

Characterization to Species Level of *Mycobacterium avium* Complex Strains from Human Immunodeficiency Virus-Positive and -Negative Patients

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Forty human clinical *Mycobacterium avium*-*M. intracellulare* complex strains isolated in Greece were characterized to the species level by PCR with three sets of primers specific for one or both species. *M. avium* predominated in both human immunodeficiency virus-positive and -negative patients, but the frequency of *M. intracellulare* isolation appeared to be higher in the latter.

Mycobacterial species are infectious agents of serious human diseases, such as tuberculosis and leprosy (7). Environmental mycobacteria, on the other hand, such as those of the *Mycobacterium avium*-*M. intracellulare* complex (MAC), tend to cause opportunistic infections only (16). Scrofula, a disease which was already known to Hippocrates and was later defined as tuberculous cervical lymphadenitis, was initially attributed to the pathogenic action of *Mycobacterium scrofulaceum* and the *M. tuberculosis* group. Since the late 1950s, when the first cases of cervical lymphadenitis caused by MAC were diagnosed, the frequency of MAC isolation has increased, with MAC replacing *M. scrofulaceum* as the most prevalent species in the etiology of disease (19). The clinical significance of MAC infection has become more important in patients immunocompromised by AIDS (11). MAC is, in fact, the organism most commonly isolated from bacteremic patients with AIDS and is believed to greatly impair their quality of life and to reduce their life expectancy (12). In the developed world, the incidence of MAC in AIDS patients can be as high as 40% and the detection rate at the time of autopsy can be as high as 53% (11).

MAC consists of two species that are genetically distinct but difficult to discriminate: *M. avium*, which predominates among MAC isolates from both AIDS patients and non-AIDS patients (87 to 98% and 58 to 60%, respectively), and *M. intracellulare*, which is more frequently isolated among non-AIDS patients (5). Differentiation of clinical isolates into either *M. avium* or *M. intracellulare* is of clinical and epidemiological interest. Although *M. avium* and *M. intracellulare* strains generally cause indistinguishable infections, *M. avium* serotypes are more frequently isolated from the environment (6) and predominate over *M. intracellulare* in all clinical conditions in which MAC is encountered, such as AIDS and cervical lymphadenopathies in children (19, 21). On the other hand, *M. intracellulare* among AIDS patients seems to be isolated preferentially from sputum (21). However, the conventional serotyping method cannot always easily distinguish between these two closely related species and requires large amounts of

these slow-growing organisms (12). Therefore, molecular diagnostic methods, which can be performed rapidly with small numbers of cells, are particularly helpful in this case (18). For example, sequencing of the 16S rRNA gene has been used to clarify the taxonomy within MAC (8); however, this methodology is limited in practice to reference laboratories. Recently, Thierry et al. successfully used species-specific primers for the selective amplification of a 187-bp fragment within the DT6 region of the *M. avium* genome or of a 666-bp fragment within the DT1 regions of both *M. avium* and *M. intracellulare* genomes (17). Use of these primers for characterization of clinical MAC strains to the species level has been demonstrated to provide an inexpensive and at least equally sensitive alternative to identification by commercially available DNA probes (3).

The aim of the present study was to determine the frequency of isolation of *M. avium* and *M. intracellulare* from AIDS patients and from human immunodeficiency virus (HIV)-negative patients in Greece, a part of the world not previously surveyed in this manner. During the period 1991 to 1996, 430 specimens from HIV-positive patients were referred to the Sotiria Hospital for Chest Disease. A total of 45 (10%) were positive for MAC, compared with 32 (7%) that were positive for *Mycobacterium tuberculosis*. These MAC strains were isolated from the following clinical specimens: blood (35 specimens), sputum (8 specimens), gastric lavage (1 specimen), and urine (1 specimen). During 1991 to 1995, 194 lymph node biopsies were taken from HIV-negative children with cervical lymphadenopathy. A total of 22 (11%) were positive for MAC, compared to 17 (9%) that were positive for *M. tuberculosis* and 2 (1%) that were positive for other, fast-growing mycobacteria. Finally, during 1991 to 1993, 38 MAC strains were isolated from 1,238 specimens (mostly sputum, pericardial tissue, urine, feces, bronchoalveolar lavage, and blood) originating from HIV-negative adults.

In the present study, 40 human clinical isolates that were identified as MAC by a combination of biochemical tests and a DNA-rRNA hybridization test (SNAP; Syngene) were tested. Of these, 20 and 20 had been isolated from AIDS patients and HIV-seronegative patients, respectively. The latter consisted of 12 children suffering from cervical lymphadenopathy and 8 adults with pulmonary or intra-abdominal disease. Multiple sweeps from each isolate grown in Loewenstein-Jensen agar were inoculated in 10 ml of Middlebrook 7H9 broth (BBL),

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TABLE 1. Origin of MAC strains and species differentiation

Patient status	Origin of specimen	No. of strains		
		Total	<i>M. avium</i>	<i>M. intracellulare</i>
AIDS	Blood culture	20	19	1
HIV negative				
Children with cervical lymphadenopathy	Lymph node biopsy	12	11	1
Adults with pulmonary disease	Sputum	7	7	
Adults with intra-abdominal disease	Gastric lavage	1	1	

which was supplemented with albumin fraction V, dextrose, and catalase (BBL), and the mixture was incubated for 5 to 10 days at 37°C with agitation. Resulting growth was tested by the Ziehl Neelsen stain for the presence of acid-fast bacilli and by the Gram stain for the presence of contaminants. For DNA extraction, 2 ml from each broth was centrifuged. The cell pellet was resuspended in 1 ml of Middlebrook 7H9 containing 100 µg of D-cycloserine (Sigma) per ml in order to weaken the mycobacterial cell wall (17) and 200 µg of lysozyme (Sigma) per ml, and the suspension was incubated for 48 h at 37°C. After centrifugation, the cell pellet was resuspended in 500 µl of a solution containing 50 mM Tris (pH 8.0), 50 mM EDTA, 10 mM NaCl, 0.5% sodium dodecyl sulfate, and 60 µg of proteinase K (Sigma) per ml and the suspension was incubated at 55°C for a further 18 h. Following standard phenol-chloroform extraction and ethanol precipitation, DNA was dissolved in 50 µl of sterile distilled water and stored at -20°C until needed. For PCR, the following primers were used: AV6 (5'-GATGGCCGGGAGACGATCTATGCCGGCGTAC-3') and AV7 (5'-TCGAGGAAGGCCTGTTCCGACCGTGTTCGC-3') for the amplification of a 187-bp fragment within the DT6 region of the *M. avium* genome, IN38 (5'-GAACGCCCGTTGGCTGGCCATTCACGAAGGAG-3') and IN41 (5'-TCGAGGAAGGCCTGTTCCGACCGTGTTCGC-3') for the amplification of a 666-bp fragment within the DT1 region present in both the *M. avium* and the *M. intracellulare* genomes (17), and P1 (5'-GCCGCCGAAACGATCTAC-3') and P2 (5'-AGGTGGCGTCGAGGAAGAC-3') for the amplification of a 487-bp fragment within the IS1245 insertion sequence, which was shown to be present in several copies in *M. avium* but not in *M. intracellulare* clinical isolates (9). Amplification reactions were performed in 50-µl reaction mixtures containing 50 mM Tris-HCl (pH 8.5), 2 mM MgCl₂, 100 pmol of each primer, 200 µM (each) the four deoxyribonucleoside triphosphates (dATP, dGTP, dTTP, and dCTP), 5 µl of template DNA, and 2 U of *Taq* DNA polymerase (Tib Molbiol, Frankfurt, Germany). The amplification mixture was overlaid with 2 drops of paraffin oil. Amplification consisted of 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and DNA synthesis at 72°C for 1 min with a Power Block System thermocycler (Ericomp, Inc.). Each amplification experiment included control reaction mixtures without DNA and with DNA extracted from *M. avium* Pasteur Institute reference strain 140310002 and from *M. intracellulare* Pasteur Institute reference strain 14031001. Amplification products were analyzed by electrophoresis on a 2% agarose gel in 0.5× Tris-borate-EDTA. Plasmid pBR322 DNA digested with *Hinf*I (New England Biolabs) was used to provide DNA size markers. The results were photographed under UV illumination after ethidium bromide staining.

As summarized in Table 1, 19 of the 20 blood isolates from the AIDS group were *M. avium* (Fig. 1A, lanes 5 to 7) and only

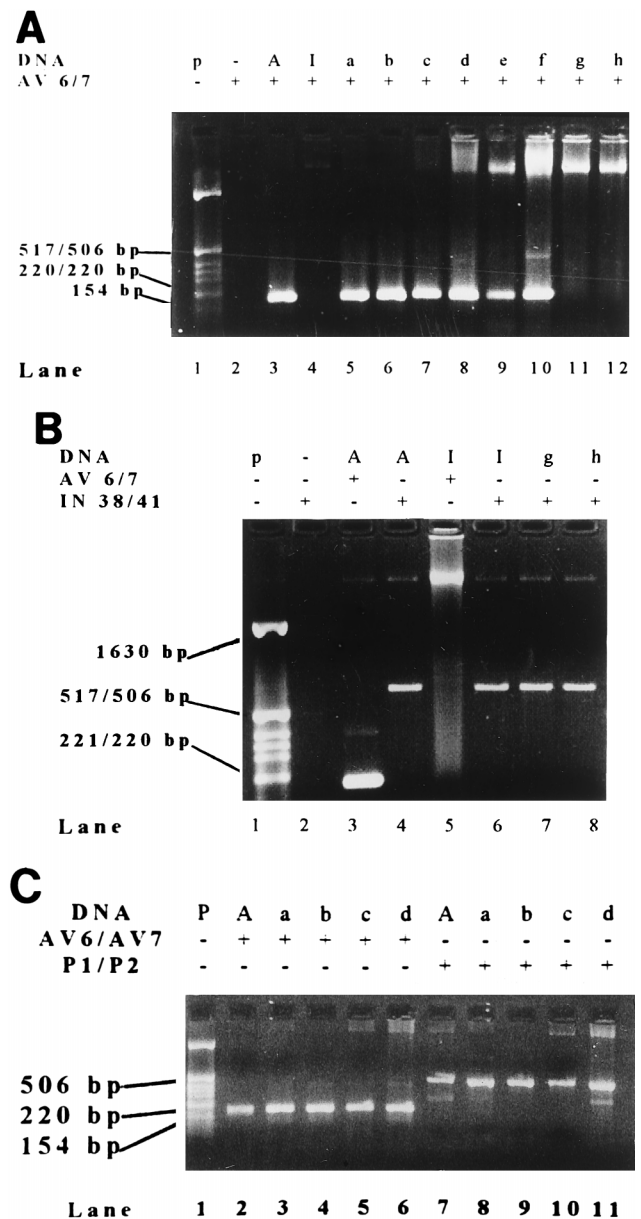


FIG. 1. (A) Identification of representative MAC strains as *M. avium* with AV6-AV7. Lanes: P, pBR322/*Hinf*I DNA size markers; A, *M. avium* reference strain; I, *M. intracellulare* reference strain; + or -, presence or absence, respectively, of the indicated primers; a to c, MAC strains from blood cultures of AIDS patients; d to f, MAC strains from HIV-negative patients (d, lymphadenitis biopsy; e, sputum; and f, gastric lavage); g, blood culture of AIDS patient; h, lymphadenitis biopsy. The AV6-AV7-specific amplification fragment is 187 bp long. (B) Identification of MAC strains as *M. intracellulare* with IN38-IN41. Lanes: P, pBR322/*Hinf*I DNA size marker; A, *M. avium* reference strain; I, *M. intracellulare* reference strain; + or -, presence or absence, respectively, of the indicated primers; g and h, MAC strains as described for panel A. The AV6-AV7- and IN38-IN41-specific amplicons are 187 and 666 bp long, respectively. (C) Presence of IS1245 in selected *M. avium* strains. Lanes: P, pBR322/*Hinf*I DNA size markers; A, *M. avium* reference strain; + or -, presence or absence, respectively, of indicated primers; a to d, *M. avium* strains (see panel A). The AV6-AV7- and P1-P2-specific amplicons are 187 and 487 bp long, respectively.

one was *M. intracellulare* (Fig. 1B, lane 7). Similarly, as summarized in Table 1, *M. avium* was present in 11 lymph node biopsies from children with cervical lymphadenopathy (Fig. 1A, lanes 8 to 10), whereas *M. intracellulare* was present only in 1 (Fig. 1B, lane 8). No amplification products with either set of

primers were obtained when target DNA from *M. tuberculosis* type strain ATCC 25618 or a clinical *Pseudomonas aeruginosa* strain was used (not shown). Finally, *M. avium* was present in all eight patients with pulmonary and intra-abdominal disease, from whom no *M. intracellulare* was isolated (Table 1). Therefore, while *M. avium* was prevalent in both groups, the frequency of *M. intracellulare* isolation was almost two times higher in HIV-seronegative children than that in patients with AIDS. Although this difference is not statistically significant by Fisher's test ($P = 0.62$), it seems to suggest that the incidence of *M. intracellulare* among immunocompetent patients may be higher than that among AIDS patients; analysis of a larger sample should test the validity of this suggestion. However, our results do indicate that Greece follows the trends of relative incidence of *M. avium* and *M. intracellulare* in HIV-positive patients seen in Europe and the United States (6). Finally, we verified the presence of IS1245 in all of our *M. avium* strains (Fig. 1C). This powerful marker has been used to confirm the genetic identity of different morphotypes of a single *M. avium* strain (21) and has the same discriminatory ability whether it is used in a conventional Southern blot-based restriction fragment length polymorphism assay or in a PCR assay (15). Apart from human clinical isolates, it has also been shown to be present in porcine *M. avium* strains (1). Our results suggest that it will be useful in typing *M. avium* strains from HIV-negative as well as -positive patients.

The use of DNA amplification techniques for the diagnosis of slow-growing, fastidious infectious pathogens such as the *Mycobacterium* species belonging to MAC is essential for the timely onset of chemotherapy, which can lead to improvement of symptoms, clearing of bacilli, and, in some instances, an increased rate of survival in patients with AIDS suffering from MAC disease (10, 14). For these reasons, DNA amplification techniques are currently being optimized with respect to their sensitivity and specificity in a number of laboratories (2, 4, 13). We have used a PCR with primers specific for the *M. avium* genome or for both the *M. avium* and the *M. intracellulare* genomes (17) to differentiate MAC strains from a variety of infections and clinical specimens into either species. Our study suggests that while *M. avium* is prevalent in all cases, *M. intracellulare* may be isolated from children with cervical lymphadenopathy more frequently than from AIDS patients.

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