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Misdiagnosis of *Mycobacterium brumae* Infection†

We read with interest the paper "Catheter-Related Bloodstream Infection Caused by *Mycobacterium brumae*" by Lee and colleagues (4) because we had described this species in 1993 and no other isolates had been reported from this first description (5). So we contacted the corresponding author, Dr. Han, who gently sent us the *M. brumae* strain causing the bloodstream infection (MDA0695 strain). In order to confirm the identification of the MDA0695 strain, we performed a total of 24 biochemical tests and growing characteristics by following standard procedures (8). *In vitro* antibiotic susceptibility was performed in Middlebrook 7H10 agar by the proportion method (8). The *M. brumae* type strain and the MDA0695 strain were in disagreement in the following eight phenotypic characteristics: colony pigmentation (Fig. 1), growth at 42°C, growth in the presence of 5% NaCl, nitrate reductase (2 h), arylsulfatase (3 days), iron uptake, use of inositol as a sole carbon source, and sensitivity to streptomycin (4 μg/ml) (Table 1).

For mycolic acid analyses, the two strains were grown on Middlebrook 7H10 agar at 30°C for 2 weeks. Mycolic acids were liberated by saponification, methylated with diazomethane, and analyzed by thin-layer chromatography as previously described (2). All the *M. brumae* strains contain only α-mycolates (5); nevertheless, the MDA0695 strain showed small amounts of α-mycolates and large amounts of dicarboxymycolates with their corresponding long-chain alcohols. The pattern of mycolic acids is widely used in taxonomy and identification of mycobacterial species. With few exceptions (1, 6), all the strains that belong to the same species share the same pattern of mycolic acids. So a different pattern of mycolic acids must arouse suspicions on whether the strains compared really belong to the same species. The sequencing of the 16S rRNA gene was performed with PCR amplicons (7) and analyzed using EditSeq and MegAlign software (DNAStar). By comparing the species-specific hypervariable regions A (39 bp) and B (45 bp) of the 16S rRNA gene (3), we found mismatches in 5 and 3 nucleotides, respectively. The MDA0695 isolate showed only 85% and 93% similarity for both regions with the *M. brumae* type strain (GenBank/EMBL/DDBJ accession number AF547907). Thus, the nucleotide sequence of the MDA0695 strain shows substantial differences from the corresponding sequence of the type strain. The sequences of hypervariable regions are very much conserved within species. The homology should be 100%, or very close to it, in strains of the same species. However, we found significant differences in both of the hypervariable regions. The results of the genotype study show that the MDA0695 strain is not an *M. brumae* strain. Hypervariable A or B 16S rRNA region's sequence alignments of the MDA0695 strain revealed it to be closer to noncultivable mycobacteria species and other known rapid-grower mycobacteria than to *M. brumae* (see Fig. S1 in the supplemental material). An unrooted neighbor-joining tree based on the concatenated hypervariable A and B sequences of MDA0695 and concatenated sequences of other related *Mycobacterium* species indicated a close relationship between MDA0695 and *Mycobacterium flavescens*, *Mycobacterium monacense*, *Mycobacterium novocastrense*, *Mycobacterium smegmatis*, and *Mycobacterium goodii* (see Fig. S2 in the supplemental material).

Therefore, the genotypic differences found between the *M. brumae* type strain and the MDA0695 strain, together with differences found in mycolic acid patterns and the other phenotypic characteristics, allow us to conclude that the MDA0695 strain cannot be considered a strain of the *M. brumae* species.

As a result of the misidentification of the MDA0695 strain, the species *M. brumae* is now considered a bacterial pathogen and has been classified as biosafety level 2 by the ATCC. Except for the MDA0695 strain, no other *M. brumae* strains

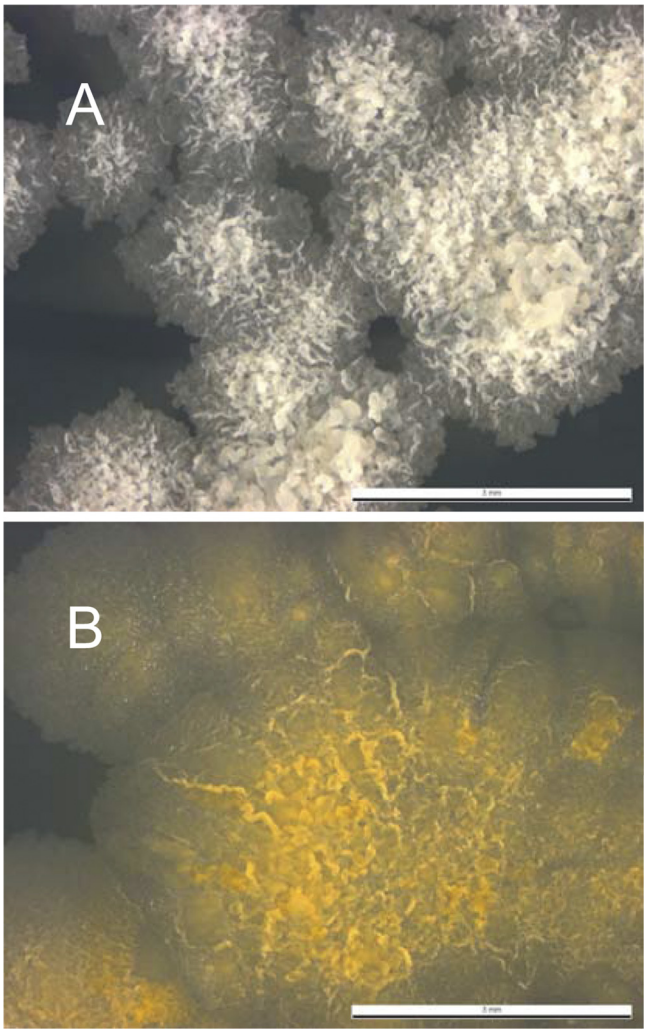

FIG. 1. The MDA0695 strain displays yellow colonies that are very different from the nonpigmented colonies of the *M. brumae* type strain (CIP103465). Colonies of the *M. brumae* type strain (A) and the MDA0695 strain (B) on Middlebrook 7H10 medium.

TABLE 1. Properties of the *M. brumae* type strain and the MDA0695 strain

Characteristics studied	Strain[d]	
	M. brumae CIP103465^T	MDA0695
Smooth colonies	−	−
Rods of >1 μm long	+	+
Pigmentation in the dark	−	+
Pigmentation in the light	−	+
Growth in <5 days	+	+
Growth at:		
25°C	+	+
30°C	+	+
37°C	+	+
42°C	−	+
Growth in nutrient agar	+	+
Growth on MacConkey[a]	−	−
Growth with 5% NaCl	−	+
Niacin	−	−
Catalase at 68°C	+	+
Nitrate reductase (2 h)	+	−
Arylsulfatase (3 days)	−	+
Tween hydrolysis	+	+
Iron uptake	+	−
Sodium citrate[b]	+	+
Mannitol[b]	−	−
Inositol[b]	+	−
L-Arabinose[c]	−	−
Dulcitol[c]	−	−
D-Xylose[c]	−	−

[a] MacConkey agar without crystal violet.
[b] Use of these compounds as sole carbon sources.
[c] Acid produced.
[d] +, positive result; −, negative result.

have been associated with infections up to the present; thus, *M. brumae* must recover its nonpathogenic species status.

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Author's Reply

I thank and applaud Dr. Jiménez and colleagues' thorough analyses of our strain MDA0695 in the foregoing letter. The new data, particularly on mycolic acids and extensive biochemical results, suggest that MDA0695 could well be a new species that clusters closely with *Mycobacterium brumae*, a rapidly growing mycobacterium.

We isolated MDA0695 from the blood of a patient with central venous catheter-related infection in 2003, and I was the one who performed the initial characterization (3). Based on the 99.6% match (847 of 850 bp) of the 16S rRNA gene, rapid growth, and several phenotypic and biochemical features, I called MDA0695 a variant of *M. brumae*. With this closest call, I did notice some differences, such as the yellow pigmentation of our strain (a scotochromogen) as opposed to the lack of pigmentation of the *M. brumae* type strain.

During my workup of MDA0695, it was difficult to fully resolve the hypervariable region A of the 16S rRNA gene, and thus, this region was not included in the initial 850-bp segment used for the GenBank match. During recent attempts and by examining the chromatograms, I noticed two possible polymorphic sites in this region A. In addition, the sequences from hypervariable region B of MDA0695 also showed differences between those obtained by my laboratory and those obtained by Dr. Jiménez's. My version matched completely with the *M. brumae* type strain, whereas hers showed three mismatches. Therefore, in order to ascertain these polymorphic sites, it would be necessary to clone the 16S gene into a plasmid vector and then sequence multiple clones. It is known that almost all mycobacteria, with rare exceptions, have multiple copies of the 16S gene, and polymorphic copies have been noted for *Mycobacterium celatum* (6), a *Mycobacterium* sp. (5), and *Mycobacterium kansasii* (X. Y. Han, unpublished observation). Similarly, polymorphic 16S genes have also been seen in *Nocardia yamanshiensis* (2), *Helicobacter pylori* (strain J99; GenBank accession no. AE001439), and *Vibrio* spp. (4). Such polymorphism may have an effect on phylogenetic analysis at the species level (1).

Based on our single case report, the classification of *M. brumae* as a biosafety level 2 pathogen by the ATCC, as mentioned by Dr. Jiménez and colleagues, seems premature to me. With the increasing population of immunocompromised patients and/or those carrying foreign objects, such as venous catheters, prostheses, and others, many environmental microbes of very low virulence find their way into those patients and, thus, clinical microbiology laboratories. Being in a cancer center, our laboratory encounters such environmental organisms often. As a general rule in microbiology, every microbe should be treated as a potential pathogen, depending on who

is exposed to them, exposure route, and the number of organisms exposed.

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† Supplemental material for this article may be found at http://jcm.asm.org/.