Infection due to a novel mycobacterium, mimicking multidrug-resistant *Mycobacterium tuberculosis*

E. Tortoli[1], P. G. Rogasi[2], E. Fantoni[2], C. Beltrami[2], A. De Francisci[3] and A. Mariottini[4]

1) *Regional Reference Centre for Mycobacteria,* 2) *Infectious Diseases Unit,* 3) *Radiodiagnostics Unit and* 4) *Cytogenetics and Genetics Laboratory, Careggi University Hospital, Florence, Italy*

Abstract

The treatment of multidrug-resistant tuberculosis (TB) requires the use, for long periods, of drugs liable to cause significant side effects. In the case of misdiagnosis of multidrug-resistant TB, the patient is exposed to toxic substances without any benefit. In low-income countries, where the microbiological diagnosis of TB relies on microscopy only, the misdiagnosis of multidrug-resistant TB is very frequent in patients persistently smear-positive despite anti-TB treatment, with the possibility of an infection due to non-tuberculous mycobacteria (NTM) being neglected. The isolation of a mycobacterium from the sputum of a Somali patient apparently confirmed the previous diagnosis of cavitary pulmonary disease. Preliminary investigations led, at first, to the strain being identified as multidrug-resistant *Mycobacterium tuberculosis*, with findings fully in agreement with the patient's history, which was characterized by repeated interruptions of anti-TB treatment. Thorough phenotypic and genotypic analyses led subsequently to the recognition that the strain was a previously unreported non-tuberculous mycobacterium. The patient, who was unresponsive to the anti-TB treatment, dramatically improved once a drug combination active against NTM was used. A major objective of this article is to alert the medical community to the risk, present also in settings in which sophisticated diagnostic techniques are used, that a cavitary infection due to NTM, and consequently not responding to the anti-TB standard regimen, will be mistaken for multidrug-resistant TB.

Keywords: Case report, line probe assay, multidrug-resistant tuberculosis, *Mycobacterium simulans*, non-tuberculous mycobacteria
Original Submission: 20 August 2009; **Revised Submission:** 2 September 2009; **Accepted:** 14 September 2009
Editor: M. Drancourt
Article published online: 14 October 2009
Clin Microbiol Infect 2010; **16**: 1130–1134
10.1111/j.1469-0691.2009.03063.x

Corresponding author and reprint requests: E. Tortoli, Regional Reference Centre for Mycobacteria, Piastra dei Servizi, A. O. U. Careggi, viale Morgagni 85, 50134 Firenze, Italy
E-mail: e.tortoli@libero.it

Introduction

A belief that is still held by infectious diseases physicians and microbiologists is that the incidence of non-tuberculous mycobacteria (NTM) is increasing in countries with low tuberculosis (TB) endemicity, whereas it is close to zero in developing countries characterized by a high TB burden. It is much more likely that the increased isolation of NTM in the developed world is a consequence of the advanced diagnostic technologies that can be afforded, and the apparent absence of NTM in developing countries is imputable to the very limited use of culture for diagnosis in the large majority of such settings [1]. The legitimacy of the latter hypothesis is confirmed by a number of studies reporting the isolation of NTM in Africa once sensitive cultural methods had been adopted [2–5].

We report here the case of a African patient who was repeatedly, and unsuccessfully, treated for pulmonary TB, and who rapidly improved once the non-tuberculous aetiology was acknowledged and suitable therapy was introduced.

Case report

A 62-year-old Somali man was admitted to the Infectious Diseases Unit because of a long-standing history of low-grade fever, weakness, weight loss, dyspnoea and productive cough. He had joined his son and his daughter in Italy 2 months previously. His medical history included malaria, approximately 40 years before, and pulmonary TB. The diagnosis of TB had been made in Somalia, when the patient was 58 years of age; he carried no medical records, but reported that he had

©2009 The Authors
Journal Compilation ©2009 European Society of Clinical Microbiology and Infectious Diseases

TABLE 1. Susceptibility testing pattern of FI-09026 obtained using the proportion method in liquid MGIT medium and the broth microdilution for the determination of the MICs

Drugs	Proportion method	MIC (mg/L) (interpretation)
Amikacin	S	8 (S)
Ciprofloxacin		2 (I)
Clarithromycin		0.5 (S)
Ethambutol	S	4 (I)
Gatifloxacin		0.5 (S)
Linezolid	S	0.5 (S)
Minocycline		4 (I)
Moxifloxacin	S	0.5 (S)
Rifabutin	R	0.25 (S)
Rifampicin	R	4 (I)
Streptomycin	R	>64 (R)
Trimethoprim–sulphamethoxazole		4–76 (R)
Capreomycin	S	
Ethionamide	S	
Isoniazid	R	
Kanamycin	R	
Pyrazinamide	S	

been given chemotherapy. Although he was unable to provide details about the drugs he had been taking, he reported at least five interruptions to the therapy because of the war in progress in his country.

Computed tomography showed extensive bilateral lesions, consistent with the diagnosis of pulmonary TB. This diagnosis, apparently supported by sputum smears strongly positive for acid-fast bacilli and by the first culture data, led to standard treatment with isoniazid, rifampicin, pyrazinamide and ethambutol. After 4 weeks of such treatment, which was well tolerated, the results of the susceptibility testing (Table 1) led to discontinuation of rifampicin, isoniazid and pyrazinamide (the susceptibility test result for the last of these was not yet available) and the addition of amikacin, ethionamide, moxifloxacin and linezolid to ethambutol [6].

The general condition of the patient rapidly improved and, again, the treatment was well tolerated. Three weeks later, the sputum smears became negative and the patient was discharged. Following the final recognition of the strain as a non-tuberculous mycobacterium, clarithromycin (shown, in the meantime, to be effective *in vitro*) was substituted for linezolid because of the high risk of toxicity associated with prolonged treatment with the latter drug. Two months later, amikacin was discontinued. The patient is now receiving clinical follow-up, and his biochemical and microbiological parameters are being checked regularly; continuation of the treatment is planned for at least 12 months.

Radiology

The chest computed tomography scan showed marked retraction of the right lung, with deviation of the mediastinum and consolidation of the right pulmonary lobes. These showed lesions characteristic of tuberculous infection, with multiple cavitations in a breadcrumb-like pattern, and a large cavity, communicating with the bronchial tree, clearly visible in the upper lobe (Fig. 1). In the upper left lobe, parenchymal infiltrates with aerial bronchogram were present, and opacities in a 'tree in bud' pattern [7] affected the lingula (Fig. 2).

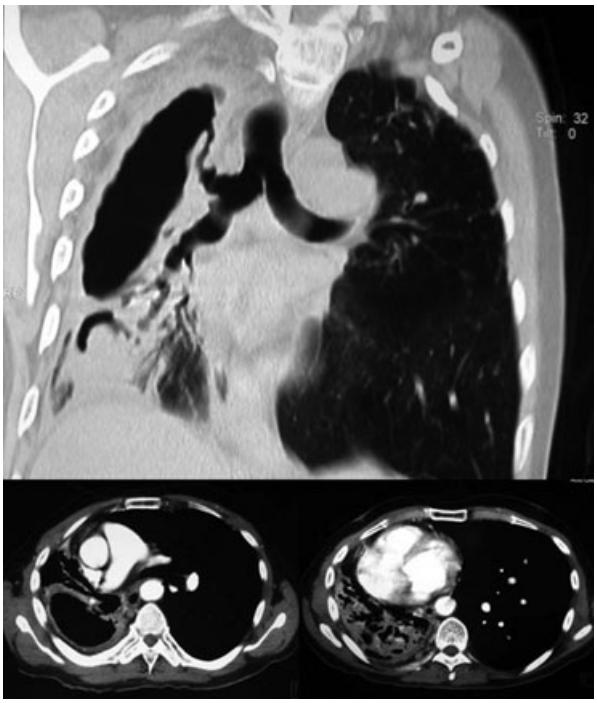

FIG. 1. Computed tomography scan showing, in the right lung, a large cavity communicating with the bronchial tree and tuberculous inflammation of the lobes with multiple cavitation.

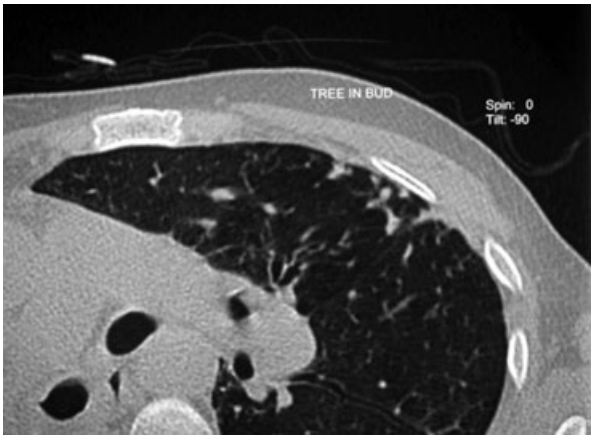

FIG. 2. Computed tomography scan showing, in the lingula (right lung), opacities in a 'tree in bud' pattern.

©2009 The Authors

Microbiology

Microbiological investigations were initially characterized by contradictory results. Acid-fast microscopy (auramine–rhodamine stain) revealed a heavy load of elongated and curved bacilli, in morphology fully compatible with *Mycobacterium tuberculosis*. Real-time PCR (COBAS TaqMan; Roche Diagnostics, Basel, Switzerland) for *M. tuberculosis* complex (MTBC), performed directly on the sputum, gave a negative result. Various cultures in liquid medium (MGIT; Becton Dickinson, Towson, MD, USA) [8] grew acid-fast bacilli in 5–9 days, and showed the corded arrangement typical of *M. tuberculosis*. On Lowenstein–Jensen medium, non-pigmented rough colonies, indistinguishable from *M. tuberculosis*, grew in approximately 20 days.

The identification of the strain (FI-09026) as belonging to the MTBC was apparently confirmed by the reverse hybridization line probe assay GenoType Mycobacterium CM (Hain Lifescience, Nehren, Germany) [9] targeting the 23S rDNA. The use of a further reverse hybridization test, GenoType MTBC (Hain Lifescience) [10], suitable for differentiating the species included in the MTBC, however, produced further confusion. Again, a positive result was obtained with the MTBC-specific line probe, but none of the nine *gyrB* stretches harbouring polymorphisms characterizing single MTBC species could be amplified.

Genetic investigation of mutations responsible for rifampicin and isoniazid resistance, performed using GenoType MTBDR*plus* (Hain Lifescience) [11], to verify the suspicion of multidrug-resistant TB, was not able to detect any, either mutant or wild type, of the specific targets (*rpoB*, *inhA* and *katG*). The test confirmed, however, the presence of a sequence of 23S rRNA specific for the MTBC.

Antimicrobial susceptibility testing was at first performed according to the proportion method (validated for *M. tuberculosis* strains only) in MGIT medium, using a wide panel of first-line and second-line drugs [12]. It revealed a pattern (Table 1) suggestive of multidrug-resistant TB.

When the MICs were determined using the microdilution method recommended by the CLSI for NTM [13], most of the second-line drugs, among them linezolid and amikacin, were shown to be effective (Table 1).

HPLC of cell wall mycolic acids, undertaken to try to resolve these identification uncertainties, revealed a pattern characterized by a single, late cluster of peaks, which differed from those obtained with *M. tuberculosis* [14] in the relative heights of several peaks (Fig. 3).

Despite the multiple indications that the strain belonged to the MTBC, several findings did not seem to support such a hypothesis. A genetic sequencing approach was therefore attempted to try to resolve the problem.

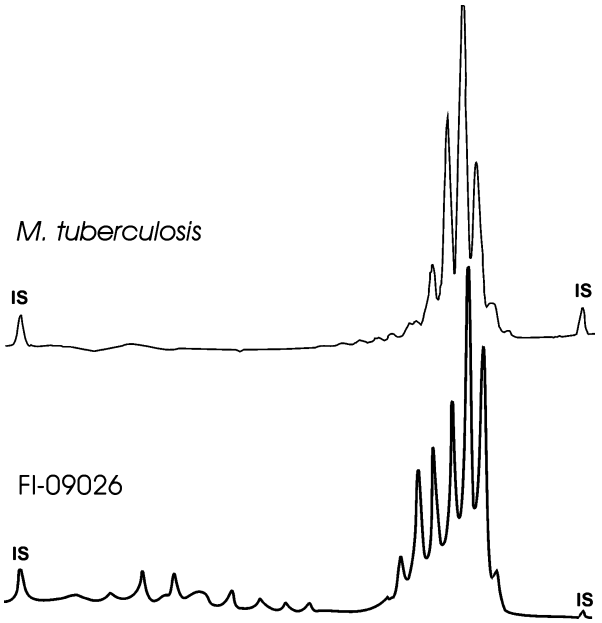

FIG. 3. Representative HPLC pattern of FI-09026 in comparison with that of *Mycobacterium tuberculosis* (IS, internal standard).

Double-strand sequencing was implemented using BigDye Terminator chemistry and an AB3730 DNA sequencer (Applied Biosystems, Foster City, CA, USA) on four genetic species markers of primary importance for the taxonomic and phylogenetic analysis of mycobacteria. In addition to the 16S rDNA gene, universally considered to be the first choice [15], the hypervariable region of the gene encoding for the 65-kDa heat shock protein (*hsp65*) [16] and the internal transcribed spacer (ITS) interposed between the 16S and the 23S rDNAs [17] were sequenced. A recently proposed fragment of the *rpoB* gene [18] was also investigated.

All of the sequences above turned out to be unique and more closely related to the recently described species *Mycobacterium riyadhense* [19]. In the almost complete sequence of the 16S rDNA of the current isolate, FI-09026 (GenBank accession number FJ786255), the similarity to the latter species was 99%, with nine mismatches in 1438 bp. In the hypervariable region of *hsp65* [20] (accession number FJ786253), there were 13 mismatches (in 423 bp; similarity 96%). In the ITS (accession number FJ786255), the presence of 13 mismatches in 278 bp was responsible for a similarity of 95%. In the 744-bp stretch of *rpoB* (FJ786254), *M. riyadhense* did not appear, at first, to be the closest species; however, once we realized that, for the latter species, this genetic region was not yet present in GenBank and we had determined such a sequence in the type strain (accession number FJ786256), *M. riyadhense* again turned out to present the closest similarity (95%, with 37 mismatches). In the same regions, the

©2009 The Authors
Journal Compilation ©2009 European Society of Clinical Microbiology and Infectious Diseases, *CMI*, **16**, 1130–1134

similarities with *M. tuberculosis* were clearly lower: 98% in the 16S rDNA, 86% in *hsp65*, 88% in the ITS, and 91% in *rpoB*.

Surprisingly, in the first part of the 23S rDNA (accession number FJ786255), which is known to include the target on which the GenoType identification probes rely, the similarity of FI-09066 to *M. tuberculosis* was also low (<97%).

The phylogenetic analysis conducted using the neighbour-joining algorithm (MEGA 4 software) [21] on the 1730-bp sequence, including the 16S rDNA and ITS, demonstrated the relatedness of FI-09026 and *M. riyadhense* (with the two strains belonging, however, to clearly distinct branches) and the sharp divergence from *M. tuberculosis* (Fig. 4).

Discussion

This is the second report of a mycobacterial strain, phenotypically mimicking *M. tuberculosis* but nevertheless clearly distinct from it, being responsible for severe disease that is indistinguishable from TB. The first case concerned a 19-year-old male with painful swelling of the left side of his face due to a tumour in the maxillary sinus extending into the nasal septum and the left orbit. Because of the isolation of a mycobacterium from lavage of maxillary sinuses, the patient was treated with a 9-month anti-TB regimen, which produced a clear improvement. A thorough characterization of the strain revealed it to be a non-tuberculous mycobacterium, and led to the description of the new species *M. riyadhense* [19].

Major features shared by FI-09026 and *M. riyadhense* include the bacillary morphology, the colony appearance, the misidentification as *M. tuberculosis* by GenoType CM, and the unquestionable pathogenicity. They differ, however, in antimicrobial susceptibility, both *in vitro* and *in vivo*: the infection due to *M. riyadhense* was successfully treated with standard anti-TB therapy that was ineffective against FI-09026.

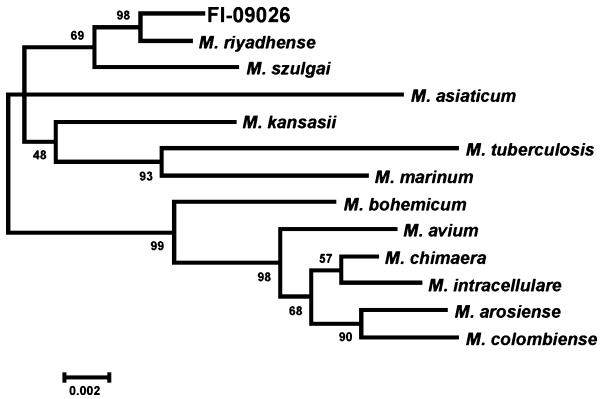

FIG. 4. Phylogenetic tree of selected *Mycobacterium* species based on sequences (1730 bp) of the ribosomal operon. The scale bar represents a 0.2% difference in nucleotide sequences. Bootstrap values are indicated at the nodes. FJ786255 is the accession number of the current strain (FI-09024).

The similarity of FI-09026 to *M. tuberculosis* is striking. Microscopically, FI-09026 is characterized by lengthened and curved bacillary morphology, similarly to *M. tuberculosis*. Likewise, colonies grown on solid media are rough and non-pigmented, similar to colonies of *M. tuberculosis*, and require comparable incubation periods to become visible to the naked eye. The arrangement of bacilli in liquid MGIT medium is characterized by the cord morphology that is considered to be a distinctive feature of *M. tuberculosis* [22]. The pattern of results of the main biochemical tests used for species identifications is very close (data not shown), with only the niacin result being discrepant: consistently positive in *M. tuberculosis* (but not in other species of the MTBC) and negative in FI-09026. The HPLC profile of cell wall mycolic acids did not allow unquestionable differentiation. The antimicrobial susceptibility is clearly different from that of *M. tuberculosis* but, paradoxically, the resistance of FI-09026 to the first-line anti-TB drugs, instead of suggesting a possible non-tuberculous mycobacterium, brought to mind a multidrug-resistant *M. tuberculosis* strain. The hybridization pattern obtained using the popular molecular identification system GenoType CM does not distinguish FI-09026 from the MTBC.

As a consequence of the above-mentioned features, the probability of misdiagnosis of multidrug-resistant TB is very high. This is true for low-income countries, in which the diagnosis of TB relies on smear microscopy only [23], but is equally true for a high proportion of laboratories in the developed world, including practically all those that base the identification of mycobacteria on methods other than genetic sequencing.

At present, we can only speculate about the real distribution of strains with the features of FI-09026. They may, indeed, not be very rare, and the major objective of this article is to alert the medical community to the possibility that some isolates considered to be multidrug-resistant *M. tuberculosis* may not actually be so.

Although we are unwilling to describe a new species until additional strains are detected, we propose, for the strain characterized here, the name '*Mycobacterium simulans*' (*simulans* meaning 'imitative'). The strain has been deposed in the German Collection of Microorganisms and Cell Cultures with the accession number DSM45395.

Transparency Declaration

No funding was used for this study, which was generated as part of routine activities. No commercial relationship or conflict of interest is expected for the present study.

©2009 The Authors

References

1. Tortoli E. Impact of genotypic studies on mycobacterial taxonomy: the new mycobacteria of the 1990s. *Clin Microbiol Rev* 2003; 16: 319–354.
2. Buijtels PC, Petit PL, Verbrugh HA *et al.* Isolation of nontuberculous mycobacteria in Zambia: eight case reports. *J Clin Microbiol* 2005; 43: 6020–6026.
3. Buijtels PC, Van Der Sande MA, De Graaff CS *et al.* Nontuberculous mycobacteria, Zambia. *Emerg Infect Dis* 2009; 15: 242–249.
4. Crump JA, Van Ingen J, Morrissey AB *et al.* Invasive disease caused by nontuberculous mycobacteria, Tanzania. *Emerg Infect Dis* 2009; 15: 53–55.
5. Bonard D, Messou E, Seyler C *et al.* High incidence of atypical mycobacteriosis in African HIV-infected adults with low CD4 cell counts: a 6-year cohort study in Côte d'Ivoire. *AIDS* 2004; 18: 1961–1964.
6. World Health Organization. *Guidelines for the programmatic management of drug-resistant tuberculosis*. Geneva: WHO, 2008.
7. Jeong YJ, Lee KS, Koh WJ *et al.* Nontuberculous mycobacterial pulmonary infection in immunocompetent patients: comparison of thin-section CT and histopathologic findings. *Radiology* 2004; 231: 880–886.
8. Badak FZ, Kiska DL, Setterquist S *et al.* Comparison of mycobacteria growth indicator tube with BACTEC 460 for detection and recovery of mycobacteria from clinical specimens. *J Clin Microbiol* 1996; 34: 2236–2239.
9. Russo C, Tortoli E, Menichella D. Evaluation of the new GenoType Mycobacterium assay for identification of mycobacterial species. *J Clin Microbiol* 2006; 44: 334–339.
10. Richter E, Weizenegger M, Rüsch-Gerdes S *et al.* Evaluation of GenoType MTBC assay for differentiation of clinical *Mycobacterium tuberculosis* complex isolates. *J Clin Microbiol* 2003; 41: 2672–2675.
11. Brossier F, Veziris N, Truffot-Pernot C *et al.* Performance of the genotype MTBDR line probe assay for detection of resistance to rifampin and isoniazid in strains of *Mycobacterium tuberculosis* with low- and high-level resistance. *J Clin Microbiol* 2006; 44: 3659–3664.
12. Rusch-Gerdes S, Pfyffer GE, Casal M *et al.* Multicenter laboratory validation of the BACTEC MGIT 960 technique for testing susceptibilities of *Mycobacterium tuberculosis* to classical second-line drugs and newer antimicrobials. *J Clin Microbiol* 2006; 44: 688–692.
13. National Committee on Clinical Laboratory Standards. *Susceptibility testing for mycobacteria, nocardiae and other aerobic actinomycetes; approved standard M24-A*. Wayne, PA: NCCLS, 2003.
14. Centers for Disease Control and Prevention. *Standardized method for HPLC identification of mycobacteria*. Atlanta, GA: US Department of Health and Human Services, Public Health Service, 1996.
15. Böttger EC. Approaches for identification of microorganisms. Despite longer experience with fatty acid profiles, DNA-based analysis offers several advantages. *ASM News* 1996; 62: 247–250.
16. McNabb A, Adie K, Rodrigues M *et al.* Direct identification of mycobacteria in primary liquid detection media by partial sequencing of the 65-kilodalton heat shock protein gene. *J Clin Microbiol* 2004; 44: 60–66.
17. Roth A, Fisher M, Hamid ME *et al.* Differentiation of phylogenetically related slowly growing mycobacteria based on 16S–23S rRNA gene internal transcribed spacer sequences. *J Clin Microbiol* 1998; 36: 139–147.
18. Adékambi T, Drancourt M. Dissection of phylogenetic relationships among 19 rapidly growing *Mycobacterium* species by 16S rRNA. *hsp65*, *sodA*, *recA*, and *rpoB* gene sequencing. *Int J Syst Evol Microbiol* 2004; 54: 2095–2105.
19. Van Ingen J, Al Hajoj SAM, Boere M *et al. Mycobacterium riyadhense* sp. nov.; a non-tuberculous species identified as *Mycobacterium tuberculosis* by a commercial line-probe assay. *Int J Syst Evol Microbiol* 2009; 59: 1049–1053.
20. Telenti A, Marchesi F, Balz M *et al.* Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. *J Clin Microbiol* 1993; 31: 175–178.
21. Tamura K, Dudley J, Nei M *et al.* MEGA 4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* 2007; 24: 1596–1599.
22. Yagupsky PV, Kaminski DA, Palmer KM *et al.* Cord formation in BACTEC 7H12 medium for rapid, presumptive identification of *Mycobacterium tuberculosis* complex. *J Clin Microbiol* 1990; 28: 1451–1453.
23. Tabarsi P, Baghaei P, Farnia P *et al.* Nontuberculous mycobacteria among patients who are suspected for multidrug-resistant tuberculosis—need for earlier identification of nontuberculosis mycobacteria. *Am J Med Sci* 2009; 337: 182–184.

©2009 The Authors
Journal Compilation ©2009 European Society of Clinical Microbiology and Infectious Diseases, *CMI*, **16**, 1130–1134