Mycobacterium szulgai identification by *hsp*65 gene sequencing in an HIV-positive patient with non-Hodgkin's lymphoma

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SUMMARY

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Mycobacterium szulgai, described for the first time in 1972, is a rare human pathogen that mainly causes pulmonary non-tubercular mycobacteriosis. We report its isolation and identification from a bronchoalveolar lavage specimen by *hsp65* gene sequencing analysis in an HIV-positive patient with non-Hodgkin's lymphoma.

KEY WORDS: Mycobacterium szulgai, INNO-LiPA, sequence analysis

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CASE REPORT

A 49-year-old homeless male smoker with a history of alcohol abuse was admitted to the Department of Infectious Diseases of our hospital. The patient had an HIV-positive partner with whom he had unprotected sex. On admission, the patient was afebrile. Abdominal examination disclosed hepatomegaly and abdomen tenderness in the epigastrium and in the right hypochondrium. Chest radiograph was normal. HIV serology testing yielded a positive result. The CD4 cell count showed a severe immunodeficiency (28 cells/µl), while HIV-RNA was 78,637 copies/ml. Highly active antiretroviral therapy (HAART) was then administered, with lamivudine, zidovudine, and lopinavir/ritonavir. On day 2 after admission, a gastroscopy with biopsy was performed, revealing large non-Hodgkin lymphoma (NHL) cells. Total body CT scan showed multiple minute pulmonary nodules, small bilateral pleural effusion, large mediastinal and mesenteric lymph nodes. Bronchoalveolar lavage (BAL) was performed and showed no detectable atypical cells or acid-fast bacilli by auramine-rhodamine staining. The patient was treated using a modified CHOP-like chemotherapy scheme (cyclophosphamide, adriblastin, vincristine and prednisone) combined with rituximab (anti-CD20 monoclonal antibody). Granulocyte colony stimulating factor (GCSF), HAART and opportunistic infection prophylaxis were administered concomitantly. After 12 days of treatment with prednisone, the drug was discontinued. Over the subsequent 24 hours, high fever recurred and chest radiography showed a nodular lesion in the middle lobe and air-space disease in the peri-hilar right region. A CT chest scan was performed again and disclosed several ill-defined nodular masses and widespread honeycomb interstitial pattern. Given the worsening clinical condition associated with strong thrombocytopenia, bron-

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choscopy was avoided. Due to a strong suspicion of pulmonary aspergillosis, despite the fact that Aspergillus galactomannan antigen in serum twice tested negative, intravenous voriconazole (200 mg bid IV, after the loading dose) was administered. The patient showed rapid cognitive decline and meningismus, associated with left-sided weakness mainly involving his left arm. On days 22 and 24, the liquid and solid culture of BAL for mycobacterial growth both became positive. The INNO-LiPA test showed positivity for the presence of *Mycobacterium* spp. A DNA. A lumbar puncture was performed on day 37 and CSF analysis was normal: no microorganisms were identified on Gram and auramine-rhodamine stains. The patient's clinical condition continued to deteriorate and he died 44 days after admission to the hospital. At this stage, sequence analysis conducted on DNA extracted from liquid positive culture identified the mycobacterium as *M. szulgai*.

Auramine-rhodamine staining on a BAL specimen was negative for the presence of mycobacteria. BAL cultures were performed on liquid medium (MIGIT- Mycobacteria Growth Indicator Media, Becton Dickinson, Atlanta, GA) and on Löwenstein-Jensen solid medium according to the standard procedure. For the growth characteristics study, the isolate was incubated at 37°C and 25°C, and daily checked for microbial growth for 7 days and every three days thereafter. Moderate growth of scotochromogenic and smooth colonies was noted after three weeks of incubation at 37°C; whilst at 25°C the colonies appeared photochromogenic. Moreover, no growth was observed at 45°C. Identification of the isolated microrganism was attempted by conventional biochemical tests (Nolte and Metchock, 1995) and by cellular fatty acid gas-chromatography (Luquin *et al.*, 1991). Urease, nitrate reduction, catalase (semiquantitative and at 68°C), Tween 80 hydrolysis, inhibition by TCH (thiophene-2-carboxylic acid hydrazide), thiacetazone and isoniazide tests were positive. Niacin accumulation, NaCl tolerance, growth on MacConkey without crystal violet and reduction tellurite tests were negative. The gas-chromatographic pattern presented two main peaks (C 16:0, 22% and C 18:1, 19%); other fatty acids were also detected (3 to 6%): C 14:0, C 16:1, C 18:0 and TBSA. Both biochemical tests and gas-chromatographic pattern failed to yield a definitive result. For these reasons, we continued investigation by molecular biology, using DNA-DNA hybridization tests for simultaneous detection of different mycobacterial species (INNO-LiPA v.2, Innogenetics, Ghent, Belgium). The INNO-LiPA assay was unable to identify the mycobaterium species, revealing only *Mycobacterium* spp. To further investigate the isolate, an aliquot of purified DNA was used for *hsp65* gene sequence analysis, using two oligonucleotide primers (Tb11 and Tb12) to amplify a 439-bp fragment of the mycobacterial *hsp65* gene (Ringuet *et al.*, 1999; Telenti *et al.*, 1993). The PCR product of *hsp65* was sequenced with a Big Dye terminator sequencing kit (Applied Biosystems, Foster City, CA). The alignment of sequence of the isolated strain was compared with those of other mycobacterial species in the GenBank and BIBI database (http://pbil.univ-lyon1.fr), showing concordance with three *M. szulgai* strains: AF547878 (420/421 bases) and AF434731 (399/401 bases) 99%; strain AJ307654 (385/390 bases) 98%. With the other Mycobacteria species the observed homology was lower than 96%. Figure 1 reports the phylogenetic tree obtained with hsp65 sequences of the sample under investigation and those of reference chromogen slow-grower mycobacteria (ClustalW software).

Since the first description of *Mycobacterium szulgai* in 1972 (Marks *et al.*, 1972), few cases of infection have been described. The first case in Italy was reported in 1998 in a patient with pulmonary mycobacteriosis (Tortoli *et al.*, 1998). This mycobacterium is a rare human pathogen that mainly causes pulmonary non-tubercular mycobacteriosis. The main risk factors for pulmonary infection appear to be chronic obstructive pulmonary pathologies, as well as alcohol abuse and smoking (Falkinham *et al.*, 1996). Only a few cases of extrapulmonary diseases have been reported: e.g. olecranon bursitis, skin infections, cervical adenitis, osteomyelitis and renal disease. Several of these cases involved immunocompromised patients. Two cases of infection have been observed in patients with carpal tunnel syndrome by isolating the Mycobacterium in synovial culture (Horusitzky *et al.*, 2000). More recently, a case of *M. szulgai* causing septic arthritis was reported in a patient with immunodeficiency virus (Hakawi *et al.*, 2005).

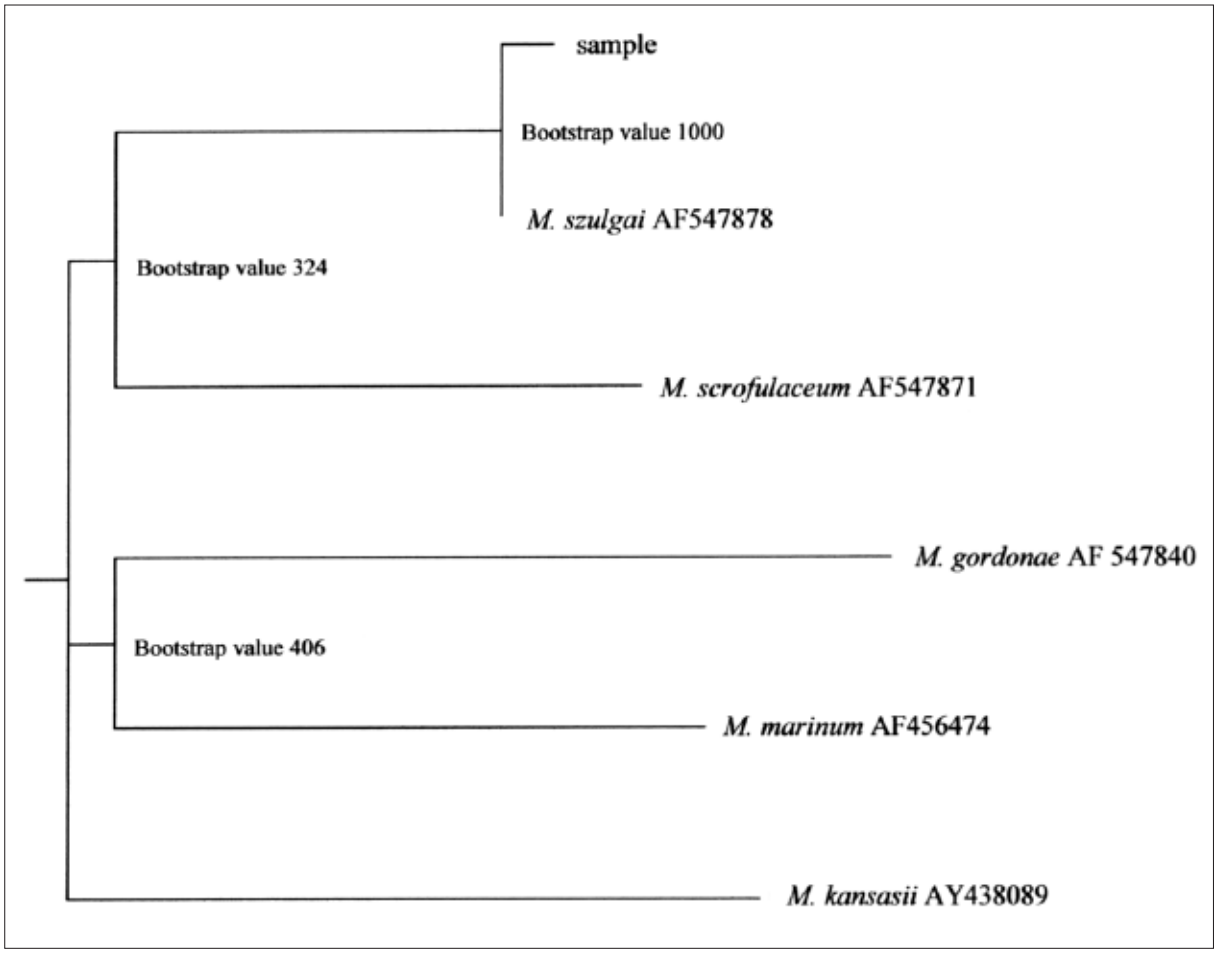

FIGURE 1 - *Phylogenetic tree obtained using hsp65 sequences of the clinical isolate and the reference chromogen slow-grower mycobacteria.*

Accurate identification of mycobacteria is an important step in patient management, which affects diagnosis and may also influence the choice of suitable treatment. *M. szulgai* identification, however, is difficult to perform on the basis of conventional biochemical and cultural tests. A characteristic feature of *M. szulgai* is its unique pigmentation: it is scotochromogen (producing pigment in both light and darkness) at 37 °C but photochromogen (forming pigment only after exposure to light) at 25°C. Some isolates from clinical specimens, although identified as *M. szulgai* by molecular analysis, were atypical in phenotypic terms because non pigmented (Zhang *et al*., 2002).

Several new identification procedures have been developed in recent years. Molecular methods such as PCR-based techniques and DNA probes have gradually become useful tools for mycobacterial differentiation. The commercial INNO-LiPA assay used in this study is easy to perform and is less prone to methodological variation and misinterpretation than conventional procedures. It has the potential to provide more accurate identification, especially for isolates with atypical phenotypic profiles (Tortoli *et al*., 2003). However, the kits based on this technology are set up only to recognize specific mycobacterium species, i.e. usually the most common mycobacteria occurring in clinical samples. This is the case of the INNO-LiPA assay, as it can identify a range of mycobacteria which (among others) do not include *M. szulgai*, though it is able to disclose mycobacteria at genus level. To overcome this

problem, other molecular approaches have been suggested, such as sequence analysis of specific genes (i.e. rRNA 16S, *hsp65*, *rpob*, *sod* and *gyrb*). The resulting sequence can be easily compared in the GeneBank database for accurate identification. However, such methods require adequate experience and specific instruments which are not always available in clinical laboratories.

In the case reported here, the reliance on phenotypic and biochemical characteristics alone predicted only that the isolated bacteria could be a slow-growing scotochromogenic Mycobacterium. The INNO-LiPA assay established positivity for *Mycobacterium spp*. but only *hsp65* gene sequencing allowed us to identify the strain as *M. szulgai*. Our study emphasizes the importance of combining conventional methods with molecular techniques for accurate identification.

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