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Isolation of the Newly Described Species *Mycobacterium celatum* from AIDS Patients

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***Mycobacterium celatum* is a recently described species which, on the basis of conventional tests, may be misidentified as *Mycobacterium xenopi* or as belonging to the *Mycobacterium avium* complex. Only genomic sequencing or high-performance liquid chromatography of cell wall mycolic acids can presently allow a correct identification of this mycobacterium. Two cases of infection due to *M. celatum*, in AIDS patients, are described here. The quantitative susceptibility pattern of the isolates to a wide spectrum of drugs is also reported.**

Disseminated infections due to nontuberculous mycobacteria are frequent complications in AIDS patients and are responsible for the evident worsening in the quality of their lives (18). Usually, mycobacteria other than *Mycobacterium tuberculosis* behave quite opportunistically and affect patients whose immune systems are severely weakened, mainly after the $CD4^+$ lymphocyte levels of these patients have fallen below 50/μl (12). Although *Mycobacterium avium* is the most frequent cause of such disease (14), many other mycobacterial species have been reported to be involved in disseminated infections in the course of AIDS.

Mycobacterium celatum has been recognized recently as a new species in a study based on 24 clinical isolates (5), 8 of which were from human immunodeficiency virus (HIV)-infected patients; it is thus possible that its role as a HIV-correlated opportunistic agent is more important than commonly accepted. We recovered two *M. celatum* strains from seriously immunocompromised AIDS patients. In this paper, we describe the clinical and microbiological features of our cases, focusing on identification procedures and drug susceptibility data.

MATERIALS AND METHODS

Case reports. Case 1. *M. celatum* was isolated seven times, between August 1993 and July 1994, from the blood of a 28-year-old woman, the partner of an AIDS patient, with a $CD4^+$ cell count of 27/μl. A partial clinical record has been reported recently (17). A first bacteriemic phase (four cultures positive) was followed by a 9-month period in which all blood cultures were negative, but then a relapse of mycobacteremia (three positive cultures) occurred. The patient died in August 1994, and an autopsy was not done.

Case 2. A 39-year-old, HIV-positive male without any major AIDS-related predisposing condition in his history was admitted in November 1991 with fever, anemia, weight loss, and diarrhea; his $CD4^+$ cell count was 33/μl. He had been treated since 1990 for AIDS-related opportunistic infections (*Salmonella enteritidis* bacteremia and neurotoxoplasmosis). An abdominal echografic scan revealed hepatosplenomegaly, and a chest film showed an upper left lung infiltrate with small cavitations. All blood cultures for mycobacteria were negative; smears from one bronchoalveolar lavage showed acid-fast bacilli; and the presence of mycobacteria in other body sites was not investigated. Therapy with clarithromycin, ciprofloxacin, and amikacin was begun; symptoms were controlled with this regimen, and the lung lesion appeared to improve. The patient refused to undergo follow-up procedures, and he died 1 year later of a progressive wasting syndrome.

No relationship between the two cases exists; the patients lived in areas far more than 1,000 kilometers apart, and no occupational or other kind of commonality has been noted.

Microbiology. Blood was seeded, after lysis-centrifugation treatment (Isolator-10 system; Wampole, Cranbury, N.J.), on radiometric broth Bactec 13A (Becton Dickinson, Towson, Md.), on biphasic medium MB Check (Becton Dickinson), and on Lowenstein-Jensen medium. Positive results were regularly achieved solely on the first medium; only two Lowenstein-Jensen cultures grew colonies: one grew 14 and the other grew 25. The bronchoalveolar lavage specimen was inoculated only on Lowenstein-Jensen medium, on which numerous colonies grew.

For conventional identification, an extended panel of biochemical and cultural tests (Table 1) was used (2, 7, 15, 19, 26, 27). The hybridization test was done with chemiluminescent DNA probes (AccuProbe; Gen-Probe, San Diego, Calif.) specific for *M. avium*, *Mycobacterium intracellulare*, and *M. tuberculosis* complex, according to the recommendations in reference 22, but using both the 5- and the 10-min "selection times." The chemiluminescence emitted was estimated with a PAL/AccuLDR luminometer (Gen-Probe) and quantified as PAL light units (PLUs).

Mycolic acid analysis by mean of high-performance liquid chromatography (HPLC) was performed according to the Centers for Disease Control and Prevention technique (3). Briefly, after a loopful of colonies grown on Middlebrook 7H11 agar was saponified with ethanolic potassium hydroxide, mycolic acids were extracted with chloroform, derivatized to *p*-bromophenacyl esters (8), mixed with 5 μl of a high-molecular-weight internal standard (Ribi ImmunoChem Research, Hamilton, Mont.), and injected into the HPLC system. Mycolic acid separation was achieved with a C_{18} Ultrasphere-XL analytical cartridge column (Beckman, Berkeley, Calif.), using a methanol-methylene chloride gradient. After equilibration with a 98%–2% mixture, the concentrations were changed, over 1 min, to 80%–20% and than linearly, over 9 min, to 35%–65%.

The Bactec NAP test (Becton Dickinson) was performed according to the recommendations of the manufacturer (20).

The susceptibility pattern of our strains was determined by using the radiometric system according to a recently proposed macrodilution method developed for *M. avium* complex (MAC) (21). In short, 100 μl of twofold dilutions of each drug was added to as many Bactec 12B vials (Becton Dickinson). A radiometric broth, seeded with the test strain, was incubated at 37°C, and its growth index

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TABLE 1. Results of standard biochemical, cultural, and inhibition tests on two isolates of *M. celatum*

Test	Result[a]
Niacin	−
Nitrate reduction	−
Thermostable catalase	+
β-Glucosidase	−
Tween 80 hydrolysis (10 days)	−
Tellurite reduction	+
Arylsulfatase (3 days)	v
Urease	−
Catalase (over 45 mm of foam)	−
Acid phosphatase	+
Photochromogenicity	−
Scotochromogenicity	−
Growth at 25°C	+
Growth at 45°C	+
Growth on MacConkey agar	−
Tolerance to:	
p-Nitrobenzoate (500 μg/ml)	+
NaCl (5%)	−
Thiophene-2-carboxylic hydrazide (5 μg/ml)	+
Thiacetazone (10 μg/ml)	+
Hydroxylamine (500 μg/ml)	−
Isoniazid (1 μg/ml)	+
Oleate (250 μg/ml)	−
Ethambutol (1 μg/ml)	+
p-Aminosalicylate (1 μg/ml)	v
Toluidine blue (300 μg/ml)	+
Growth rate	Slow
Colonial morphology	Smooth

[a] −, negative; +, positive; v, variable (one strain was positive and one was negative).

(GI) was read daily with the Bactec 460TB instrument until it reached the maximum on the scale (GI, ≥ 999); it was then diluted 1/100, and 100 μl was inoculated into all antibiotic-containing vials and one drug-free control vial. This inoculum, which provides an initial concentration of roughly 10^4 to 10^5 CFU/ml (11), was further diluted 1/100 and used to seed a second drug-free bottle. The vials were incubated at 37°C, and the GIs were recorded daily. According to previous studies (11), the MIC is defined as the lowest drug concentration in the presence of which, throughout the test, the daily GI does not score more than 50, and a final reading is achieved when the GI for the 1/100-diluted control has remained greater than 20 for 3 consecutive days; at the same time, the GI of the undiluted control must have reached 999.

The 16S rRNA gene fragment sequencing was performed as described previously (16).

RESULTS

Mature colonies were unpigmented and not substantially different from "opaque" MAC colonies; like MAC strains, they tended to become pale yellow with age. On agar-based media, the very early observation under low magnification of microscopic colonial morphology revealed a "bird's nest" appearance closely resembling that of *M. xenopi* microcolonies. Liquid broth cultures remained clear except for the presence of a fine granulation. On the basis of conventional tests results (Table 1), the most likely identification, obtained with a program for the computerized identification of mycobacteria (24), was *M. avium-intracellulare*.

Hybridization tests performed with DNA probes specific for either *M. avium* or *M. intracellulare* had negative results. Also, values for hybridization with the probe for the *M. tuberculosis* complex, done in duplicate, remained below the recommended cutoff (900 PLUs), although they were higher than the average with unrelated mycobacteria, especially when the 5-min selection step was used: 312 and 285 PLUs, respectively, versus a mean of 45.8 PLUs, with a standard deviation of 23.0, on 81 strains belonging to various mycobacterial species other then *M. tuberculosis* and *M. celatum*.

The HPLC profiles for our two isolates appeared identical and very similar to that of *M. xenopi*. A closer comparison of the eluate profiles (Fig. 1) does, however, show minor differences: in the second cluster of *M. celatum*, peak B9 is higher than E2 and peak B8 is present, whereas the contrary is true for *M. xenopi*. The pattern presented by our *M. celatum* isolates was like that found by Butler et al. in a cluster of mycobacteria unidentified at that time and defined as "*xenopi* like" (6).

No inhibition by NAP was noticed.

Sequencing of the 16S rRNA signature region definitively assigned our two strains to the recently described new species *M. celatum*, type 1.

Only minor differences in results of conventional tests between our two strains and the ones investigated in the new species description are present (5). They were, in fact, able to grow, even if poorly, not only at 45°C (like type strains) but also at 25°C, like the strains (biochemically and morphologically similar to *M. celatum*) described by Brander et al. (1). The arylsulfatase activity at 3 days, determined in duplicate on Wayne arylsulfatase agar (19), was only weak in one strain and was absent in the other.

The close similarity of growth kinetics between *M. celatum* and *M. avium* easily allowed us to perform the susceptibility testing with the procedure validated for MAC strains (21), which has been routinely adopted with satisfaction in our laboratories; the time requested for the final reading was 8 days for both strains. The MICs able to inhibit the growth of 99% of the mycobacteria are reported in Table 2.

DISCUSSION

Several clinically significant mycobacterial species (*M. genavense*, *M. haemophilum*, and *M. malmoense*) have been described only in recent years, mainly because their particular metabolic requirements had long delayed their isolation.

This is not the case for *M. celatum*, which grows easily; its recognition has been delayed because for a long time it was hidden (*celatum*, Latin) among strains of MAC or *M. xenopi*, with which it shares most of the features conventionally investigated for the identification of mycobacteria. Only recently introduced techniques, e.g., DNA probes and HPLC, have unveiled the important differences between these organisms and the species with which they were confused, finally leading to the recognition of the new species.

Various mycobacterial species display a wide range of roles in AIDS; while the incidence of MAC and *M. tuberculosis* infections in HIV-positive patients is very high, diseases related to other mycobacterial species are less frequent and, for some, very rare. The incidence of *M. celatum* in AIDS patients is unknown; at present, no other isolation from blood of such patients has been reported (only 1 of 24 strains studied for the new species description [5] originated from blood, but the HIV status of the patient was unknown), but we expect that *M. celatum* will be found responsible for a share of the infections due to what presently appear to be nonhybridizing MAC strains. The repeated isolation from blood in one of our patients confirms the clinical importance of *M. celatum*, and the consequences of its impact on immunocompromised patients deserve attention.

Cross-hybridization of commercial probes for *M. tuberculosis* with *M. celatum* type 1 has been reported recently (4, 23); in our tests, no positivity was achieved, but the signal of both strains did appear unusually high.

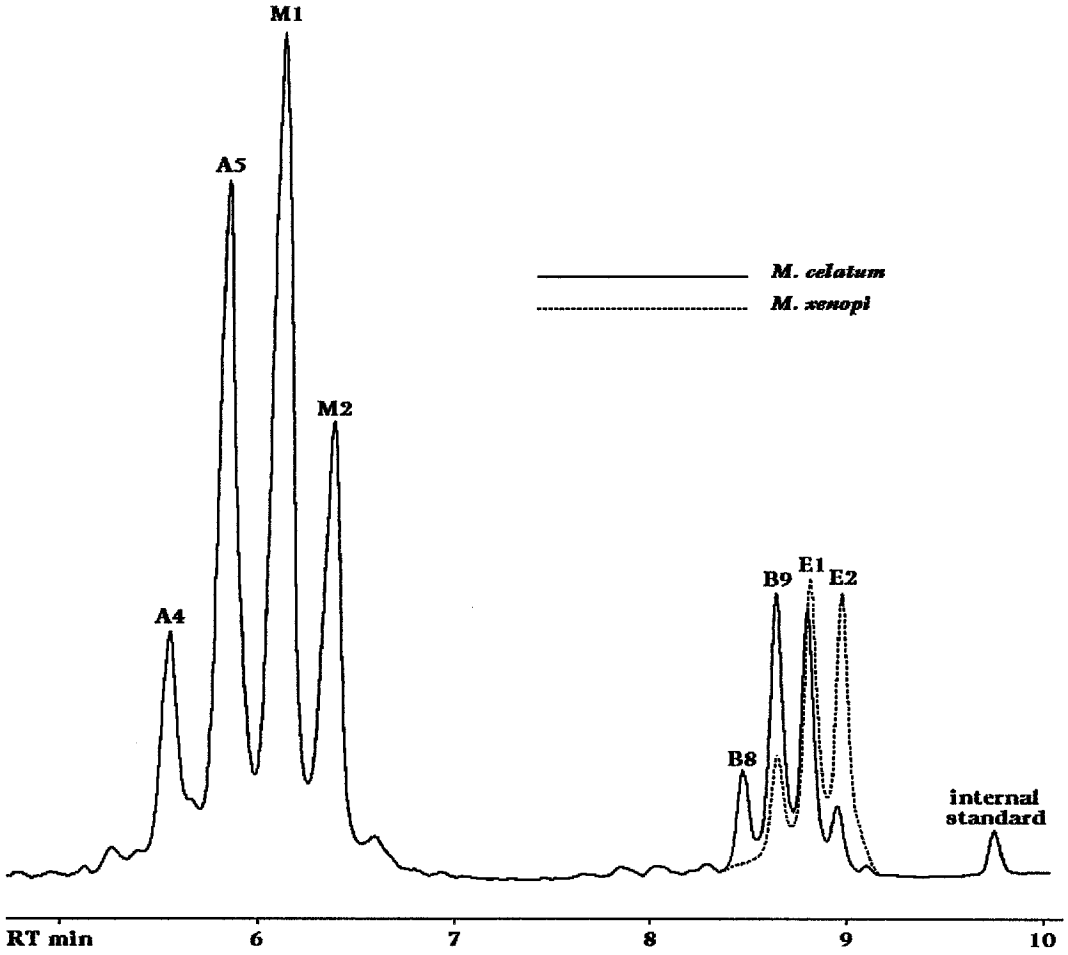

FIG. 1. Comparison of HPLC mycolic acid bromophenacyl esters of *M. celatum* and *M. xenopi* (the identification of peaks follows the scheme proposed by Glickman et al. [9]).

Our susceptibility tests do not confirm the multiple drug resistance suggested for this species in the only available report (5); the relevant results from the latter are shown in the fourth column of Table 2, for comparison. The differences can, at least in part, be attributed to the use of different testing procedures, as it is well known that MICs for slowly growing mycobacteria are lower in liquid media than on solid media. As stressed by Heifets (10) and Inderlied (13), the broth dilution method is the most accurate. Nevertheless, it can be confirmed that the susceptibility of *M. celatum* to most of the major antimycobacterial drugs is halfway between those of MAC and *M. xenopi* (5); in fact, only ethambutol is consistently less active against *M. xenopi* (25). The full susceptibility to quinolones, clarithromycin, and rifabutin and the complete resistance to rifampin seem to be the salient features.

Although at present only genetic and mycolic acid HPLC analyses can confirm the identification of *M. celatum*, this organism should be suspected whenever a nonpigmented mycobacterium that behaves like MAC or *M. xenopi* in conventional tests fails hybridization with probes specific for MAC. Useful hints for a presumptive identification should include the concurrent presence of a strong resistance to rifampin, a bird's nest microscopic morphology of colonies, and a temperature optimum of 37°C instead of 42°C.

TABLE 2. Susceptibility pattern of *M. celatum*[a]

Drug	MIC (μg/ml)		Breakpoint concn (μg/ml) tested (% resistance)[b]
	Strain A	Strain B	
Amikacin	0.5	0.25	
Azithromycin	4	2	
Capreomycin	2	0.5	10 (100)
Ciprofloxacin	0.5	0.5	2 (0)
Clarithromycin	0.25	0.25	
Clofazimine	0.12	0.12	
Ethambutol	1	2	5 (33)
Isoniazid	0.5	0.5	1 (100)
Kanamycin	4	1	5 (16)
Ofloxacin	1	1	
p-Aminosalicylic acid	0.25	0.06	
Pyrazinamide[c]	>400	>400	25 (100)
Rifabutin	0.5	0.5	2 (83)
Rifampin	256	128	1 (100)
Sparfloxacin	0.25	0.12	
Streptomycin	0.5	0.5	2 (29)

[a] Comparison of the MICs in liquid medium for our two strains and the published results of breakpoint agar dilution tests on 24 strains (5).

[b] When more than one concentration had been tested, the one closer to our MIC was used for comparison.

[c] MICs were estimated according to the Bactec recommendations for this drug.

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