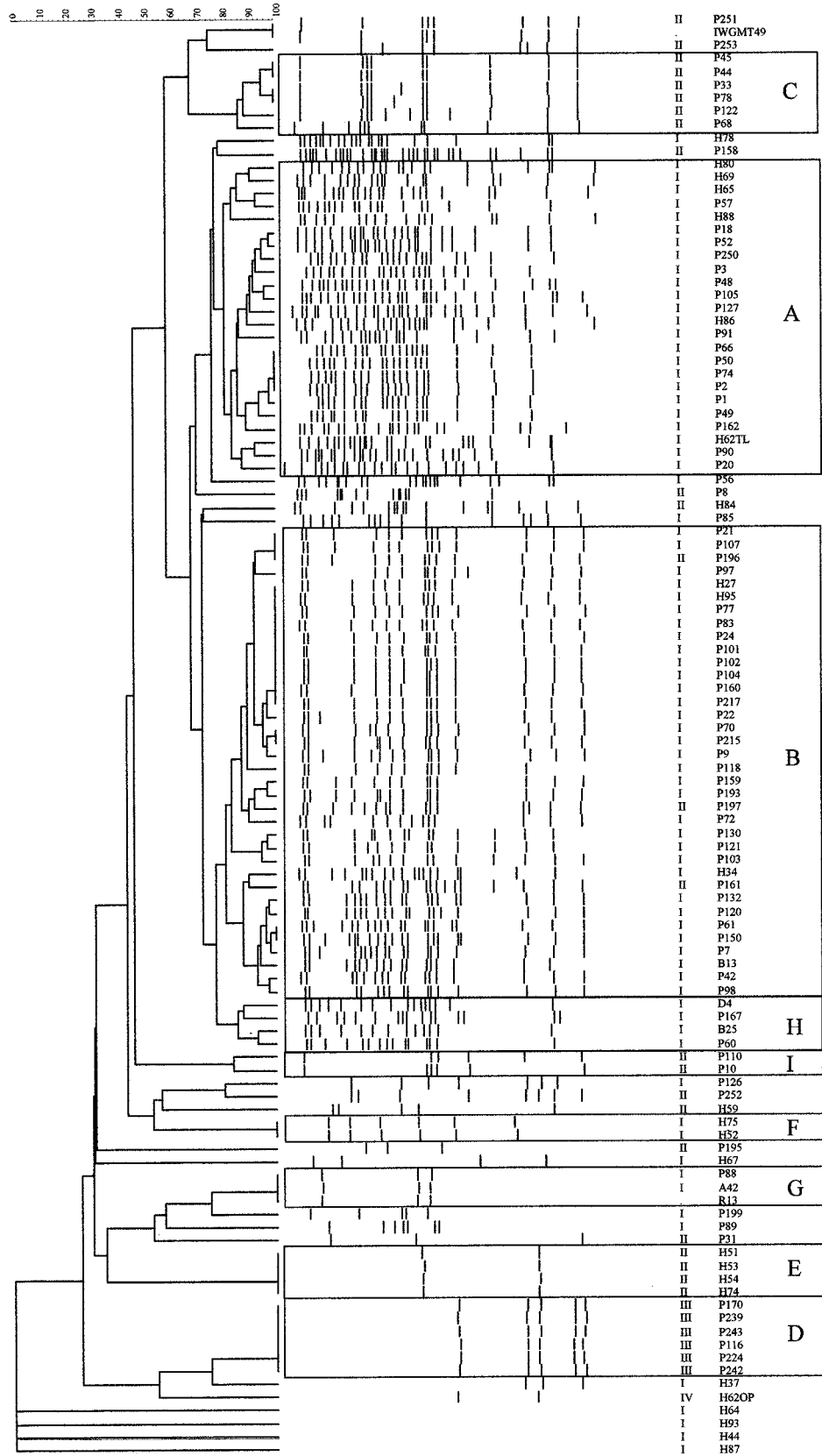
FIG. 1. Dendrogram of the 108 IS*1245*-based RFLP profiles of *M. avium* isolates from pigs, humans, birds, and bovines. The numbers at the top represent percent relatedness. Isolates showing more than 80% similarity are boxed. Letters indicate the cluster to which the isolates belong. The two columns on the right present PRA patterns and isolate names, respectively.
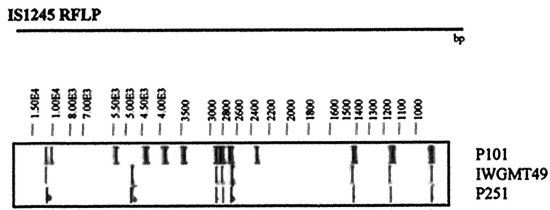
FIG. 2. IS*1245*-based RFLP patterns of strain IWGMT49 (serotype 10) and isolate P251, showing identical band patterns, and isolate P101 from cluster B, which shows seven hybridizing bands at the same positions (indicated by arrowheads) as strains IWGMT49 and P251.

FIG. 4. Variations among IS*1245*-based RFLP patterns of single colonies from isolates H78, H80, P104, and P116. Arrows indicate the positions where changes occurred.

previously (11, 12, 17, 18) and are summarized in Table 1. Seventy-seven isolates (71.3%) were identified as *M. avium* PRA allelic variant I, 24 (22.2%) were identified as *M. avium* PRA allelic variant II, 6 (5.5%) were identified as *M. avium* PRA allelic variant III, and 1 (0.9%) was identified as *M. avium* PRA allelic variant IV. Positive amplification with primers from IS*1245*, IS*1311*, and DT6 sequences was obtained with DNA from all isolates; and no positive amplification was obtained with primers from DT1 sequence. Biochemical identification and the AccuProbe test with an *M. avium*-specific probe were performed with selected isolates of each PRA allelic variant. Taken together, the results confirmed the identification of all isolates as *M. avium*.

Analysis of the RFLP profiles for all isolates performed with the GelCompar II program provided the dendrogram shown in Fig. 1. Nine clusters of isolates whose RFLP profiles showed more than 80% similarity were detected. The largest cluster (cluster B) included 32 of 79 pig isolates (40.5%), 3 of 25 human isolates (12%), and 1 of 2 bovine isolates, comprising 33% of all isolates. The RFLP pattern of cluster B included a motif of seven bands present in the RFLP pattern of pig isolate IWGMT49, which belongs to serovar 10 (14) (Fig. 2). The PRA patterns for all but three isolates were *M. avium* PRA variant I. The second largest cluster (cluster A) included 18 of 79 pig isolates (22.8%) and 6 human isolates (24%), all of which were *M. avium* PRA pattern I. Six smaller clusters included six isolates from pigs (clusters C and D), four and two
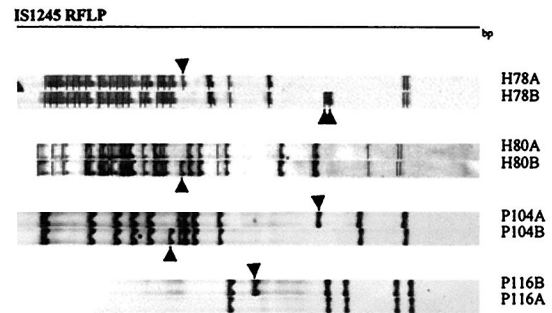
human isolates (clusters E and F, respectively), two pig isolates (cluster I), and two pig isolates plus one bovine isolate and the avian purified protein derivative strain (cluster H). The clustering of isolates with low IS*1245* copy numbers in clusters D, E, and F was confirmed by hybridization with the IS*1311*-specific probe and PRA (Fig. 3). Cluster G represented the bird-type profile and included, besides reference strain R13, the crane isolate and one pig isolate. Four human isolates lacked IS*1245*-specific bands in the RFLP experiments, but the result of PCR with IS*1245*-specific primers was positive and confirmed by sequencing. Isolated colonies of these cultures were retested, with the same results. Lack of hybridization was not related to problems with DNA, as hybridization with the DT6-specific probe was positive (data not shown).

During the replicate RFLP analyses, minor one- or two-band changes were detected among individual colonies of four strains (strains H78, H80, P104, and P44) (Fig. 4).

DISCUSSION

Previous studies of *M. avium* isolates by molecular typing by IS*1245*-RFLP analysis or pulsed-field gel electrophoresis have emphasized the remarkable diversity of genotypes. In this study, a less stringent cutoff of 80% was chosen to facilitate identification of clusters of isolates that differ at a modest fraction of the insertion sequence loci. In fact, although this
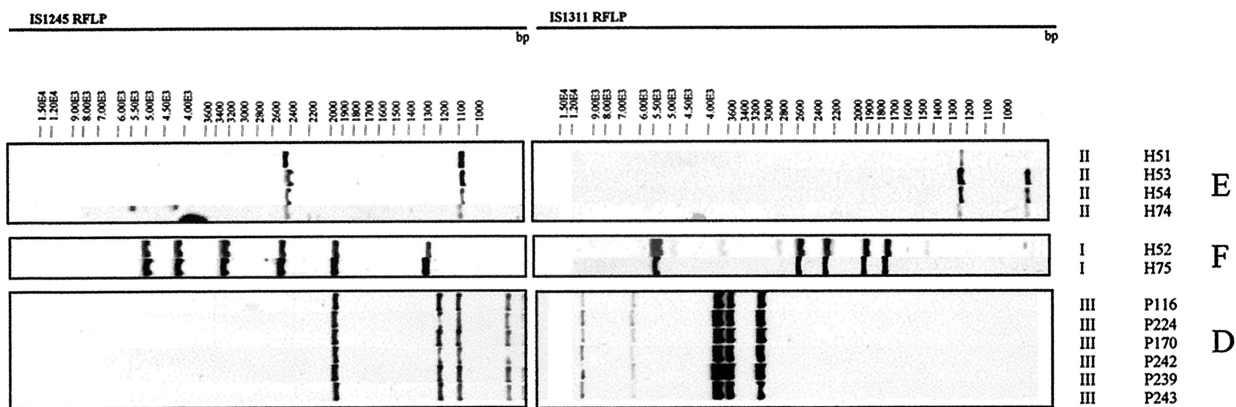

FIG. 3. Isolates with low IS*1245* copy numbers belonging to clusters E, F, and D were probed with IS*1311*. In each cluster, indistinguishable patterns were obtained by RFLP with both probes and by PRA, confirming that the isolates in each cluster represent isolates with closely related genotypes.

study did not systematically investigate the frequency of recent transposition events, we observed one- or two-band changes in the IS*1245*-based RFLP profiles among subcultures of single colonies of four strains (Fig. 4). Pestel-Caron and Arbeit (13) observed the frequent transposition of IS*1245* in vivo among independent isolates collected at a single time, isolates collected from individual patients over time, and isolates collected from multiple patients. Bauer and Andersen (1) confirmed the frequent observation of one- to two-band changes when they analyzed single colonies of *M. avium* from cultures. Thus, among isolates with ≥80% similarity, at least some of the variations in RFLP profiles could represent relatively recent events.

Although few isolates had indistinguishable IS*1245* profiles, the RFLP analysis defined nine clusters that included 87 (80.6%) of the isolates studied. The most prevalent cluster (cluster B) included more than one-third of all isolates and infected pigs, bovines, and humans. Two human isolates and eight pig isolates showed indistinguishable RFLP and PRA patterns. All but three isolates presented *M. avium* PRA pattern I. Three isolates showing *M. avium* PRA pattern II could represent convergent evolution of PRA types, which seems implausible for synonymous alleles, or inappropriate assignment of the isolates to cluster B. Sequencing of additional loci might help resolve this issue.

The pattern for the strains in cluster B included the basic seven-band motif found for serotype 10 strains (14). The RFLP profile of the serotype 10 prototype strain was previously found for two of three isolates from pigs from Brazil by Ritacco et al. (14) and in this work (isolate P251). A similar pattern was also previously seen for a dog isolate (6). Patterns like that found for the serotype 10 strains were also found for human and animal isolates from Europe, where this serotype does not seem to be highly prevalent (2, 9, 14). Isolates with this RFLP profile were found in pigs, bovines, and humans and were predominant in our study. The high frequency could be influenced by the inclusion of strains from an epidemic outbreak.

The second most prevalent RFLP profile (the profile for strains in cluster A) was similar to the multiband profiles described in previous studies (2, 9, 14). The majority of human and pig *M. avium* isolates in those studies belonged in this group, suggesting that strains with genotypes that result in multiband profiles are particularly pathogenic for animals and humans (Fig. 1).

Cluster D represented a set of interesting isolates characterized by a particular PRA pattern (*M. avium* PRA variant III) and a five-band IS*1245*-based RFLP pattern; Ritacco et al. (14) did not observe these patterns among the *M. avium* serovars that they studied. In the present study, all these isolates were obtained from pigs in a restricted geographical region in the south of Brazil, raising the possibility that they represent the local spread of a distinct MAC isolate. However, Smole et al. (19) recently reported on human and environmental isolates that had *M. avium* PRA pattern III and variable number of bands by IS*1245*-based RFLP analysis, suggesting that *M. avium* PRA pattern III isolates have a wider distribution.

In a previous publication (11) we reported that PCR with IS*1245*-specific primers and lysates of cluster D isolates prepared by the freeze-boil method was unsuccessful. Similar negative amplification of IS*1245* was also observed in human iso-

late H62OP, which exhibited a different PRA pattern (*M. avium* PRA variant IV) and a nonclustered two-band IS*1245*-RFLP pattern. The presence of nonspecific PCR inhibitors was ruled out by positive amplifications for other loci (*hsp65*, DT6, IS*1311*). In the present studies, we successfully performed PCR with IS*1245*-specific primers using purified DNA as the template, but we have not determined why our initial attempts were repeatedly negative.

Four isolates that reacted with the AccuProbe reagent specific for *M. avium* and that were positive for amplification by PCR with IS*1245*- and IS*1311*-specific primers were nevertheless repeatedly negative by IS*1245*-based RFLP analysis. The same membranes with all four isolates were positive when they were subsequently probed by RFLP analysis with a PCR-derived DT6-specific fragment (data not shown), confirming that the restriction digests and transfers were technically successful. Different investigators have described the absence of hybridization with the IS*1245*-specific probe in RFLP experiments with *M. avium* isolates, but PCR results have not been reported (2, 14). Komijn et al. (9) stated that the DNA of 25% of human isolates did not hybridize to IS*1245*, suggesting the existence of subgroups within MAC. At present, we do not have a definitive explanation for the negative hybridization with the IS*1245*-specific probe, but it will be investigated further.

The bird-type profile was found for the R13 reference strain, an isolate from a bird in a zoo (a crane [*Grus virgo* Linnaeus 1758]), and a pig isolate. It has been proposed that isolates with this profile represent a distinct subset of *M. avium* (9, 14). Komijn et al. (9) studied 47 *M. avium* isolates from birds and showed that they all belonged to a separate taxon within MAC and presented this three-band RFLP pattern. The bird-type RFLP pattern was observed only as an exception among isolates from other hosts. Our results are in accordance with the hypothesis that isolates with this profile represent a distinct subset of *M. avium*. *M. avium* isolates from pigs were obtained in a region dedicated to pig and fowl production. Despite an outbreak of *M. avium* infection in pigs, no concomitant infections were detected in poultry and only one pig isolate presented the bird-type RFLP profile.

In molecular typing studies of *M. tuberculosis* strains, clustered isolates with the same or highly similar IS*6110*-based RFLP patterns have typically been interpreted to represent recently transmitted isolates that are epidemiologically related. In contrast, nonclustered isolates have been considered consistent with reactivation from previous infections. In *M. avium* infections, person-to-person or animal-to-person transmission, as well as the occurrence of latent infections and reactivations, has never been proven. Therefore, the clustering of *M. avium* RFLP patterns must have different interpretations. Isolates in clusters could represent strains with increased virulence. Otherwise, isolates that are clustered could have better resistance to environmental stress, resulting in greater exposure.

The virulence potential of selected isolates belonging to four major clusters (clusters A, B, C, and D) was analyzed by inoculation of the isolates into hamsters (*Mesocricetus auratus*). Preliminary results suggest that isolates from clusters A and B (*M. avium* PRA pattern I) were more virulent than isolates from clusters C and D (*M. avium* PRA patterns II and III) (data not shown). A recent independent publication reported that isolates with *M. avium* PRA pattern I had an increased

propensity to cause invasive, disseminated infection and that isolates with *M. avium* PRA patterns II and III were more predominant among environmental isolates (19). These results suggest that the different *M. avium* genotypes identified by these methods may differ in terms of their virulence determinants that influence the transmission and occurrence of disease.

In conclusion, molecular typing techniques such as IS*1245*-RFLP analysis and PRA effectively identified *M. avium* isolates with distinct traits and defined genetic lineages of interest for future pathogenicity studies.

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