Short Communication

Report of Two Cases of *Mycobacterium europaeum* from Iran

Fazel Pourahmad^{1*}, Hasan Shojaei², Parvin Heidarieh³,
Azardokht Khosravi³, and Abodolrazagh Hashemi³

¹*School of Veterinary Medicine, Ilam University, Ilam;*

²*Department of Microbiology, School of Medicine, Isfahan University of
Medical Sciences, Isfahan; and*

³*Department of Microbiology, School of Infectious and Tropical Diseases,
Jundishapur University of Medical Sciences, Ahvaz, Iran*

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SUMMARY: Herein, we report repeated isolation of *Mycobacterium europaeum* from the sputum samples of an Iranian human immunodeficiency virus-infected patient and a cystic fibrosis patient with chronic pulmonary disease. To our knowledge, this is the first isolation of *M. europaeum* from human clinical specimens in Asia to be reported.

Mycobacterium europaeum, a scotochromogenic species related to the *Mycobacterium simiae* complex, has only recently been described as a novel *Mycobacterium* sp. based on 5 irrelevant clinical isolates from Europe (1). The present study describes 2 independent cases of *M. europaeum* infection diagnosed using phenotypic and molecular methods. To our knowledge, this is the first report of isolation and identification of this species from Asia, which may provide further evidence of the clinical relevance of this newly characterized species.

Case 1: A 48-year-old man with a history of human immunodeficiency virus (HIV) infection and intravenous drug use was admitted to hospital because of high temperature and cough. HIV infection had been diagnosed 1 year prior to admission when he presented with fever, weight loss, cough, dyspnea, and chest pain. His CD4 lymphocyte count was 18 cells/mm³.

Upon admission, he appeared severely ill, with a productive cough and fever of 39.6°C. His tuberculin test was negative. Other laboratory testing revealed lymphopenia, an elevated C-reactive protein level of 33 mg/l, an erythrocyte sedimentation rate of 48 mm/h, and an HIV viral load of 300 copies/ml. He was negative for hepatitis B virus and hepatitis C virus antigens. Direct smear examination of sputum revealed the presence of acid-fast bacilli (AFB), and sputum culture revealed the formation of slow-growing colonies on Löwenstein-Jensen (LJ) medium after 2 weeks' incubation at 37°C. Based on these laboratory results and the clinical findings, the patient was administered anti-mycobacterial therapy.

After 3 months, the patient was referred back to our hospital because of fever and cough. He reported taking his anti-mycobacterial medications for only 2 weeks after he was discharged from hospital. A second sputum examination was positive for AFB, and as with the previous laboratory tests, slow-growing colonies were observed on LJ medium.

The initial and subsequent isolates were respectively designated as AFP-0001 and AFP-0002. These isolates were submitted to our laboratory for identification and antimicrobial susceptibility testing. The morphology of the colonies, their pigment production, and the niacin test led to presumptive identification as non-tuberculosis *Mycobacterium* sp. Therefore, the patient was treated with amikacin (15 mg/kg body weight/day), and he made a good recovery and improved markedly.

Case 2: Strain AFP-0008 was isolated in 2009 in pure culture from 3 different sputum samples from a 13-year-old Iranian girl with chronic pulmonary disease. She had a history of weight loss, persistent cough, and repeated lung and sinus infections, and had been diagnosed with cystic fibrosis from the identification of mutations in p.F508del (Δ F508) of the cystic fibrosis transmembrane conductance regulator (CFTR) gene.

On admission, physical examination revealed weight loss and wheezing. For a few months, she had been exhibiting signs of chronic pulmonary disease with episodes of intermediate fever. A series of culture examinations from throat swabs and sputum samples led to the isolation of *Pseudomonas aeruginosa*. The patient was therefore treated with several courses of antibiotics, including tobramycin and ceftazidime, without any clinical improvement.

Three separate expectorated sputum samples analyzed for mycobacterial infection yielded 3 pure cultures of slow-growing mycobacteria after 18 days' incubation at 37°C. Accordingly, the patient was administered anti-mycobacterial drugs, including isoniazid, rifampicin, kanamycin, and ethambutol, for 5 months. The efficacy of the treatment was confirmed by negative results for AFB staining and culture, as well as clinical recovery.

The isolates AFP-0001, AFP-0002, and AFP-0008 were phenotypically characterized by the following analyses: acid-fast staining, growth at 37°C and 42°C on LJ medium, growth on MacConkey agar without crystal violet, pigment production, arylsulfatase test, nitrate reduction, tolerance to 5% NaCl, iron uptake, urease activity, tellurite reduction, semi-quantitative and heat-stable (68°C) catalase test, and niacin production, according to standard procedures described previ-

*Corresponding author: Mailing address: School of Veterinary Medicine, Ilam University, Ilam, Iran. Tel & Fax: +98 841 2224308, E-mail: f.pourahmad@ilam.ac.ir

ously (2).

The susceptibility of the isolates to common antimycobacterial agents was determined by the microdilution method in accordance with the methodology of the Clinical and Laboratory Standards Institute (CLSI).

Molecular identification included the use of PCR amplification followed by direct sequence analyses of almost full-length 16S rRNA (3,4), partial sequences of *hsp65* (5), *rpoB* (6), and the 16S-23S internal transcribed spacer (ITS) region (7), following the protocols described in those publications. A concatenated phylogenetic tree was constructed by neighbor-joining clustering, which included the sequences aligned to the sequences obtained from related *Mycobacterium* spp. (8,9).

The isolates grew on LJ medium, producing yellow-pigmented scotochromogenic colonies after 2 weeks' incubation at 37°C. The isolates, however, did not grow on LJ medium incubated at 42°C, MacConkey agar without crystal violet, or LJ medium containing 5% NaCl.

The isolates were positive for the semi-quantitative and heat-stable (68°C) catalase and tellurite tests, but negative for Tween hydrolysis, arylsulfatase activity in 3 days, nitrate reduction, urease activity, niacin production, and iron uptake.

The isolates were susceptible to amikacin, clarithromycin, doxycycline, sulfamethoxazole, rifampicin, streptomycin, and isoniazid, and were resistant only to ethambutol.

The sequences obtained were compared with published sequences in the GenBank database using the BLASTN algorithm (<http://www.ncbi.nlm.nih.gov/blast>). The almost complete 16S rRNA gene sequence (1,441 bp) of isolates AFP-0002 and AFP-0008 exhibited 100% identity with those of *M. europaeum* DSM45397^T (GenBank accession no. HM022196) and 99.6% identity (6-bp mismatches) with *Mycobacterium parascrofulaceum* ATCC BAA-614^T (GenBank acces-

sion no. GQ153273), while the 16S rRNA sequence of isolate AFP-0001 also exhibited the greatest similarity with those aforementioned species (99.9% with 2-bp mismatches and 99.4% with 8-bp mismatches, respectively) over the same length.

The identical sequences of the isolates' ITS (311 bp), *hsp65* (597 bp), and *rpoB* (702 bp) genes exhibited 98.4%, 99.7%, and 99.7% similarity to those of *M. europaeum* DSM45397^T (GenBank accession nos. HM022196, HM022220, and HM022215, respectively). These values corresponded to 5, 1, and 2 nucleotide differences over 311-bp, 361-bp, and 680-bp sequences, respectively. The relationship between our isolates and *M. europaeum* was supported by the concatenated phylogenetic tree of the 16S rRNA, *hsp65*, and *rpoB* genes (2,532 bp in total), and by the high bootstrap value obtained using the neighbor-joining method (Fig. 1).

M. europaeum, a newly identified member of the *M. simiae* complex, was recently detected by polyphasic analyses of 5 independent strains from various European countries. It was described as an acid-fast, Gram-positive, slow-growing, and yellow-pigmented species (1).

Based on the paraclinical manifestations, recovery of several identical isolates from independently obtained sputum samples, and the results from combinations of conventional and molecular methods, we concluded that the causative agent in both cases was *M. europaeum*. Noticeably, the application of molecular techniques including sequence analyses of the 16S rRNA, *hsp65*, *rpoB*, and ITS regions yielded complete or near-complete identity, confirming species identification.

The majority of cases of *M. simiae* complex infection reported in the literature have been in immunocompromised patients, in whom disseminated disease can occur, primarily with respiratory and reticuloendothelial system involvement (10,11). The Iranian HIV-infected patient described here presented with pulmonary in-

Fig. 1. Phylogenetic positioning of the Iranian isolates of *M. europaeum* and their closest related species based on concatenated 16S rRNA, *hsp65*, and *rpoB* gene sequences by the neighbor-joining method and Kimura's two-parameter model. The *M. tuberculosis* (ATCC 27294) sequence was used as the outgroup. Bootstrap values were calculated from 1,000 replicates. Bootstrap values $\geq 70\%$ are stated at the nodes. The scale bar indicates the estimated number of substitutions per 100 bases.

fection. Two of 4 *M. europaeum* strains (no information was available for the fifth) reported by Tortoli et al. were also isolated from patients with respiratory tract diseases (1). Further studies are required to determine whether *M. europaeum* is responsible for infection in immunocompetent individuals, although it can be presumed that this colonizing organism has the potential to cause pulmonary infection.

The present report describes 2 independent clinical cases where disease was attributed to *M. europaeum*, consistent with the descriptions reported in the scarce literature on the clinical significance of pulmonary disease caused by this organism.

The GenBank/EMBL accession numbers for the 16S rRNA gene sequence of isolates AFP-0001 and AFP-0002 are FR686461–2, those for the ITS region are FR686464–5, those for the *hsp65* gene are FR682914–5, and those for the *rpoB* gene are FR695851–2, respectively.

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Conflict of interest None to declare.

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