

First Isolation of *Mycobacterium kyorinense* from Clinical Specimens in Brazil

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In this article, the first isolation of *Mycobacterium kyorinense* specimens in Brazil is described. *M. kyorinense* is a recently identified species, with a few strains reported only in Japan. The Brazilian isolates were initially identified as *Mycobacterium celatum* by PCR restriction enzyme pattern analysis (PRA) with *hsp65*. However, biochemical tests indicated the same profile of *M. kyorinense* and distinguished them from *M. celatum* and *Mycobacterium branderi*. The sequencing of the *hsp65*, *rpoB*, and 16S rRNA genes allowed the accurate identification of isolates as *M. kyorinense*.

Mycobacterium kyorinense is a nonpigmented slowly growing mycobacterium that was first isolated from sputum of a patient with pneumonia. The species was described in 2009, and to date a few strains have been isolated, all from patients in Japan (5, 9). All patients were immunocompetent, and most of them had a history of pulmonary disease. *M. kyorinense* was considered a possible cause of clinically significant respiratory disease. The sequences of 16S rRNA genes, *hsp65*, and *rpoB* were identical in the strains tested but different from those of the two phylogenetically most related species, *Mycobacterium celatum* and *Mycobacterium branderi* (1, 4). Biochemical tests can also distinguish *M. kyorinense* from *M. celatum* and *M. branderi* (1, 4, 5, 8).

The aim of this work is to report the first isolation of *M. kyorinense* in Brazil and, to our knowledge, its first isolation outside Japan and to characterize its *hsp65* restriction profile by PCR restriction enzyme pattern analysis (PRA).

This study includes two pulmonary specimens (HF1629 and HF1836) that were initially identified as *M. celatum* in 2007 by PRA with *hsp65* in the National Reference Laboratory for Tuberculosis of the Centro de Referência Professor Hélio Fraga/ENSP/FIOCRUZ. These isolates were obtained from two separate expectorated sputum samples from a patient from Rio de Janeiro State, Brazil, according to microbiological diagnostic criteria for nontuberculous mycobacterial lung disease according to the American Thoracic Society guidelines (3).

At admission to the health authority, the patient presented with complete destruction of the left lung by fibrotic lesions, which over the course of the disease extended to the right lung. Treatment with rifampin, isoniazid, pyrazinamide, and ethambutol temporarily improved the patient's condition, but after less than 1 year, symptoms reappeared, leading to death.

The DNA extraction protocol used in this work was developed in our laboratory and is being introduced here. Briefly, one loopful of bacteria grown on Lowenstein-Jensen medium was mixed in 300 μ l of 1% Triton X-100 and 100 μ l of acid-washed glass beads with a diameter of 106 μ m. The cells were shaken vigorously in a Vortex mixer for 1 min and incubated at room temperature for 8 min. After this incubation, 50 μ l of mixture was placed in a microtube with 100 μ l of lysis buffer

(equal amounts of 15% Chelex 100, 0.5% Tween 20, and Tris-EDTA). The mixture was heated to 100°C for 20 min and shaken vigorously in a vortex mixer for 1 min, followed by centrifugation at 13,000 \times g for 8 min. The supernatant was then transferred to a microtube and stored at 4°C.

Amplification and digestion of *hsp65* fragments were performed as described by Telenti et al. (7), and the resulting restriction digest pattern was compared to those in the PRASite database (<http://app.chuv.ch/prasite/index.html>). The full *rpoB* gene was amplified using 20 primers (see Table S1 in the supplemental material), and the 16S rRNA gene was amplified using the MicroSEQ full-gene 16S rRNA gene PCR kit (Life Technologies). All sequences obtained were compared on the basis of similarity to those in the GenBank database by using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The *hsp65* 441-bp fragment, the almost complete 16S rRNA gene, and the full *rpoB* gene sequences of strain HF1629 (the older of the two isolates) were used to construct a concatenated neighbor-joining tree with MEGA version 5 (2, 6).

Cleavage of the *hsp65* PCR product obtained from strains HF1629 and HF1836 with BstEII produced fragments of about 230, 130, and 80 bp, and with HaeIII, the digestion resulted in fragments of about 125, 105, 80, 40, 35 and 25 bp (see Fig. S1 in the supplemental material). The closest match in the PRASite database was the pattern of *M. celatum* type 2 (BstEII, 235, 130, and 85 bp; HaeIII, 130, 105, and 80 bp) with a score of 9, but very distinct from *M. celatum* type 1 (BstEII, 235 and 210 bp; HaeIII, 130, 80, and 60 bp). Thus, the isolates were initially identified as *M. celatum* type 2, because the *M. kyorinense* profile was not available in

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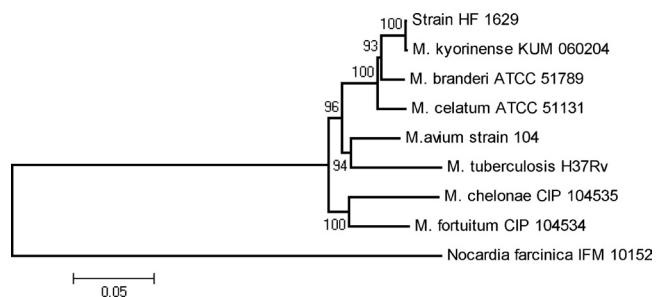


FIG 1 Phylogenetic tree computed from the concatenation of 16S rRNA gene, *rpoB*, and *hsp65* sequences by the neighbor-joining method and with Kimura's two-parameter model as the substitution model. The significance of branches is indicated by bootstrap values calculated for 1,000 replicates. *Nocardia farcinica* sequence was used as an outgroup.

PRAsite. To remedy this, the novel profile has been deposited in the PRAsite database.

Sequences of *hsp65*, *rpoB*, and 16S rRNA genes from HF1629 and HF1836 were obtained to further explore the identity of the isolates. First, the sequences of these three genes were identical in HF1629 and HF1836, indicating that those two isolates belong to the same species. A BLAST search showed that the *hsp65* 441-bp fragment is 100% identical to the *M. kyorinense* type strain KUM060204 sequence. Alignment of the full *rpoB* sequence from the Japanese and Brazilian isolates (unpublished data) showed them to be 100% identical. The 16S rRNA gene sequence displaying the highest identity score was that of *M. kyorinense* type strain KUM060204, with 4 mismatches (99.73%). *M. celatum* type strain ATCC 51131 showed 98.28% identity (25 mismatches in 1,450 bases), and *M. branderi* type strain ATCC 51789 showed 97.9% identity (31 mismatches in 1,475 bases). The concatenated phylogenetic tree revealed that the HF1629 strain was tightly clustered (with 100% bootstrap value) to the type strain of *M. kyorinense*, but distinct from those of *M. celatum* and *M. branderi* or all other sequences tested (Fig. 1).

To complete the identification of HF1629 and HF1836 as *M. kyorinense*, we carried out microbiological and biochemical tests. Isolates presented slow growth, and pigment was not observed. The biochemical results agree with those obtained for *M. kyorinense* type strain KUM060204 (see Table S2 in the supplemental material). The arylsulfatase activity and tellurite reduction assays distinguish *M. kyorinense* from *M. celatum*, and the heat-stable catalase test allows *M. kyorinense* to be distinguished from *M. branderi* (1, 4, 5, 8).

The results of molecular, microbiological, and biochemical

analyses of two Brazilian mycobacterial isolates from a patient with lung disease have allowed their identification as *M. kyorinense*. Comparison of genetic similarity and phylogenetic analysis show that these isolates are very closely related to *M. kyorinense*, with only a slight difference from the 16S rRNA gene sequence of this species' type strain. Thus, all results confirm the identification of our isolates as *M. kyorinense*.

As we used the American Thoracic Society (ATS) microbiologic criteria that are important to make a diagnosis of nontuberculous mycobacterial (NTM) lung disease and there are *M. kyorinense* disease reports previously reported in Japan, we speculate that *M. kyorinense* infection is also the cause of the pulmonary deterioration in the Brazilian patient.

Nucleotide sequence accession numbers. The 16S rRNA, *hsp65*, and *rpoB* gene sequences from the oldest isolate of this study have been deposited in GenBank for strain HF1629 under accession no. [JN634643](#), [JN974461](#), and [JQ744020](#), respectively. All accession numbers of sequences retrieved from GenBank are available in the supplemental material.

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