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INVESTIGATION ON SEVERAL PHENOTYPIC FEATURES IN TWO STRAINS OF *MYCOBACTERIUM GENAVENSE*

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SUMMARY

The newly recognized species *Mycobacterium genavense* causes disseminated infections in AIDS patients, but its prevalence is difficult to assess because of its inability to grow on standard solid media. For the same reason, very little is known about the phenotypic traits of its isolates.
We report here the results of our studies on two such strains isolated from AIDS patients and subcultured on a non-standard solid medium. Besides several features conventionally explored for mycobacterial speciation, we tested the isolates for 19 enzymatic activities and determined their mycolic acids profiles by means of high performance liquid chromatography. We also compare our findings with the scanty literature data on the laboratory characteristic and antimicrobial susceptibility of *M. genavense*.

KEY WORDS Mycobacterium genavense, *culture media, enzymatic activities, HPLC, mycolic acids, susceptibility testing*

INTRODUCTION

Mycobacterium genavense is a recently described (Böttger *et al.*, 1993) new species of mycobacterium which can cause disseminated infections in severely immunocompromised AIDS patients (Bessesen *et al.*, 1993; Böttger *et al.*, 1992; Hirschel *et al.*, 1990; Wald *et al.*, 1992; Jackson *et al.*, 1992; Coyle *et al.*, 1992; Nadal *et al.*, 1993; Heiken *et al.*, 1993; Tortoli *et al.*, 1994).

M. genavense, which grows slowly in liquid media (Bactec radiometric broths and Middlebrook 7H9 broth), is reported to be unable to grow on standard solid media for mycobacteria, a feature which, from the laboratory point of view, is of paramount importance, and a serious obstacle to the phenotypic characterization of this novel species. An extremly slow development of colonies of *M. genavense* is however achievable on Middlebrook 7H11 solid medium enriched with mycobactine (Coyle *et al.*, 1992).

We report here on several phenotypic features investigated on two strains of *M. genavense* previously isolated on radiometric medium from AIDS patients (Tortoli *et al.*, 1994) and subsequently subcultured on mycobactine-enriched Middlebrook 7H11. The tests performed concern cultural features, both on solid and in liquid media, biochemical activities, inhibition tests, antimicrobial susceptibility, commercial DNA-probe reactivity and high performance liquid chromatography (HPLC) profile of cell wall mycolic acids.

MATERIALS AND METHODS

The two strains on which we report here had been isolated from blood cultures on Bactec 13A medium (Becton Dickinson, Towson, USA) from separate AIDS patients, one year apart. They were definitely identified as *M. genavense* by E Böttger (Institut für Medizinische Mikrobiologie, Hannover, Germany), who kindly performed the direct sequence determination of their PCR-amplified 16S rRNAs (Böttger *et al.*, 1992).

From each strongly positive broth culture 100 µl were spread on Middlebrook 7H11 agar plates supplemented with 2 µg/ml of mycobactine J (Rhône-Mérieux, Lyon, France) which were then incubated in polyethylene bags at 37° C in a CO_2 enriched (5-10%) atmosphere.

Subcultures were also performed in liquid radiometric broths at pH 6.8 (Bactec 12B) and pH 6 (Bactec Pza test medium, Becton Dickinson) which were read daily in the Bactec 460TB instrument.

Radiometric media at both the above mentioned pH conditions were incubated at various temperatures (25°, 32°, 37°, 42° and 45° C).

In a search for growth enhancing substances we investigated the following materials, which were added to liquid media at two different pHs: polyoxyethylene stearate (100 µg/ml) (Bactec reconstituting fluid, Becton Dickinson), mycobactine (2 µg/ml), 0.2% hemoglobin (BBL, Cockeysville, USA), 10% Middlebrook ADC enrichment (Difco, Detroit, USA), 10% Middlebrook OADC enrichment (Difco), 2.5% fresh egg yolk.

To test the hypothesis that *M. genavense* could produce, during its growth, either growth factors or substances neutralizing hypothetical toxic components of the medium, broth-filtrates from previous logarithmic-phase cultures were added to new subcultures of the organism, at final concentrations ranging from 2.5 to 10%.

The inhibition assay by p-Nitro-α-acetylamino-β-hydroxypropiophenone (NAP test, Becton Dickinson) was also performed in both acidic and neutral broths, and otherwise carried out according to the manufacturer's directions.

Nineteen enzymatic activities were investigated using a semiquantitative micromethod (Api Zym, bioMérieux, Marcy l'Etoile, France). A heavy suspension in distilled water (with a turbidity between the McFarland standards no. 5 and no. 6) was prepared from colonies of each *M. genavense* isolate and was used to inoculate all the substrate-containing cupoles of the gallery. The system was then incubated overnight at 37° C, in the humid chamber provided. For the interpretation of the results, 10 min after the addition of Zym A and Zym B reagents (fast blue BB reaction) the colors developed in each cupole were compared with the color-chart to estimate the approximate number of free nanomoles.

Several tests usually employed for the speciation of mycobacteria (Tween 80 hydrolysis, urease, nitrate reduction, heat stable catalase, 3-days arylsufatase, β-glucosidase, tellurite reduction) were performed according to the standard procedures (Roberts *et al.*, 1991).

The ability of *M. genavense* to resist inhibition by p-nitrobenzoate (400 µg/ml), isoniazid (1 µg/ml), thiacetazone (10 µg/ml), hydroxylamine (600 µg/ml) and oleate (250 µg/ml) was investigated on mycobactine-enriched 7H11 containing the respective substances. The plates were incubated at 37° C in 5 to 10% CO_2 for two months and read also with the aid of a low magnification (100x) microscope.

The HPLC analysis of mycolic acids was performed according to the CDC technique (Butler *et al.*, 1992). In short, a loopful of colonies was saponified in the autoclave (1 h at 121° C) with 2 ml of ethanolic potassium hydroxide (25% KOH in 50% ethanol); after acidification mycolic acids were extracted with chloroform and derivatized to p-bromophenacyl esters (Durst *et al.*, 1975). To 100 µl of extract 5 µl of a high molecular-weight internal standard (Ribi ImmunoChem Research, Hamilton, USA) were added before the injection (10 µl sample). The separation was performed on an HPLC model System Gold (Beckman Italia, Milan, Italy) with a C-18 reverse phase cartridge column (particle size 5 µm) 25 cm by 2 mm (Beckman) with a methanol-dichloromethane gradient elution; the bromophenacyl esters were detected with a model 166 (Beckman) UV detector set at 254 nm. The solvent conditions were changed linearly, after the injection, from 90%/10% (methanol/dichloromethane) to 80%/20%, in 1 min, and to 35%/65% over the next 19 min (flow rate = 0.6 ml/min).

Hybridization tests were performed from colonies, according to recommended directions, with AccuProbe systems (Gen-Probe, San Diego, USA) for *Mycobacterium tuberculosis* complex, *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium gordonae* and *Mycobacterium kansasii*.

RESULTS

The growth of our two isolates on the original cultures in 13A medium required 33 and 55 days respectively before reaching a growth index (GI)>10 and a further 12 and 20 days to reach a value ≥100. All attempts to subculture the isolates on conventional solid media, both egg- and agar-based, gave negative results.

Subcultures in liquid media (Bactec 12B, Bactec 13A, Pza test medium, Dubos broth) were uniformly successful, and we noticed that acid broths supported a clearly faster rate of growth.

Most of the substances tested as additives (polyoxyethylene stearate, mycobactine, Middlebrook ADC enrichment, Middlebrook OADC enrichment, egg yolk) turned out to be inhibitory rather than stimulatory of growth; only the addition of hemoglobin revealed a moderate stimulatory effect. The addition of spent broth filtrates from log phase cultures had no effect.

Tiny colonies appeared after two months on mycobactine-enriched 7H11 plates; with further incubation the colonies turned out to be white, creamy, domed and smooth. No pigment induction was noted after light exposure. The optimal growth temperature was 37° C; only in acid broth was a moderate rise in GI also appreciable at 32° and 42° C.

NAP clearly inhibited the growth of *M. genavense*, both in acid and in neutral broth.

Results of biochemical and inhibition tests are listed in Table 1, while Table 2 reports the enzymatic activities. The HPLC profile (Figure 1) was similar in the two isolates, and was characterized by three clusters of peaks. All hybridization tests gave negative results.

DISCUSSION

The paucity of *M. genavense* isolates warrants reporting whatever information is obtained on them. We regret that we could study only two strains, but their uniform behavior and the agreement of results in those tests previously performed on a larger number of strains (Coyle *et al.*, 1992) corroborate the significance of our findings.

Phylogenetic studies based on 16s rRNA sequences place *M. genavense* in a cluster

TABLE 1
Results of standard biochemical, cultural an inhibition tests on M. genavense

Test	This study [a]	Coyle et al. (1992) [b]
Nitrate reduction	-	-
Thermostable catalase	+	+
β-glucosidase	-	
Tween 80 hydrolysis (10 days)	-	-
Tellurite reduction	-	
Arylsulfatase (3 days)	-	-
Urease	+	+
Niacin		-
Catalase (over 45 mm of foam)		+
Pyrazinamidase		+
Photochromogenicity	-	-
Scotochromogenicity	-	-
Tolerance to:		
p-nitrobenzoate (500 μg/ml)	-	
Thiophene-2-carboxylic hydrazide (5 μg/ml)	-	
Thiacetazone (10 μg/ml)	-	
Hydroxylamine (500 μg/ml)	-	
Isoniazid (1 μg/ml)	+	
Oleate (250 μg/ml)	-	
Growth rate	slow	slow
Colonial morphology	creamy	creamy or dry

[a] two strains.
[b] seven strains.

clearly distinct from all other slowly growing mycobacterial species and closely related only to *Mycobacterium simiae* (Böttger *et al.*, 1992). Several phenotypic characters, but above all the mycolic acid composition of the cell wall, seem to confirm such relatedness: HPLC profiles of mycolic acid bromophenacyl-esters share with *M. simiae* a highly characteristic three-clustered shape, with *M. simiae* showing a supplemental peak at the end of the sequence.

On the contrary other features appear to be distinctive of *M. genavense* and differentiate it from other nontuberculous mycobacteria, in particular its inhibition by NAP and its substantially high susceptibility (Table 3) to almost all the antimicrobials tested (Tortoli *et al.*, 1994; Siddqi *et al.*, 1993).

The importance of *M. genavense* as a cause of disseminated infections in AIDS patients has been stressed by the reports of several cases in recent months (Bessesen *et al.*, 1993; Böttger *et al.*, 1992; Wald *et al.*, 1992; Jackson *et al.*, 1992; Nadal *et al.*, 1993; Heiken *et al.*, 1993; Tortoli *et al.*, 1994; Kirschner *et al.*, 1994), and surely the prevalence of this organism is underestimated owing to the problem of isolation.

A better knowledge of its characteristics will minimize the misidentifications of fastidious mycobacteria unable to grow on solid media. As other species like *Mycobacterium haemophilum* and several strains of *M. avium* also fail to grow on conventional media, this feature alone is not sufficient for a presumptive indentification of *M. genavense*. Our study confirms that, be-

TABLE 2
Enzymatic profile of our two strains of M. genavense

Enzymatic activity	Result
Alkaline phosphatase	-
Esterase (C4)	+
Esterase lipase (C8)	+
Lipase (C14)	v[a]
Leucine arylamidase	-
Valine arylamidase	-
Cystine arylamidase	-
Trypsin	+
Chymotrypsin	-
Acid phosphatase	+
Naphthol-AS-BI-phosphohydrolase	-
α-galactosidase	-
β-galactosidase	-
β-glucuronidase	-
α-glucosidase	+
β-glucosidase	-
N-acetyl-β-glucosaminidase	-
α-mannosidase	-
α-fucosidase	-

[a]variable

TABLE 3
Susceptibility pattern of M. genavense[a]

Antimicrobial	Tortoli et al. (1994)[b]	Siddiqi et al. (1993)[c]
Amikacin	S	
Azithromycin [d]	S	
Ciprofloxacin	I	
Clarithromycin	S	
Clofazimine	S	
Ethambutol	R	R
Isoniazid	R	R
Kanamycin	S	
Ofloxacin	S	
Pyrazinamide	R	R
Rifabutin	S	
Rifampin	S	S
Sparfloxacin	S	
Streptomycin	S	S

[a]R=resistant; I=intermediate; S=susceptible.
[b]two strains.
[c]six strains.
[d]not included in the cited study.

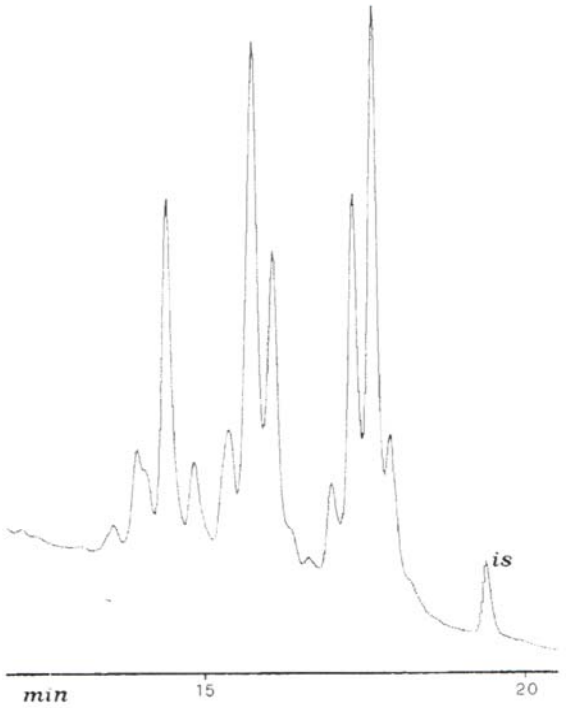

FIGURE 1 - Chromatogram of mycolic acids p-bromophenacyl esters of *M. genavense* (*is* = internal standard).

sides the rRNA sequencing, mycolic acids HPLC can provide a step towards the laboratory identification of this organism and suggests a series of tests that may be useful to presumptively identify this novel opportunistic agent.

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