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Characterization of an Isolate of the Newly Described Species *Mycobacterium interjectum*

ENRICO TORTOLI[1], ALESSANDRO BARTOLONI[2], CLAUDIO BURRINI[3], DOMENICO COLOMBRITA[4], ANTONIA MANTELLA[2], GABRIELE PINSI[4], M. TULLIA SIMONETTI[1], GIOVANNI SWIERCZYNSKI[5], and ERIK C. BÖTTGER[6]

[1] Bacteriological and Virological Laboratory, Careggi Hospital, 50139 Firenze, Italy
[2] Institute of Infectious Diseases, University of Florence, 50121 Firenze, Italy
[3] Institute for Clinical Researches, 50129 Firenze, Italy
[4] Bacteriological Laboratory, Spedali Civili, 25100 Brescia, Italy
[5] Clinical Laboratory, Desenzano Hospital, 25015 Desenzano del Garda, Italy
[6] Institut für Medizinische Mikrobiologie, Medizinische Hochschule, 30623 Hannover, Germany

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Summary

The phenotypic features of a clinical isolate of the new species *Mycobacterium interjectum*, identified on the basis of its 16S rRNA gene sequence, are compared with those of the type strain. The differentiation of *M. interjectum* from *Mycobacterium gordonae* or *Mycobacterium scrofulaceum* is not achievable on the basis of phenotypic traits usually tested for mycobacterial speciation, but it can be reached by 16S rRNA gene sequencing or by high performance liquid chromatography (HPLC) of cell wall mycolic acids. The former reveals sequence identity with the signature region of the type species, and the latter yields a profile which is easily differentiated from those of the other two species. The unique HPLC profile of *M. interjectum* is reported here for the first time and so are the MICs of a wide spectrum of drugs.

Introduction

Recently developed technology based on the sequencing of conserved and variable regions of bacterial genomes permits the detection of new species previously misidentified owing to their similarity in phenotypic characters with other formerly recognized organisms (11).

Within the genus *Mycobacterium*, two clinically significant species have been recently differentiated on the basis of their 16S rRNA sequences: *Mycobacterium celatum* (1) and *Mycobacterium interjectum* (14). The majority of the strains belonging to

these two novel species have probably been identified previously as *Mycobacterium avium-intracellulare* (with which *M. celatum* shares most features in tests conventionally performed for mycobacterial speciation), *Mycobacterium gordonae*, or *Mycobacterium scrofulaceum* (the species which present more phenotypic similarities with *M. interjectum*).

Material and Methods

The strain was isolated from the urine of an asymptomatic 71-year-old woman, affected by hydronephrosis of the right kidney due to ureteral stenosis; the patient had been previously treated with radiotherapy for an unspecified gynecological pathology.

For the identification of the organism, conventional tests (10), HPLC analysis of cell wall mycolic acids and 16S rRNA gene sequencing were performed. All investigations were conducted in the same way both on our clinical isolate and on the type strain from the German Collection of Microorganisms and Cell Cultures (DSM 44064).

The Bactec NAP-test (Becton Dickinson, USA) was performed following the standard procedure (12). An hybridization assay was performed with a probe specific of *M. gordonae* (AccuProbe *M. gordonae* culture identification test, Gen-Probe, USA) (17), according to the manufacturer's recommendation.

The complete set of phenotypic characters investigated for the conventional identification (10) is reported in Table 1.

Nineteen different enzymatic activities (Table 2) were investigated using a commercial semiquantitative micromethod (Api Zym, BioMérieux, France). Each substrate-containing well in the strip was inoculated with 65 µl of a heavy mycobacterial suspension (no. 5 McFarland or more) and incubated overnight at 37 °C; the results were read after addition of the reagents provided (fast blue BB reaction) and semi-quantitatively interpreted on the basis of their color intensities (9).

The HPLC profile of mycolic acid bromophenacyl esters was determined according to the technique of *Butler* et al. (2). Briefly, a loopful of colonies grown on Middlebrook 7H11 was saponified with ethanolic potassium hydroxide. Mycolic acids were extracted with chloroform, derivatized to p-bromophenacyl esters (4), mixed with 5 µL of a high-molecular weight internal standard (Ribi ImmunoChem Research, USA) and injected into the HPLC system. Mycolic acid separation was achieved with a C-18 Ultrasphere-XL analytical cartridge column (Beckman, USA), 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%.

Seqencing of the gene encoding the 16S rRNA was performed as reported previously (8). Briefly, nucleic acids were extracted by simple mechanical lysis of cells, using a tissue homogenizer. PCR was performed with a 50 µL reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% (wt/vol), gelatin, 200 µM each deoxynucleoside triphosphate, 1.25 U of Taq polymerase (Perkin-Elmer, Cetus, Überlingen, Germany), 30 pmol of primer 264 (5'TGC ACA CAG GCC ACA AGG GA 3', corresponding to *Escherichia coli* 16S rRNA from position 1046 to 1027), and 10 pmol of biotinylated primer 285 (5' GAG AGT TTG ATC CTG GCT CAG 3'; corresponding to *E. coli* 16S rRNA from position 9 to 30). The thermal profile involved 39 cycles with a 1 min denaturation step at 94 °C and a 3 min annealing and extension step at 68 °C. Successful amplification was controlled by agarose gel electrophoresis.

The biotinylated single-stranded DNA template was prepared using Dynabeads M-280-streptavidin (Dynal, Hamburg, Germany) and a Dynal MPC-E magnetic separator. A 20 µL Dynabeads solution (10 mg/mL) was used for each PCR. Following preparation of the biotinylated DNA strand, essentially as described by the manufacturer, the beads were finally resuspended in 20 µL of H_2O. Sequencing was performed with 2 to 5 µL of the Dynabeads

Table 1. Comparison of standard testing of the present and previous isolates of *M. interjectum*; literature data on *M. gordonae*, *M. scrofulaceum*, and *M. simiae*

Test	our strain	type strain[a]		Emler et al. (5)	*M. gordonae*[b]	*M. scrofulaceum*[b]	*M. simiae*[b]
		A	B				
Niacin	–	–	–	–	–	–	v
Nitrate reduction	–	–	–	–	–	–	–
Thermostable catalase	+	+	+	+	+	+	+
β-glucosidase	–	–		–	–	–	–
Tween 80 hydrolysis (10 days)	+	+	–	–	+	–	–
Tellurite reduction	+	+			–	+	+
Arylsulfatase (3 days)	–	–	–	–	v	–	–
Urease	–	+	+	–	–	+	+
Catalase (over 45 mm of foam)	–	–		+	+	+	+
Photochromogenicity	–	–	–	–	–	–	+
Scotochromogenicity	+	+	+	+	+	+	–
Growth at 25 °C	+	+		+	+	+	+
Growth at 37 °C	+	+	+	+	+	+	+
Growth at 45 °C	–	–	–	–	–	–	–
MacConkey	–	–			–	–	v
Tolerance to:							
p-nitrobenzoate (500μg/ml)	+	+			+	+	+
NaCl (5%)	–	–		–	–	–	–
Thiophene-2-carboxylic hydrazide (5μg/ml)	+	+		+	+	+	+
Thiacetazone (10 μg/ml)	+	+		+	+	–	+
Hydroxylamine (500 μg/ml)	–	–			–	+	+
Isoniazid (1 μg/ml)	+	+		+	+	+	+
Oleate (250 μg/ml)	+	+			+	+	+
Ethambutol (1 μg/ml)	+	+	+	+	+	v	+
p-Aminosalicylate (1 μg/ml)	+	+		+	–	–	v
Toluidine blue (300 μg/ml)	–	–			v	v	+
Growth rate[c]	Sl	Sl	Sl	Sl	Sl	Sl	Sl
Colonial morphology[d]	Sm	Sm	Sm	Sm	Sm	Sm	Sm

[a] A: our results; B: *Springer* et al. (14). [b] v = variable. [c] Sl = slow. [d] Sm = smooth.

single-stranded DNA solution, 2 pmol of sequencing primer 244 (5′CCC ACT GCT GCC TCC CGT AG 3′: corresponding to *E. coli* 16S rRNA from positions 361 to 341) per reaction, 0.5 to 1.0 μCi of [α^{-32}P] dCTP at 3,000 Ci/mmol (Amersham Buchler, Braunschweig, Germany), and Sequenase version 2.0 (USB, Bad Homburg, Germany) according to standard procedures (USB and manuals).

The susceptibility of the isolate to a wide panel of drugs was quantitatively tested, using a radiometric macrodilution method based on the technique recently proposed for the *M. avium* complex (13). Briefly, a vial of Bactec 12B (Becton Dickinson) inoculated with the bacterial suspension (turbidity = No. 1 McFarland) was incubated and read daily on the Bactec 460TB instrument unit a growth index (GI) ≥ 999 was reached. A 1/100 dilution was used to inoculate (100 μL/vial) a number of Bactec 12B broths containing twofold dilutions of various antimicrobials and one antibiotic-free vial (undiluted control); a further 1/100 dilution was used to inoculate a second (diluted) control bottle. When the GI of the diluted control had remained > 20 for three consecutive days, the MIC of each antimicrobial was determined as the drug concentration present in the vial which had never scored a GI greater than 50 during all the days of incubation.

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129                                 172
TGA TCT GCC CTG CAC TTC / TAC CGG ATA GG-ACCA CGG GAT GCA TGTCT-TGT GGT  M. tuberculosis
.A. ... ... ... ... ...   ... ... ... ..-...T ..A .GC ... ..C..-... ...  M. interjectum
.A. ... ... ... ... ...   ... ... ... ..-...T ..A .GC ... ..C..-... ...  our strain
.A. ... ... ... ... ...   ... ... ... ..-.... .TT .GC ... ..G..-... ...  M. simiae
... ... ... ... ... ..T   ... ..A ... TTCC.TA TT. .TC ... ..G.CTG.. A.G  M. flavescens
... ... ... ... ... ..T   ... ..A ... CACC.TG .T. .TC ... ..G.CTG.. A.G  M. smegmatis
... ... ... ... ... ..T   ... ..A ... ..-...G .AT .C. ... ..GTG-... ...  M. nonchromogenicum
... ... ... ... ... .CT   ... ... ... ..-.... T.. ... ... ...TC-... ...  M. terrae
... C.. ... ... ... ...   ... ... ... ..-.... TTC TGC ... ...GG-G.. ...  M. xenopi
.A. ... ... ... ... A..   ... ..A ... ..-.... .A. ..C A.. ....C-... ...  M. gordonae
C.. ... ... ... ... ...   ... ... ... ..-.... ... ... T.. ....C-... ...  M. marinum
CA. ... ... ... ... ...   ... ... ... ..-.... .TT .GC ... ..C..-... ...  M. scrofulaceum
.A. ... ... ... ... ...   ... ... ... ..-...C ..A .GC ... ..C..-..G ...  M. szulgai
.A. ... ... ... ... ...   ... ..A ... ..-...C ..A .GC ... ..C..-..G ...  M. malmoense
CA. ... ... ... ... AC.   ... ... ... ..-.... .TT .GC ... ..C..-... ...  M. gastri/M. kansasii
CA. ... A.. ... ... ...   ... ... ... ..-...T .AA ..C ... .....-.C. ...  M. avium
CA. ... ... ... ... ...   ... ... ... ..-...T TTA .GC ... .....-.TA ...  M. intracellulare
.A. ... ... ... ... ACT   ... ... ... ..-...T .TC .GC ... ..C..AG.A ...  M. intermedium
.A. ... ... ... ... ...   ... ... ... T.-.... ... A.C ... ...T.-... ...  M. genavense
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Fig. 1. Alignment of selected mycobacterial 16S rRNA sequences. *Mycobacterium tuberculosis* was used as the reference sequence. Only nucleotides different from those in the *M. tuberculosis* sequence are shown; dashes indicate deletions. The respective *Escherichia coli* 16S rRNA position are indicated.

Results

The strain which had not been inhibited by NAP, appeared to be a slowly growing (about three weeks) scotochromogenic mycobacterium appearing as smooth and creamy yellow colonies.

The maximum likelihood identification on the basis of conventional tests (Table 1), obtained with a software developed by one of the authors (16), was *M. gordonae,* but several results (mainly the scanty catalase activity and the tellurite reduction) significantly lowered the reliability of this identification (i. e., the isolate was referred to as a very atypical *M. gordonae*).

Sequence determination of the 16S rRNA revealed the presence of a short helix 18, a feature characteristic of rapidly growing mycobacteria but present also in a group of slowly growing ones, the most representative species of which is *Mycobacterium simiae.* The regions where species-specific signature nucleotides are present were identical to the ones of the recently described species *M. interjectum* (14) (Fig. 1).

Results of enzymatic characterization are reported in Table 2.

No hybridization was achieved with a commercial probe specific of *M. gordonae.*

The HPLC profile of mycolic acids (Fig. 2) was identical for our isolate and for the type strain of *M. interjectum,* and clearly different from those reported for *M. gordonae. M. gordonae* is the only mycobacterial species for which two different HPLC profiles have been reported (3): the first one, presented by the majority of the strains (78% of those studied by *Cage* (3) and 23 out of 25 in our laboratory collection), is characterized by a single, late group of peaks while a minority of strains show two clusters of peaks. The profile of *M. interjectum* appears to be completely different from the most common one

Table 2. Enzymatic profile of *Mycobacterium interjectum*

Enzymatic activity	our strain	type strain[a]
Alkaline phosphatase	−	−
C4 esterase	+	±
C8 esterase lipase	+	+
C14 lipase	+	−
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] ± = very weak activity.

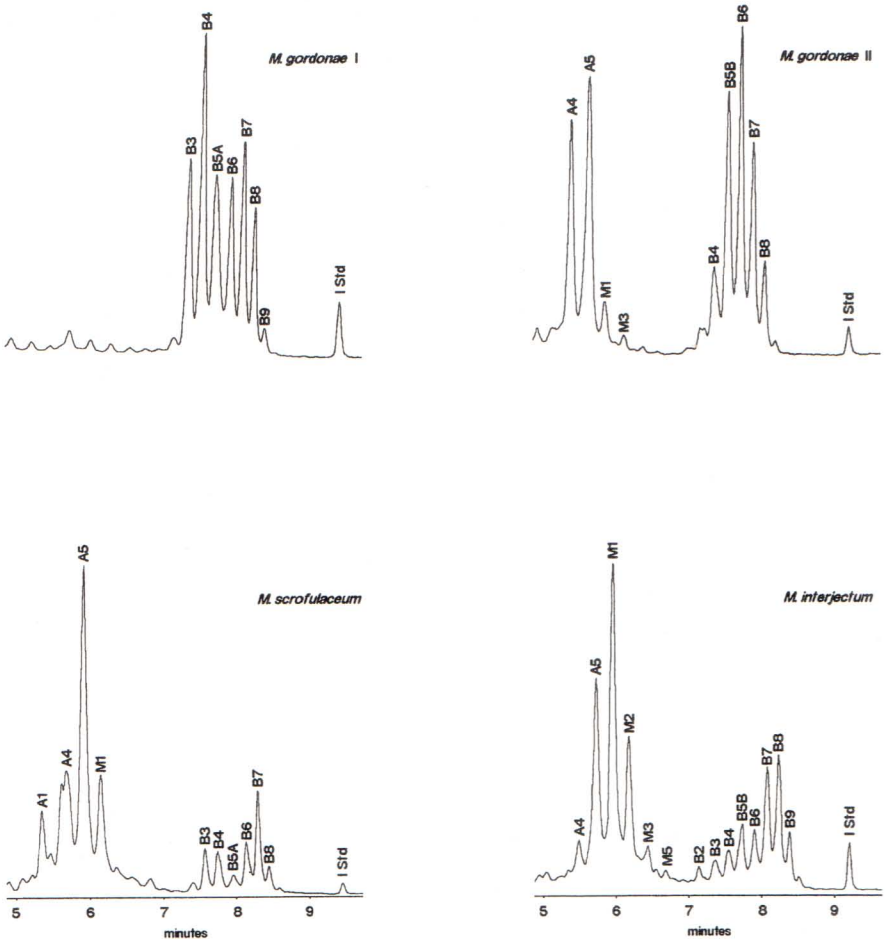

Fig. 2. Representative chromatograms of mycolic acids bromophenacyl esters of *M. gordonae, M. scrofulaceum* and *M. interjectum*. I Std = internal Standard; peaks labelled according to *Glickman* et al. (6).

of *M. gordonae,* and clearly distinguishable both from the second one of *M. gordonae,* and from the one of *M. scrofulaceum.* In fact, the latter three profiles differ both for the retention times of the most prominent peaks, and for the fact that, in *M. interjectum,* there is an uninterrupted sequence of peaks which almost fills the gap between the two clusters of peaks shown by the other two species. The mycolic acids profile of *M. interjectum* was unique to the species and quite different from any other we have seen.

On account of the close similarity with *M. avium* with respect to growth kinetics in liquid radiometric medium, the strict requirements of the method for susceptibility testing were easily fulfilled. Our isolate tended to show higher MICs than the reference strain (Table 3).

Table 3. Susceptibility patttern of *M. interjectum*. Comparison of MICs and break-points determined on a previously reported strain

Drugs	MICs (μg/mL)		Break-points[a] *Emler* et al. (5)
	our strain	type strain	
Amikacin	4	1	≤ 6
Azithromycin	8	16	
Capreomycin	8	4	
Ciprofloxacin	4	0.5	≤ 1
Clarithromycin	0.5	0.25	≤ 4
Clofazimine	0.5	0.06	≤ 1
Ethambutol	8	4	≤ 7.5
Isoniazid	4	4	> 0.1
Kanamycin	8	0.25	
Ofloxacin	8	1	
p-Aminosalicylic acid	8	4	
Pyrazinamide[b]	> 400	> 400	
Rifabutin	0.25	0.5	
Rifampin	0.25	0.5	
Sparfloxacin	2	0.06	≤ 4
Streptomycin	4	2	≤ 6

[a] As the test was performed on various isolates from the same patient collected over time, the results on the first isolate are reported.
[b] Test performed according to the Bactec recommendations for this drug.

Discussion

The use of new molecular biology tools allows species differentiation otherwise not feasible on the basis of phenotypic characters. Due to its unique 16S rRNA sequence, *M. interjectum* has been recently recognized as a new species (14). Whereas phenotypic traits of the new species resemble those of *M. scrofulaceum* and *M. gordonae,* its 16S rRNA reveals the highest sequence homology (98.9%) with *Mycobacterium simiae* (14). A differentiation from the latter with conventional tests is, however, less problematic (Table 1) thanks to its photochromogenicity and, often, to its ability of accumulating niacin. Furthermore the very characteristic three-clustered HPLC profile of *M. simiae* (15) excludes its assignment to other species.

A definite set of characteristic phenotypic features for this novel species cannot be detected among the ones normally used for the identification of mycobacteria. So far, only three strains (albeit two of them isolated repeatedly from the same patients) have been investigated. Compared with the type strain (14), our isolate has shown differences in Tween hydrolysis and urease results while tests for semiquantitative catalase and Tween hydrolysis were in disagreement with the other reported isolate of *M. interjectum* (5) (Table 1). On the basis of these discrepancies, our isolate closely resembled *M. gordonae,* while the type strain appeared to be more like *M. scrofulaceum* (14). A reevaluation of the biochemical features of the type strain however, revealed positivity for Tween hydrolysis (Table 1). In addition, we obtained a positive acid phosphatase result for both strains; in this case, however, our method (Api Zym) differed

from the standard procedure for this test (7). Not only the genetic sequencing but also the analysis of cell wall fatty acids has the potential for differentiating *M. interjectum;* its pattern of mycolic acids phenacyl esters differs in fact from that of every other species.

MICs of our isolate are slightly higher than those of the reference strain; most remarkable are the differences with regard to the quinolones. Even if not fully comparable, the susceptibility pattern determined in radiometric medium using a break-point method after *Emler* et al. (5) did not substantially differ from our MICs (Table 3).

We abstain from making conjectures about the clinical significance of our isolation as no other *M. interjectum* was grown in subsequent urine cultures performed during a period of six months. The finding of nontuberculous mycobacteria in the urine is often difficult to interpret, much more in this case, in which clinical data were very scanty. *M. interjectum* was, however, playing a pathogenetic role in the previously reported cases of infection (5, 14), which has drawn attention to this novel species. The prevalence of *M. interjectum* is certainly underestimated, as it is normally misidentified as *M. scrofulaceum* or *M. gordonae*. Unfortunately, conventional tests are insufficient for its identification, whereas genetic sequencing and HPLC analysis are out of the reach of the majority of clinical microbiology laboratories. To minimize misidentification of *M. interjectum* we suggest at least to confirm all identifications of *M. gordonae* with commercial probes.

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Dr. *Enrico Tortoli,* Laboratorio di Batteriologia e Virologia, viale Pieraccini 24, 50139 Firenze, Italy