

## Species Identification of *Mycobacterium avium* Complex Isolates by a Variety of Molecular Techniques

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**Organisms in the *Mycobacterium avium* complex (MAC; *M. avium*, *M. intracellulare*, and “nonspecific or X” MAC) are emerging pathogens among individual organisms of which significant genetic variability is displayed. The objective of the present study was to evaluate various molecular methods for the rapid and definitive identification of MAC species. Isolates were obtained from both human immunodeficiency virus (HIV)-positive patients and HIV-negative patients with and without known predisposing conditions. The isolates were initially hybridized with nucleic acid probes complementary to the rRNA of the respective mycobacterial species (AccuProbe Culture Confirmation kits for *M. avium*, *M. intracellulare*, and MAC species; Gen-Probe). Isolates were also examined by PCR and in some cases by Southern blot hybridization for the insertion element IS1245. Two other techniques included a PCR assay that amplifies the *mig* gene, a putative virulence factor for MAC, and *hsp65* gene amplification and sequencing. This study led to the following observations. Eighty-five percent of the isolates from HIV-positive patients were *M. avium* and 86% of the isolates from HIV-negative patients were *M. intracellulare*. Fifteen of the *M. avium* isolates did not contain IS1245 and 7% of the *M. intracellulare* isolates were found to carry IS1245. All of the *M. avium* strains were *mig* positive, and all of the *M. intracellulare* strains were *mig* negative.**

Organisms in the *Mycobacterium avium* complex (MAC) are opportunistic pathogens that can be isolated from soil and water and cause infection and clinical disease in a wide variety of animals and humans (9). Many *M. avium* infections are seen in patients who are immunocompromised, such as those with AIDS (5). These infections are usually disseminated and have been shown to contribute significantly to the morbidity of AIDS patients (7). Prior to the advent of AIDS, most patients in the United States with MAC pulmonary disease were elderly males who had underlying lung diseases, such as bronchitis, emphysema, prior tuberculosis, and other fibrotic disorders. More recently, MAC pulmonary disease has been diagnosed in an increasing number of women who have no history of underlying lung disorders and who appear to be immunocompetent (10, 17, 18, 24, 25).

The objective of this study was to evaluate various molecular methods for the rapid and definitive identification of MAC species (*M. avium*, *M. intracellulare*, and “nonspecific or X” MAC). MAC isolates from the three human populations described above were included in the study. The isolates were initially hybridized with nucleic acid probes complementary to the rRNA of the respective mycobacterial species (AccuProbe Culture Confirmation kits for *M. avium*, *M. intracellulare*, and MAC species; Gen-Probe Inc., San Diego, Calif.). These kits are routinely used in clinical mycobacteriology laboratories for the rapid identification of mycobacterial species. Isolates were also examined by PCR and in some cases by Southern blot hybridization for the insertion element IS1245 because it has been suggested to be specific for *M. avium* (6). Two other techniques included a PCR assay that amplifies the *mig* gene, a putative virulence factor for MAC, and *hsp65* gene amplifi-

cation and sequencing (16, 23). The combination of these techniques has yielded new information concerning clinical MAC isolates.

### MATERIALS AND METHODS

**MAC isolates.** A patient profile sheet was obtained for each human isolate. The strains used in this study were obtained from the following sources: Walter Pace, University of Arkansas for Medical Sciences clinical laboratory and the John L. McClellan Memorial Veteran's Hospital clinical laboratory; Richard Wallace and Barbara Brown, Department of Microbiology, University of Texas Health Center at Tyler; Leonid Heifets and Gwen Huit, National Jewish Center for Immunology and Respiratory Medicine; Richard J. Blinkhorn, MetroHealth Medical Center Clinical Laboratory, Cleveland, Ohio; Hiroe Shiratsuchi, Case Western Reserve University, Cleveland, Ohio; and William Stead, Arkansas State Health Laboratory. The isolates from the United Kingdom were received from Jack Crawford, Centers for Disease Control and Prevention, Atlanta, Ga., and were isolated by Tobin Hellyer, Becton-Dickinson Corp., Baltimore, Md.

In some cases the isolates had been streaked for isolation and single colonies had been selected before being sent to us. All other isolates, upon receipt, were streaked onto 7H11 plates and were examined for colonial morphology in our laboratory. Single colonies were selected and cultivated in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI), two aliquots were frozen, and the remainder of the culture was used for DNA isolation and pulsed-field gel electrophoresis (PFGE).

**Testing with Gen-Probe kits.** Culture confirmation kits (AccuProbe; Gen-Probe) were used to determine the species of each isolate. The *M. avium* probe kit detects *M. avium* isolates, the *M. intracellulare* probe kit detects *M. intracellulare* isolates, and the MAC probe kit detects *M. avium*, *M. intracellulare*, and MAC isolates which are designated X or nonspecific MAC and which are not detected by either species-specific probe (3). Each test was performed by following the manufacturer's instructions and with the inclusion of controls.

Each isolate was tested with the MAC AccuProbe kit to confirm that the isolates belonged to MAC. Isolates were then tested with the *M. avium*-specific AccuProbe, and all positive isolates were given a number preceded by an “A” to identify them as *M. avium* isolates. Isolates that were positive with the MAC-specific AccuProbe but negative with the *M. avium*-specific probe were tested with the *M. intracellulare*-specific AccuProbe. Isolates that were positive with this probe were given a number preceded by “I” to identify them as *M. intracellulare* isolates. Isolates that were positive with the MAC-specific probe but that were negative with the *M. avium*-specific or the *M. intracellulare*-specific probe were given a number preceded by “X” to identify them as nonspecific MAC isolates.

**Preparation of genomic DNAs from mycobacteria.** The following large-scale DNA preparation method was developed in our laboratory. For DNA preparation, mycobacterial cells (10 to 100 ml) were incubated to a Klett reading of 100 to 150, which represents logarithmic-phase growth, and were treated with D-

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cycloserine (1 mg/ml) for 24 h. After sedimentation in the centrifuge, the cell pellets were heat killed, resuspended in 1 ml of lysing buffer (4 mM Tris HCl [pH 8.0], 4% sodium dodecyl sulfate, 10 mM NaCl, 0.001 mM EDTA, 0.1 mg of proteinase K per ml), and incubated at 37°C overnight or at 68°C for 2 h. The lysate was cooled to room temperature, and the debris was pelleted by centrifugation. The supernatant was extracted two times with phenol-chloroform in phase-lock gel tubes (Five Prime to Three Prime Inc., Boulder, Colo.). After a final extraction in chloroform the aqueous phase was precipitated with 0.3 M sodium acetate (pH 5.5) and 2 volumes of ethanol for 15 min at -70°C. Preparations were centrifuged for 20 min at 4°C, washed one time with 70% ethanol, dried, and reconstituted in TE (Tris-EDTA) buffer. Yields were approximately 200 to 1,000 ng/ $\mu$ l.

**PCR assays.** DNA amplification was performed on a GeneAmp PCR system 9600 (Perkin-Elmer, Norwalk, Conn.) in a 50- $\mu$ l reaction mixture containing 10 pmol of each primer, 1 ng of genomic DNA, 200  $\mu$ M deoxyribonucleoside triphosphates, 1 $\times$  PCR buffer (1.5 mM MgCl<sub>2</sub> [pH 8.3]; Perkin-Elmer), and 1 U of *Taq* DNA polymerase (Perkin-Elmer). Occasionally, the organisms were tested directly in the PCR without prior DNA extraction. In this case, a sterile toothpick was used to obtain cells from a colony, and the cells were added directly to 49  $\mu$ l of the complete PCR mixture. Primers and conditions for *IS1245* PCR were as reported and amplify *IS1245* and the closely related element *IS1311* (6, 20). The primers specific for the *mig* gene were designed to amplify a 737-bp fragment within the coding region of the gene (16). The *mig*-specific primer sequences were as follows: *mig* upper, 5'-CCC GTT CAA CGT CAA CTT CC-3'; *mig* lower, 5'-GGG CTC GCC GGT CAT CAG GT-3'. The cycling conditions were as follows: initial denaturation at 95°C for 5 min and 30 cycles of 30 s of denaturation at 95°C and 2.0 min of annealing and extension at 68°C, followed by a single 5-min extension at 72°C. The PCR products were electrophoresed on a 2% agarose gel, with a 1-kb DNA ladder used for size determination (Gibco Life Technologies, Grand Island, N.Y.), stained with ethidium bromide, and photographed by using the Eagle Eye II gel documentation system (Stratagene, La Jolla, Calif.).

**Fingerprinting with *IS1245*.** For fingerprinting with *IS1245* (6), isolated genomic DNA (500 ng) was digested with the restriction enzyme *Pvu*II, electrophoresed on a 0.7% gel, alkaline transferred to Hybond (Amersham Pharmacia Biotech, Piscataway, N.J.), probed with a 427-bp fragment of *IS1245* produced by PCR, and labeled with [<sup>32</sup>P]dCTP with the Random Primed DNA Labeling Kit (Boehringer Mannheim, Mannheim, Germany). The gel images were saved in tif format and were analyzed by using the PFGE program described below.

**PFGE and analysis.** Whole cellular DNA for the experiments was prepared essentially as described by Zhang et al. (26). Bacterial DNA slices were restricted with the low-frequency-cleavage restriction endonuclease *Xba*I and in some cases with *Spe*I (New England Biolabs, Beverly, Mass.). In order to detect large plasmids, whole cellular DNA was electrophoresed without prior digestion with a restriction endonuclease. PFGE of the digested or undigested DNA samples was performed in a contour-clamped homogeneous electric field on the CHEF-DR II system (Bio-Rad Laboratories, Richmond, Calif.) in 0.8% low-endomosis agarose gels (Pulsed Field Certified Agarose; Bio-Rad Laboratories) at a ramped pulse time from 5 to 30 s for 24 h. DNA size standards consisting of concatemers of bacteriophage lambda DNA (Bio-Rad Laboratories) were included in each gel.

After electrophoresis, the gels were stained with ethidium bromide, destained in distilled water, and visualized on a UV transilluminator (Stratagene), and a tif image of each gel was stored on a diskette. The profiles were scanned and analyzed with the Molecular Analyst Fingerprinting software (Bio-Rad Laboratories). This package enables conversion of the gel image, normalization to a universal standard, analysis of the macrorestriction patterns, and cluster analysis of the PFGE or *IS1245* patterns. By using the hierarchic unweighted pair group average method as a clustering algorithm and the Jaccard coefficient of similarity, dendrograms for the isolates from each subtype were created.

***hsp65* sequencing.** The *hsp65* gene encodes a 65-kDa heat shock protein and has been used to identify species in the MAC complex (11, 21-23). Amplification and sequencing of the 441-bp segment of the *hsp65* gene were performed for selected strains, as described elsewhere (23).

## RESULTS

**Strain identification with Gen-Probe kits.** An integral aspect of this study was identification of MAC isolates from human immunodeficiency virus (HIV)-positive and HIV-negative patients to the species level. The most common method of species identification of MAC organisms is by use of the 16S ribosomal gene sequence, which is the basis of the Gen-Probe assay. We identified 92 *M. avium* isolates 75 of which were from HIV-positive patients and 17 of which were from HIV-negative patients; 57 *M. intracellulare* isolates, 7 of which were from HIV-positive individuals and 50 of which were from HIV-negative patients; and 12 nonspecific or X isolates, 5 of which

TABLE 1. Summary of *IS1245* and *mig* gene data for all isolates

GenProbe result	No. of isolates			
	<i>IS1245</i>		<i>mig</i> gene	
	Positive	Negative	Positive	Negative
<i>M. avium</i>	78	13	92	0
<i>M. intracellulare</i>	4	53	0	57
MAC X isolate	7	5	9	3

were from HIV-positive patients and 7 of which were from HIV-negative patients.

***IS1245* fingerprinting.** During this study all isolates were checked for the presence of the insertion element *IS1245* by PCR (6) (Table 1). Fourteen *M. avium* isolates (15%) did not contain *IS1245* by PCR. These isolates were checked for the presence of *IS1245* by Southern blot hybridization, with the same negative results (data not shown).

Three of the *IS1245*-negative *M. avium* isolates were from HIV-positive patients in the United Kingdom. The remaining 11 isolates were from Cleveland, and all but 1 of these isolates were from HIV-positive patients. To verify that the Cleveland isolates were not identical, they were digested with *Xba*I, electrophoresed on a single pulsed-field gel, and analyzed (Fig. 1). The results showed that two of the isolates (isolates A67 and A73) shared identical patterns, while the remainder of the isolates had unique patterns. When A67 and A73 were run after *Spe*I digestion, there was a single band difference which has been determined to be a large plasmid in A73 (data not shown). Therefore, these isolates would be considered identical or essentially the same.

It was verified by PCR and probing of a Southern blot that four isolates of *M. intracellulare* (7%) contained *IS1245* or its related element *IS1311*. The *IS1245* patterns of all four isolates were different (Fig. 2). Isolate I4 was isolated from an HIV-negative patient in Cleveland, isolates I33 and I36 were iso-

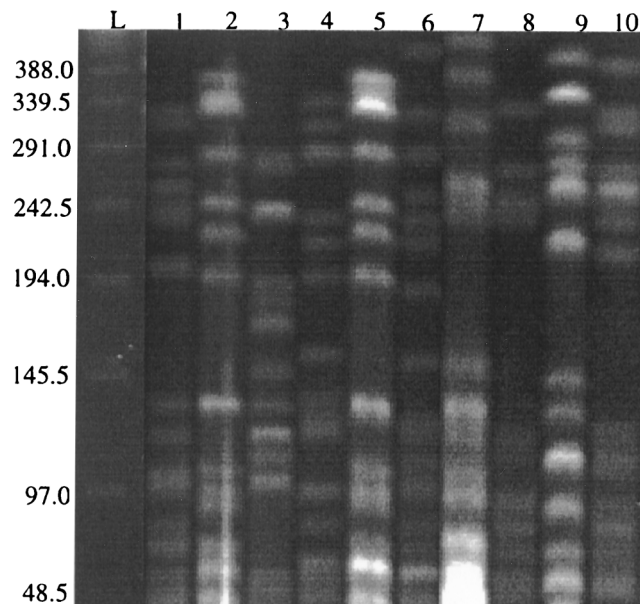


FIG. 1. PFGE patterns of *IS1245*-negative *M. avium* isolates from Cleveland. Lane L, 48.5-kbp bacteriophage lambda concatamer ladder (numbers on the right are in kilobase pairs); lane 1, A54; lane 2, A67; lane 3, A71; lane 4, A72; lane 5, A73; lane 6, A76; lane 7, A84; lane 8, A94; lane 9, A95; lane 10, A97.

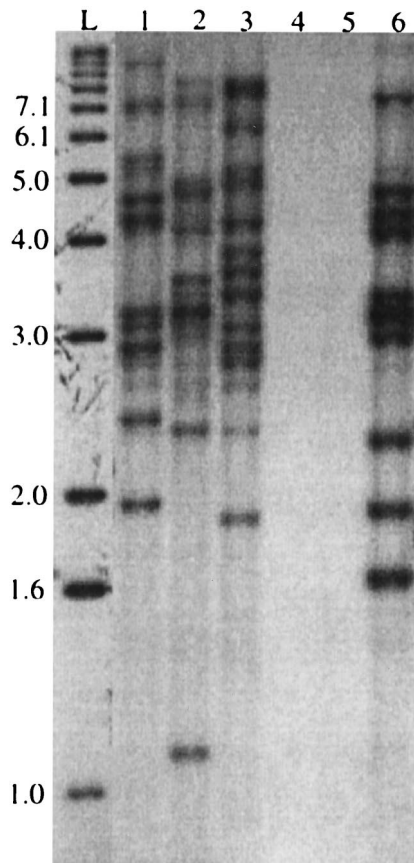


FIG. 2. *IS1245* patterns of five *M. intracellulare* isolates. Lane L, ladder (numbers on the left are in kilobase pairs); lane 1, I40; lane 2, I36; lane 3, I33; lane 4, I14; lane 5, I7; lane 6, I4. Lanes 4 and 5 contain *M. intracellulare* strains that were negative for *IS1245* by PCR.

lated from HIV-positive patients in Arkansas, and isolate I40 was isolated from an HIV-negative patient in Arkansas. The PFGE patterns of these isolates were also unique (data not shown).

***hsp65* sequencing.** *hsp65* sequencing was used as a secondary method to verify that the 13 *M. avium* isolates that did not contain *IS1245* were *M. avium* (Table 2). All of the isolates had an *M. avium* type; 10 of the isolates had the most common *M. avium* types (types 65.1 and 65.2), and the remaining 3 isolates were of type 65.4 (23). The isolates with identical PFGE patterns, isolates A67 and A73, were of *hsp65* type 65.34. The four *M. intracellulare* isolates that contained *IS1245* were all typed as *M. intracellulare* by *hsp65* sequencing. Eight of the X isolates had an *hsp65* allele, indicating that they were *M. avium*, three were typed as *M. intracellulare*, and one had the 65.29 allele, considered "other" in the MAC group (Table 3).

***mig* PCR.** The only virulence factor identified and well characterized in MAC isolates is the *mig* gene (14, 16). The *mig* PCR results indicated that all 92 of the *M. avium* isolates contained this gene and that none of the 57 *M. intracellulare* isolates contained *mig* (Table 1). Therefore, *mig* appears to be a marker for differentiation of these two species. The isolates designated X varied in their possession of the *mig* gene. The eight X isolates identified as *M. avium* and the one isolate identified as "other" by *hsp65* sequencing were *mig* positive, and of these nine isolates, seven contained *IS1245* (Table 3). The three X isolates which were negative for *mig* were also

TABLE 2. *hsp65* alleles for *IS1245*-negative *M. avium* isolates and *IS1245*-positive *M. intracellulare* isolates

Gen-Probe result	<i>hsp65</i> allele	
	<i>M. avium</i>	<i>M. intracellulare</i>
A5	65.1	
A6	65.1	
A71	65.1	
A78	65.1	
A8	65.2	
A54	65.2	
A72	65.2	
A76	65.2	
A79	65.2	
A95	65.2	
A67	65.34	
A73	65.34	
A97	65.34	
I4		65.3
I40		65.9
I33		65.9
I36		65.9

negative for *IS1245* and were identified as *M. intracellulare* by their *hsp65* sequence.

## DISCUSSION

The objective of this study was to use various molecular techniques to define the species of MAC isolated from three patient populations. The most commonly used method for species identification of MAC isolates is based on the ribosomal 16S sequence and is the principle of the Gen-Probe AccuProbes. Generally, the MAC-specific AccuProbe test is used to identify an organism as a member of this group. Most clinical laboratories do not use the species-specific kits to identify the particular species because the kits are expensive and the treatment is the same for each type of MAC infection. Therefore, the exact species causing infection is not known.

Each isolate was identified to the species level with the Gen-Probe AccuProbes. Eighty-five percent of the isolates from HIV-positive patients were identified as *M. avium*. The isolates from HIV-negative patients were predominately *M. intracellulare* (86%) and were from individuals with the clinical manifestations of cavitory disease and nodulation or bronchiectasis and a few patients with invasive disease. Thirteen of the

TABLE 3. *IS1245*, *mig*, and *hsp65* allele data for MAC X strains

Gen-Probe result	PCR result <sup>a</sup>		<i>hsp65</i> allele		
	<i>IS1245</i>	<i>mig</i>	<i>M. avium</i>	<i>M. intracellulare</i>	Other (MAC X)
X1	Neg	Pos	65.1		
X5	Neg	Pos			65.29
X6	Pos	Pos	65.1		
X7	Pos	Pos	65.1		
X8	Pos	Pos	65.2		
X10	Pos	Pos	65.10		
X11	Pos	Pos	65.34		
X12	Pos	Pos	65.1		
X16	Pos	Pos	65.1		
X9	Neg	Neg		65.7	
X14	Neg	Neg		Isolate 1373	
X17	Neg	Neg		65.7	

<sup>a</sup> Pos, positive; Neg, negative.

HIV-negative patients had no known predisposing factor. The leading predisposing factor for the majority of the other HIV-negative patients was bronchiectasis, with smoking being the next most common factor. It is not clear if bronchiectasis precedes MAC infection or is the result of this infection (12). The few studies performed to look for immune dysfunction in these patients have not identified immune dysfunction in any of the patients at this time (8, 12).

Twelve of the isolates were positive only with the MAC-specific AccuProbe, which defined these strains as being neither *M. avium* nor *M. intracellulare* but MAC strain X or nonspecific. When the *hsp65* genes of these strains were sequenced, all but one could be identified as either *M. avium* or *M. intracellulare* (23). Therefore, many isolates that would not be recognized as *M. avium* or *M. intracellulare* with the species-specific Gen-Probe kits can be given a species identification by *hsp65* sequencing (Table 3). This is due to the increased variability of the *hsp65* gene compared to that of the 16S gene and therefore its greater power of discrimination. *hsp65* sequencing was especially useful as a secondary species identification tool for verification that the *M. avium* isolates without IS1245 were in fact *M. avium* and that the *M. intracellulare* isolates with IS1245 were in fact *M. intracellulare*. There have been two other reports of the identification of *M. avium* strains without IS1245, but other than tests with Gen-Probe no other method of identification was performed with the strains (1, 19). We do not know the significance of the fact that all of the *M. avium* isolates that lacked IS1245 were isolated from patients in Great Britain or the Cleveland area. We are continuing our collection of MAC from various geographical sites in order to determine the extent to which *M. avium* isolates lack the element and *M. intracellulare* isolates carry the IS1245 element. It was interesting to find that many isolates of both *M. avium* and *M. intracellulare* contained large plasmids (100 to 300 kb) (data not shown), which, if found to be conjugative, could explain the passage of IS1245 between species (4, 15). Therefore, it appears that the presence of the IS1245 insertion element does not necessarily identify an isolate as *M. avium*, as has been suggested in other reports (2, 6, 19).

Tests for the detection of the *mig* gene by PCR were performed because it is the only well-characterized virulence factor identified in MAC (14, 16). The role of this gene has not been elucidated, but the gene has been demonstrated to enhance the growth of organisms residing inside the macrophage (14). It was interesting that all our isolates identified as *M. avium* contain this gene but that the *M. intracellulare* isolates did not. This finding also held true for the environmental and animal isolates that were examined, but those findings are not included in this report. These results suggest that the detection of the *mig* gene may be a simple and useful way of differentiating *M. avium* and *M. intracellulare*. The presence of this gene in *M. avium* strains but not *M. intracellulare* strains suggests that these two species may differ in their virulence mechanisms, which has also been suggested by Maslow et al. (13). The *mig* gene may be part of a pathogenicity island which is not easily transferred between these two species. Further studies in this regard may reveal the virulence mechanisms used by each species when causing human infections.

In summary, *hsp65* sequencing is a useful method for identification of MAC isolates whose species cannot be identified by the AccuProbe Gen-Probe assays. IS1245 is not universally found in all *M. avium* strains and can be present in *M. intracellulare* strains. The presence of the *mig* gene appears to be specific for *M. avium* strains and may indicate differences in virulence mechanisms between *M. avium* and *M. intracellulare* organisms.

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