MYCOBACTERIUM THERMORESISTIBILE: CASE REPORT OF A RARELY ISOLATED MYCOBACTERIUM FROM EUROPE AND REVIEW OF LITERATURE

Mycobacterium thermoresistibile is a non-tuberculous mycobacterium strongly associated with human infections. Since 1966, there have only been six reports of its isolation from clinical samples. We report on the first case from Europe and review all the previous cases. Identification was achieved with sequencing of the 16S rRNA and *hsp65* genes. This study presents its phenotypic and biochemical profile, susceptibilities to selected antibiotics and *hsp65* polymerase chain reaction–restriction fragment length polymorphism profile with *BsteII* and *Hae III*.

Key words: *hsp65, Mycobacterium thermoresistibile, non-tuberculous mycobacteria*

Although *Mycobacterium tuberculosis* is responsible for majority of the mycobacterial infections worldwide, many non-tuberculous mycobacteria (NTM) are also of medical relevance. Over the past four decades, more than 120 mycobacterial species have been described. A growing number of NTM isolates, often of newly described species, are submitted to laboratories for identification.[1] Members of the *M. avium* complex, *M. chelonae, M. abscessus, M. fortuitum, M. kansasii* and *M. xenopi*, have been considered responsible for most of the NTM infections like pulmonary diseases, lymphadenopathy and skin and soft tissue lesions. Among NTM, rapidly growing mycobacteria (RGM) are those which show visible growth on solid culture media within seven days.

M. thermoresistibile is an RGM that was first recovered from soil samples in Japan by Tsukamura in 1966.[2] Of the commonly known species, it is closely related to *M. phlei* and, as its name points out, has the ability to grow at 52°C. *M. thermoresistibile* has been strongly related with human infections.[3,4] However, it is rarely isolated. Since 1966, there have been only six reports of *M. thermoresistibile* isolations from human clinical samples.

We report on the isolation of *M. thermoresistibile* from a sputum culture of a patient from the island of Crete, Greece, with chronic obstructive pulmonary disease (COPD), diabetes and purpura. To our knowledge, this is the first report of *M. thermoresistibile* isolation from clinical samples in Europe. Identification was achieved with sequencing. Its biochemical profile and susceptibilities to selected antibiotics are reported and a concise review of previous cases is presented. The polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP) profile of *hsp65* using *BstEII* and *HaeIII* is also presented.

Case Report

A 67-year-old male, human immunodeficiency virus negative, who was a heavy smoker (one pack/day for 30 years) and had a history of COPD, type II respiratory distress syndrome, diverticulosis and diabetes was presented to our hospital with fever (38°C), productive cough, dyspnea, weakness and acute purpura. The chest radiograph revealed an elevated cardiothoracic ratio as well as chronic obstructive lung disease, peribronchial infiltrations, consolidations in the right middle and lower lung zones and a small blunt at the left pneumodiaphragmatic angle. The Mantoux test was positive (30 mm) and suggestive of latent tuberculosis.

Apart from cultures for common bacteria, a sputum sample was sent to our laboratory for mycobacterial culture. The sample was treated according to standard procedures.[5] Two Lowenstein–Jensen (LJ) slants were used for solid culture (one at room temperature and one at 37°C) and an MB/BacT/Alert 3D Automated System (bioMerieux, Durham, NC, USA) was used for liquid culture at 37°C. Acid fast staining was negative. The Amplified *Mycobacterium tuberculosis* Direct test (Gen-Probe, San Diego, CA, USA) performed on the clinical sample was negative. After 15 days of incubation, the LJ slant at 37°C showed growth of an acid-fast coccobacillus. Nine days later, growth was detected in the liquid culture. The Accuprobe *M. tuberculosis* complex assay (Gen-Probe) was negative, revealing the presence of an NTM. Accuprobe also yielded negative results for the *M. avium* complex and *M. gordonae*. A series of further biochemical tests were performed according to standard guidelines.[5] The strain was positive for pigmentation in the dark. No growth was obtained at 20°C whereas the mycobacterium grew at 37 and 45°C. The nitrate reductase, iron uptake, arylsulphatase (14 days) and β-galactosidase tests were negative whereas the catalase (greater than 45 mm and 68°C) and urease tests were positive. No growth on the McConkey agar was obtained at 37°C whereas the mycobacterium grew on LJ supplemented with thiophene-2-carboxylic hydrazide, on blood sheep agar and on Middlebrook 7H11 supplemented with 5% NaCl at 37°C. Because no clear-cut identification was achieved, the two commercial kits of GenoType CM and GenoType AS (Hain, Lifescience, Nehren, Germany) based on DNA strip technology for the molecular identification of NTM were used following the

manufacturer's instructions. The banding patterns obtained were not species-specific (GenoType CM: 1, 2, 3 and 10 and GenoType AS: 1, 2, 3 and 12). The GenoType results ruled out the presence of *M. phlei*.

DNA from the isolate was extracted using a guanidinium thiocyanate lysis buffer, as described previously.[6] A small number of mycobacterial colonies were suspended in 600 µl of lysis buffer (guanidinium thiocyanate 4 M, 0.5% N-lauryl sarcosine, 1 mM dithiothreitol, 25 mM sodium citrate and 50 µg of glycogen) and incubated overnight at room temperature. A total of 600 µl of ice-cold (-20°C) isopropyl alcohol was then added. After 1.5 h, the sample was centrifuged for 10 min (16,600 g at 4°C). Isopropyl alcohol was removed and the pellet was washed with 70% ethanol. The dried pellet was dissolved in 50 µl of DNAse and Rnase-free double-distilled sterile water.

A 1869-bp product containing the full length of the 16S rRNA gene and 16S–23S internal transcribed region (ITS) was amplified in an automated DNA sequencer (3730 DNA analyzer; Applied Biosystem Inc, Foster City, CA, USA) using the Big Dye terminator sequencing kit (Applied Biosystems) and the primers F16S, R16S and ITS2, as previously described.[7,8] The sequence was aligned with the sequences of the Ribosomal Differentiation of Medical Microorganisms (www.ridom.de) and GenBank (www.ncbi.nlm.nih.gov) databases.[9] It showed 100% similarity (429/429 identities) with the 16S rRNA gene of *M. thermoresistibile* DSM 44167 and 99% similarity (1398/1409 identities) with the 16S rRNA sequence of the *M. thermoresistibile* strain ATCC 19527 (X55602).

Moreover, a 439-bp fragment of the 65-kDa heat shock protein (*hsp65*) gene was amplified using the protocol and primers Tb11 and TB12 as previously described.[10] The sequence was compared with other published mycobacterial sequences in the GenBank database. It showed 99% similarity (438/439 identities) with the *hsp65* sequence of the *M. thermoresistibile* strain CIP105390 (AF547880) and 99% similarity (437/439 identities) with the *M. thermoresistibile* strain ATCC 19527 (AY299162).

The 16S rRNA-ITS and *hsp65* gene sequences have been deposited in GenBank with the accession numbers FJ236481 and FJ236482, respectively.

The PCR product of *hsp65* was further used for RFLP analysis. The PCR product (12 µl) was digested with *Hae III* (New England Biolabs Inc, Ipswich, MA, UK) and *BsteII* (New England Biolabs) and the restriction mixture was run on 3.5% metaphor agarose gel (Cambrex Bioscience Inc, Baltimore, MD, USA) at 100 V. The *BstEII* digestion produced two fragments of 235 and 210 bp and the *HaeIII* digestion produced four fragments of 180, 135, 70 and 50 bp [Figure 1].

Figure 1: Agarose gel electrophoresis of fragments produced by digestion of polymerase chain reaction amplification products of the *hsp65* gene with *BstEII* (lane B) and *HaeIII* (lane H). Lane L: GeneRuler 50 bp DNA ladder (Fermentas)

Susceptibility testing of *M. thermoresistibile* performed by the E-test methodology (AB Biodisk, Solna, Sweden) according to the manufacturer's instructions and results were evaluated as previously described for RGM.[11] The antibiotics tested and the minimum inhibitory concentrations obtained (in parenthesis) were: amikacin (0.064 µg/ml; AN), cefoxitin (1.0 µg/ml; FOX), ciprofloxacin (0.032 µg/ml; CIP), clarithromycin (0.023 µg/ml; CLA), doxycycline (0.064 µg/ml; DOX), ethionamid (256.0 µg/ml; ETN), imipenem (0.064 µg/ml; IMP), levofloxacin (0.016 µg/ml; LEV), ofloxacin (0.032 µg/ml; OFL), trimethoprim–sulfomethoxazol (0.064 µg/ml; SXT) and tobramycin (1.0 µg/ml; TOB). According to available breakpoints, susceptibility was found for AN, FOX, CIP, CLA, DOX, IMP and SXT.[11]

As the American Thoracic Society (ATS) criteria were not met, the presence of *M. thermoresistibile* was considered colonization.[12] Because the culture for common bacteria were also negative, empirical treatment for COPD exacerbation (ciprofloxacin and cefuroxime) was administered with clinical improvement.

Discussion

Weitzman *et al*, reported the isolation of *M. thermoresistibile* in a middle-aged patient, with weight

loss, cough, fever and small cavities on his chest X-ray.[3] *M. thermoresistibile* was considered pathogenic for the respiratory tract infection. Notably, the patient had visited hot springs in Hawaii before developing the clinical symptoms. Drug susceptibility testing showed that the strain was sensitive to rifampicin (RMP), ethambutol (ETH), streptomycin (10 μg; STR) and resistant to isoniazid (INH), para-amino-salicylic acid (PAS), STR (two μg) and ETN. Therapy with RMP, ETH and STR led to substantial improvement.

In 1984, Liu *et al*, reported a *M. thermoresistibile* infection in an immunocompromised host with hypogammaglobulinaemia.[4] The symptoms included cough and purulent nasal drainage, with the chest X-ray showing a left upper lobe pulmonary nodule. An open lung biopsy of the nodule was performed and the culture revealed the presence of a mycobacterium. Based on the phenotypic and biochemical profile, the strain was identified as *M. thermoresistibile*. Susceptibility testing showed sensitivity to RMP, ETH, STR and kanamycin and resistance to INH and PAS. A favourable therapeutic result was obtained with RMP, ETH and STR.

In the third case, *M. thermoresistibile* was responsible for an infection near the surgical scar of a diabetic patient who had undergone a cardiac transplantation three months earlier.[13] A slow response to the drugs given (RMP, ETH and INH) was noticed.

Wolfe and Moore reported on the formation of breast abscesses by *M. thermoresistibile* following augmentation mammaplasty.[14] Sixteen months of therapy were needed by the patient for a full recovery. A modification of the proportional susceptibility test was suggested by the authors. Their antimicrobial test showed susceptibility to AN, CIP, DOX, RMP, ETH, STR, caproemycin and tetracycline and resistance to ETN, PAS, INH and OFL.

A coinfection of *M. thermoresistibile* and *M. fortuitum* was described by Cummings *et al*.[15] It concerned a patient with a slowly expanded cutaneous lesion developed after an injury with garden shears. Identification was achieved with high-pressure liquid chromatography (HPLC). After three months of therapy with levofloxacin and DOX, the skin lesion was completely resolved.

LaBombadi *et al*, reported on a *M. thermoresistibile* infection following knee-replacement surgery.[16] The infection was masked by prior isolation of a vancomycin-resistant enterococcus. *M. thermoresistibile* was identified with biochemical tests and HPLC. Clinical improvement was achieved after seven months of therapy with MOX and linezolid (later replaced by DOX).

Discussion

To our knowledge, the present case represents the first report of *M. thermoresistibile* isolation from a clinical sample in Europe and one of the few worldwide. Over the last 42 years, it has been reported only six times, with all the cases coming from the United States of America. In the previous cases, *M. thermoresistibile* was related to either pulmonary or dermal infections. Although *M. thermoresistibile* is considered pathogenic, it is rarely isolated from clinical samples. A probable reason for this is the improper culturing conditions. Usually, the laboratories do not incubate samples at temperatures greater than 40°C, which is considered to be the optimal temperature for *M. thermoresistibile*. On the contrary, in cases of cutaneous specimens, laboratories usually incubate specimens at 30°C on media capable of growing *M. haemophilum* as well as other pathogens typically associated with skin infections.[16] Another reason for its rare isolation is that identifying *M. thermoresistibile*, based on its phenotypic and biochemical profile, is difficult as it shares common features with other mycobacteria such as *M. phlei, M. flavescens* or *M. gordonae* (not an RGM).[14,15] Moreover, the currently available commercial diagnostic kits do not include species-specific probes for *M. thermoresistibile*.[17]

One of the greatest difficulties concerning NTM is whether the isolation of an NTM is merely colonization or cause of the disease. A majority of the physicians adhere to the ATS criteria for their decision. Nevertheless, it is not usually clear whether colonization or a low-grade infection occurs.[18] Moreover, exposure to environmental mycobacteria is not considered innocent, especially in patients with chronic pulmonary diseases. Primm *et al*, noted that hypersensitivity pneumonitis results from inflammatory products released by mycobacteria, not necessarily in the process of an infection.[19] Griffith *et al*, reported that in pulmonary diseases with a parallel isolation of *M. abscessus* (which is another RGM), a true colonization does not exist and patterns with minimal symptoms have minimal disease.[20] In our case, the isolation of *M. thermoresistibile* was considered colonization on the basis of the ATS criteria. However, the treatment given included ciprofloxacin, to which *M. thermoresistibile* was susceptible. Consequently, clinical improvement was achieved.

M. thermoresistibile is strongly related to pulmonary and dermal diseases. When samples for mycobacterial cultures are associated with pulmonary or skin infections, incubation at 42°C should be performed. Furthermore, the use of molecular techniques such as sequencing and PCR–RFLP analysis to identify uncommon mycobacteria is strongly advocated.

References

1. Neonakis IK, Gitti Z, Kourbeti IS, Michelaki H, Baritaki M, Alevraki G, *et al*. Mycobacterial species diversity at a general hospital on the island of Crete: First detection of

Mycobacterium lentiflavum in Greece. Scand J Infect Dis 2007;39:875-9.
2. Tsukamura M. Adansonian classification of mycobacteria. J Gen Microb 1966;45:253-73.
3. Weitzman I, Osadczyi D, Corrado ML, Karp D. *Mycobacterium thermoresistibile*: A new pathogen for humans. J Clin Microbiol 1981;14:593-5.
4. Liu F, Andrews D, Wright DN. *Mycobacterium thermoresistibile* infection in an immunocompromised host. J Clin Microbiol 1984;19:546-7.
5. Koneman E, Allen S, Janda W, Schreckenberger P, Winn W. Colour atlas and textbook of Diagnostic Microbiology. Philadelphia: Lippincott; 1997.
6. Casas I, Klapper PE, Cleator GM, Echevarria JE, Tenorio A, Echevarria JM. Two different PCR assays to detect enteroviral RNA in CSF samples from patients with acute aseptic meningitis. J Med Virol 1995;47:378-5.
7. Hiraishi A. Direct automated sequencing of 16SrDNA amplified by polymerase chain reaction from bacterial cultures without DNA purification. Lett Appl Microbiol 1992;15:210-3.
8. Richter E, Niemann S, Rüsch-Gerdes S, Hoffner S. Identification of *Mycobacterium kansasii* by using a DNA probe (AccuProbe) and molecular techniques. J Clin Microbiol 1999;37:964-70.
9. Harmsen D, Rothganger J, Frosch M, Albert J. RIDOM: Ribosomal Differentiation of Medical Microorganisms Database. Nucleic Acids Res 2002;30:416-7.
10. Telenti A, Marchesi F, Balz M, Bally F, Bottger EC, Bodmer T. Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. J.Clin Microbiol 1993;31:175-8.
11. Woods GL, Bergmann JS, Witebsky FG, Fahle GA, Boulet B, Plaunt M, *et al*. Multisite reproducibility of Etest for susceptibility testing of *Mycobacterium abscessus*, *Mycobacterium chelonae* and *Mycobacterium fortuitum*. J Clin Microbiol 2000;38:656-1.
12. American Thoracic Society. Diagnosis and treatment of disease caused by nontuberculous mycobacteria. Am J Respir Crit Care Med 1997;156:S1-25.
13. Neeley SP, Denning DW. Cutaneous *Mycobacterium thermoresistibile* infection in a heart transplant recipient. Rev Infect Dis 1989;11:608-11.
14. Wolfe JM, Moore DF. Isolation of *Mycobacterium thermoresistibile* following augmentation mammaplasty. J Clin Microbiol 1992;30:1036-8.
15. Cummings GH, Natarajan S, Dewitt CC, Gardner TL, Garces MC. *Mycobacterium thermoresistibile* recovered from a cutaneous lesion in an otherwise healthy individual. Clin Infect Dis 2000;31:816-7.
16. LaBombardi VJ, Shastry L, Tischler H. *Mycobacterium thermoresistibile* infection following knee-replacement surgery. J Clin Microbiol 2005;43:5393-4.
17. Neonakis IK, Gitti Z, Krambovitis E, Spandidos DA. Molecular diagnostic tools in mycobacteriology. J Microbiol Methods 2008;75:1-11.
18. Catanzaro A. Diagnosis, differentiating colonization, infection, and disease. Clin Chest Med 2002;23:599-601.
19. Primm TP, Lucero CA, Falkinham JO 3rd. Health impacts of environmental mycobacteria. Clin Microbiol Rev 2004;17:98-106.
20. Griffith DE, Girard WM, Wallace RJ Jr. Clinical features of pulmonary disease caused by rapidly growing mycobacteria: An analysis of 154 patients. Am Rev Respir Dis 1993;147:1271-8.

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