Case Report

Detection of mixed infections with *Mycobacterium lentiflavum* and *Mycobacterium avium* by molecular genotyping methods

Philip Suffys,¹ Adalgiza da Silva Rocha,¹ Adeilton Brandão,² Bart Vanderborght,³ Wouter Mijs,⁴ Geert Jannes,⁴ Fernanda C. Q. Mello,³ Heloisa da Silveira Paro Pedro,⁵ Leila de Souza Fonseca,⁶ Rosângela Siqueira de Oliveira,⁷ Sylvia Cardoso Leão⁷ and Maria Helena Féres Saad¹

Correspondence

Philip Suffys

psuffys@ioc.fiocruz.br

^{1,2}Department of Mycobacterioses¹ and Department of Tropical Medicine², Oswaldo Cruz Institute, Fiocruz, Rio de Janeiro, Brazil

^{3,6}University Hospital Clementino Fraga Filho³ and Microbiology Institute⁶, Federal University of Rio de Janeiro (UFRJ), Rio de Janeiro, Brazil

⁴Innogenetics N. V., Technologiepark 6, B-9052 Ghent, Belgium

⁵Adolfo Lutz Institute, São Paulo, Brazil

⁷Department of Microbiology, Immunology and Parasitology, Paulista School of Medicine, São Paulo, Brazil

Received 29 June 2005

Accepted 19 September 2005

Three mycobacterial isolates, one from the blood of an HIV-infected patient and two consecutive isolates from a woman with unknown HIV status, had been identified as belonging to the *Mycobacterium avium* complex by conventional procedures. In both patients, using genetic analysis procedures such as PCR–restriction enzyme analysis (PRA) of the *hsp65* gene, a commercially available reverse hybridization-based assay (INNO-LiPA MYCOBACTERIA) and/or sequencing analysis of the 16S–23S internal transcribed spacer (ITS), the presence of *Mycobacterium lentiflavum* was also demonstrated. At the time of detection, both cases were also infected with *M. avium*, suggesting an underestimation of infection with *M. lentiflavum* and co-infection with different *Mycobacterium* species.

Introduction

Mycobacterium lentiflavum is a slowly growing acid-fast bacillus (AFB) that has biochemical characteristics identical to those of organisms belonging to the *Mycobacterium avium* complex (MAC) and mycolic acid and fatty acid chromatography patterns very similar to those of *Mycobacterium simiae*, so genetic analysis is necessary for conclusive identification (Springer *et al.*, 1996). This organism has been isolated from sterile clinical samples in Italy, Switzerland, Germany, France and Spain (Springer *et al.*, 1996; Tortoli *et al.*, 1997; Niobe *et al.*, 2001; Ibanez *et al.*, 2002) and from sputum samples in Brazil (da Silva Rocha *et al.*, 1999) and Italy (Molteni *et al.*, 2005) and, recently, cases of human disease have been reported, including chronic pulmonary disease (Molteni *et al.*, 2005), cervical lymphadenitis (Cabria *et al.*, 2002), liver abscess (Tortoli *et al.*, 2002) and fatal disseminated infection (Ibanez *et al.*,

2002). The main reservoir in the environment has not been firmly established, but organisms with *M. lentiflavum*-like 16S rRNA gene sequences were detected in soil samples from the UK and from France (Mendum *et al.*, 2000) and the species seems to be frequently present in drinking water distribution systems in Finland (Torvinen *et al.*, 2004). We here report the detection of a co-infection with *M. avium* and *M. lentiflavum* in the blood or lung of two different patients.

Methods

Case descriptions

Case 1. In December 1994, a 27-year-old Caucasian male was submitted to a renal biopsy at the Clementino Fraga Filho University Hospital in Rio de Janeiro and membranoproliferative glomerulonephritis type I was diagnosed. The individual was submitted to intravenous corticoid treatment and discharged from the hospital while continuing oral methylprednisone treatment that was gradually reduced until interruption in December 1995. By that time, the patient had developed lymphopenia (as shown by CD4 lymphocyte

Abbreviations: AFB, acid-fast bacillus; ITS, internal transcribed spacer; MAC, *Mycobacterium avium* complex; PRA, PCR–restriction enzyme analysis.

counts of 500 cells mm⁻³) that was held under control, but, in September 1996, he returned with weight loss, cough, fever and oral candidiasis. A chest X-ray revealed a diffuse reticulonodular infiltrate suggestive of active pulmonary disease. Sputum bacteriology showed no evidence of AFB, but pneumocystosis, a definitive marker of AIDS, was diagnosed and infection with HIV was confirmed. Treatment of pneumocystosis resulted in clinical improvement but, 3 weeks later, the patient returned with high fever and aqueous diarrhoea. A blood culture was performed and was positive for AFB. Upon treatment with streptomycin, sulphamethoxazole/trimethoprim and corticoid, the patient improved and was discharged from the hospital in October 1996. Two weeks later he returned to the hospital and received zidovudine and didanosine and was discharged with the recommendation to return for regular re-evaluation. The patient died a few months later in another hospital and no data are available on the exact cause of death.

Case 2. In 1996, a 64-year-old woman was diagnosed with tuberculosis in the State of Mato Grosso. Despite treatment for tuberculosis since February 1997, she presented to Public Health Care in Votuporanga, São Paulo, with cough, fever, coryza, weakness, lack of appetite, severe weight loss and dyspnoea. The X-ray showed bilateral thin-walled cavitations and sputum collection revealed AFB. She was therefore enrolled in 1998 at the 'Núcleo de Gestão Assistencial', a health care service. During the following 4 years, she was submitted to several treatment schemes for tuberculosis, including antibiotics such as clarithromycin, ethambutol, clofazimine, rifampicin and doxycycline. During this period, the patient did not show improvement but rather a gradual worsening of the clinical symptoms was observed with frequent positive bacilloscopy and mycobacterial cultures. In August 2002 she died of uterine cancer at 26 kg in weight.

Culture, conventional identification and drug susceptibility testing. AFB staining was performed by the Ziehl-Neelsen method. For case 1, a blood culture was performed using the lysis-centrifugation method (Fandinho *et al.*, 1997) and colonies of AFB were grown by incubation on Löwenstein-Jensen (LJ) medium. For case 2, sputum culture on LJ medium was performed after decontamination using the Petroff method and centrifugation (Kent & Kubica, 1985). Subculture was carried out on LJ medium and colonies were submitted to conventional identification (Kent & Kubica, 1985).

Genetic characterization. For genetic analysis, part of the sample was submitted to three consecutive cycles of snap freezing and boiling in 10 mM Tris/HCl, 1 mM EDTA and 1% Triton X-100. The INNO-LiPA MYCOBACTERIA assay is commercially available (Innogenetics) for detection of *Mycobacterium* and identification of members of the *Mycobacterium tuberculosis* (MTB) complex, *Mycobacterium kansasii*, *M. xenopi*, *M. goodii*, *M. avium*, *M. intracellulare*, *M. scrofulaceum* and *M. chelonae*, targeting the 16S-23S rRNA internal transcribed spacer (ITS) region (Miller *et al.*, 2000); this assay was evaluated in Brazil with excellent results (Suffys *et al.*, 2001). The PCR-restriction enzyme analysis (PRA) assay was originally described by Telenti *et al.* (1993) and is based on amplification of part of *hsp65* and restriction analysis with *Hae*III and *Bst*EII; evaluation of the assay in Brazil also demonstrated the straightforwardness of the method (da Silva Rocha *et al.*, 2002). Sequencing was performed on the amplified ITS that was used for INNO-LiPA using a QIAquick PCR purification kit (Qiagen) and sequenced using an ABI PRISM BigDye Terminator cycle sequencing ready reaction kit on an ABI PRISM 377 sequencer (Applied Biosystems). Sequence analysis was performed by comparison against a database present at Innogenetics and against GenBank using SEQED and FASTA of the Wisconsin Package (version 9.1; Genetics Computer Group). Some PCR systems for differentiation of *M. intracellulare* and *M. avium* were used: amplification of DT1 or DT6 is characteristic for

M. intracellulare and *M. avium*, respectively, according to Thierry *et al.* (1993), while the presence of the insertion sequence IS1245 is indicative of *M. avium* (Guerrero *et al.*, 1995). Because the two cases were investigated independently by different research groups, the methodologies performed on the isolates from the two patients were not identical.

Results

Case 1

Blood culture yielded small, pale-yellow, smooth-domed colonies of AFB after 28 days of incubation at 37 °C in LJ medium and the culture was identified as MAC by conventional biochemical methods (negative for Tween hydrolysis, nitrate reduction and urease and positive for catalase and tellurite reduction). Part of the sample was treated by heat shock and submitted to genetic characterization by PRA of part of the *hsp65* gene (Telenti *et al.*, 1993; da Silva Rocha *et al.*, 2002): no digestion with *Bst*EII and 145/125 bp fragments with *Hae*III were obtained (Fig. 1a). According to the literature, this pattern is identical to *M. lentiflavum* I (Springer *et al.*, 1996), but this pattern has also been described for *M. simiae* (da Silva Rocha *et al.*, 2002). The isolate was submitted to INNO-LiPA MYCOBACTERIA and hybridized with the *Mycobacterium* species probe but not with the probes specific for MAC, confirming that the isolate did not belong to this complex. Since the INNO-LiPA assay does not contain probes specific for *M. lentiflavum* or *M. simiae*, sequencing of the 16S-23S ITS was performed and the isolate was confirmed to be *M. lentiflavum*.

A subculture obtained from a small amount of the original bacterial mass in the tube containing LJ medium before submitting it to heat shock was grown in Middlebrook 7H9 and reanalysed by PRA. On this occasion, the *M. avium* II restriction digest was observed and no (not even weak) bands corresponding to the *M. lentiflavum* pattern were found.

Case 2

Among the 15 *Mycobacterium* cultures obtained from 1997 to 2001 on LJ medium, 12 were identified as MAC and 3 as *M. goodii* using conventional biochemical identification procedures (data not shown). Among these, two cultures, one obtained in June 1998 and the other in September 2000 and both identified as MAC, were submitted to molecular identification procedures. Both cultures were identified as *M. intracellulare* I by PRA; however, amplification results using primers from the *M. intracellulare*-specific DT1 fragment and the *M. avium*-specific DT6 and IS1245 fragments were contradictory. The first isolate was negative in the three PCR systems, while the more recent isolate was positive upon amplification of DT6 and IS1245; both results were therefore inconsistent with the identification as *M. intracellulare*.

For that reason, subcultures were prepared on 7H10-OADC plates and the molecular tests were repeated on isolated

Fig. 1. Ethidium-bromide-stained agarose gels showing PRA of the *hsp65* gene. (a) PRA patterns of isolates from Case 1. *Hae*III (lane 1) and *Bst*EII (2) digestion products of *M. lentiflavum* I and *Hae*III (3) and *Bst*EII (4) digestion products of *M. avium* II; lanes M contain a 50 bp ladder. (b) PRA patterns of isolated colonies of the second isolate of Case 2, including *Bst*EII of *M. avium* II (lanes 1 and 3) and of *M. lentiflavum* III (2) and *Hae*III of *M. avium* II (4 and 6) and of *M. lentiflavum* III (5); lane M contains a 50 bp ladder and lane m contains a 25 bp DNA ladder.

colonies. In the second isolate, a mixed population containing colonies with two different morphologies was observed; PRA analysis resulted in the *M. lentiflavum* III pattern for the small yellow colonies, while the pale colonies were identified as *M. avium* II. This confirms the presence of a mixed infection with two different species and explains the earlier results obtained using amplification systems for DT1, DT6 and IS1245. On the other hand, analysis of the earlier isolated culture yielded small yellow colonies only and PRA patterns were all *M. lentiflavum* III.

Discussion

In contrast to *M. avium*, a species isolated frequently from the blood of HIV-infected individuals, isolation of *M. lentiflavum* from blood was described only recently (Niobe *et al.*, 2001). Previous reports describe isolates from

contaminated bronchoscope, gastric juice and urine samples (Springer *et al.*, 1996), lymph nodes (Tortoli *et al.*, 1997; Haase *et al.*, 1997), bronchoalveolar lavage fluid and, very recently, sputum (Molteni *et al.*, 2005); the present report is to our knowledge the second to demonstrate detection of *M. lentiflavum* in Brazil. Infection with MAC is frequent in Brazilian AIDS patients (Barreto *et al.*, 1993) and because routine identification procedures do not differentiate some *Mycobacterium* species (including *M. lentiflavum*) from MAC, use of genetic methods for identification on a more routine basis would give a better idea of the prevalence of such species in this country.

Niobe *et al.* (2001) published the ITS sequences of a clinical isolate of *M. lentiflavum* and of the type strain ATCC 51985 and, although the 16S rRNA gene sequence of the clinical isolate had 100 % identity to the sequence of the type strain, the ITS sequence of the clinical isolate had only 92.6 % identity to that of strain ATCC 51985, suggesting the need to study ITS variability in this species. The ITS sequence of one *M. lentiflavum* isolate described here was identical to that published by Niobe *et al.* (2001), as was the case for another isolate obtained from a sputum sample of an HIV-positive individual from Rio de Janeiro (data not shown). This suggests that the ITS sequence variability, at least in Brazil, is limited or non-existent while, interestingly, the isolates presented different PRA patterns (Springer *et al.*, 1996). Bearing in mind that the whole ITS sequence was evaluated while restriction enzyme analysis only demonstrates creation or destruction of restriction sites, this finding suggests that mutation in the two genes seems to occur at different rates.

In both patients, mixed mycobacterial populations were present upon genetic analysis. Although an *M. lentiflavum* pattern was observed in the original sample from the first patient, *M. avium* was observed exclusively in the sample that was obtained after culturing of a fraction of that sample, suggesting the selection of *M. avium* either by fractionating the sample during collection or because of the faster growth of *M. avium*; *M. lentiflavum* has been reported to be less virulent than *M. intracellulare* in mice (Saito *et al.*, 2000), and this needs to be further investigated. This suggests the importance of submitting identical fractions to different identification procedures and considering analysis of isolated colonies, as confirmed by the results of characterization of separate colonies of the second isolate of case 2. Besides observing different colony morphology using this strategy, two genotypes belonging to different species were observed.

In the initial identification, both isolates of the second patient were identified as *M. intracellulare* by PRA. Comparing PRA patterns of *M. intracellulare* and *M. lentiflavum* III, the *Bst*EII fragments are indistinguishable and there is a one-band difference upon *Hae*III digestion, a 60 bp band in *M. intracellulare* I that is absent from *M. lentiflavum* III. Failure to observe the 60 bp band or the appearance of the 60 bp band from *M. avium* could have led to this

misinterpretation (Fig. 1b); the influence of such bands on correct pattern interpretation has been observed before (da Silva Rocha *et al.*, 1999; Leão *et al.*, 2005).

Sequencing also confirmed that the isolates were actually *M. lentiflavum* rather than *M. intracellulare*. In our earlier study, the PRA pattern *M. lentiflavum* III was also observed in about half of the *M. intracellulare* isolates, adding one more identifying pattern for the latter species (da Silva Rocha *et al.*, 1999). The fact that these two species that are hard to separate by conventional identification and have the same PRA type co-exist in Brazil demonstrates the need to use different genotype assays for some organisms. Also, *M. lentiflavum* was present in cultures from material taken with a 2 year interval, showing that this species is more than a mere colonizer.

Prior to the HIV era, mixed mycobacterial infections consisted mainly of *M. tuberculosis* and some atypical mycobacteria. However due to immune suppression, simultaneous infection with different atypical *Mycobacterium* species is now more frequent; polyclonal infection of *M. avium* strains has been reported in AIDS patients in Brazil (Oliveira *et al.*, 2000; Saad *et al.*, 2000). So far, simultaneous infection of *M. avium* and *M. lentiflavum* has not been reported, probably because the two species are phenotypically indistinguishable. Although we suspect that Case 2 could initially have been infected with *M. avium* (and *M. gordonae*), superinfection with *M. lentiflavum* probably occurred when the patient was being treated for cancer. Infection with *M. lentiflavum* has been diagnosed in the lung of a woman that had been treated with corticoids because of suspicion of lung cancer (Galarraga *et al.*, 2002), but this association is difficult to confirm here because not all isolates have been submitted to genetic analysis. Because of the lack of clinical data and the presence of different mycobacterial species, it was not possible to associate the presence of *M. lentiflavum* with the pathological states of the patients, but both disseminated disease (Niobe *et al.*, 2001) and chronic pulmonary disease (Oliveira *et al.*, 2000) caused by *M. lentiflavum* have been reported. Although *M. lentiflavum* has been reported to be susceptible to several antibiotics used for treatment of infection with mycobacteria other than *M. tuberculosis* (MOTT) (Niobe *et al.*, 2001; Molteni *et al.*, 2005), the clinical significance of susceptibility testing of these organisms has not been fully validated and complicates decisions on proper treatment of infection with *M. lentiflavum*.

The fact that *M. lentiflavum* has been reported in particular situations as one of the more frequently isolated mycobacteria not associated with disease (Idigoras *et al.*, 2004), has been isolated from drinking water (Torvinen *et al.*, 2004) and can lead to fatal disseminated disease highlights the importance of the correct identification of this species. Molecular identification tools can aid in characterization and thus in better understanding of the epidemiology of this species.

Acknowledgements

This study was supported by CNPq (PRONEX 661028/1998-4), an International Collaborations in Disease Research (ICIDR) grant and FAPESP 98/11746-1. R.S.de O. was supported by CAPES and A. da S. R. by Faperj. We thank Lucilaine Ferrazoli for patient record information and Dr Lee Riley for his thoughts on existing but not yet apparent infectious agents.

References

- Barreto, J. A., Palaci, M., Ferrazoli, L. & 7 other authors (1993). Isolation of *Mycobacterium avium* complex from bone marrow aspirates of AIDS patients in Brazil. *J Infect Dis* **168**, 777–779.
- Cabria, F., Torres, M. V., Garcia-Cia, J. I., Dominguez-Garrido, M. N., Esteban, J. & Jimenez, M. S. (2002). Cervical lymphadenitis caused by *Mycobacterium lentiflavum*. *Pediatr Infect Dis J* **21**, 574–575.
- da Silva Rocha, A., da Costa Leite, C., Torres, H. M., de Miranda, A. B., Pires Lopes, M. Q., Degraive, W. M. & Suffys, P. N. (1999). Use of PCR-restriction fragment length polymorphism analysis of the *hsp65* gene for rapid identification of mycobacteria in Brazil. *J Microbiol Methods* **37**, 223–229.
- da Silva Rocha, A., Werneck Barreto, A. M., Dias Campos, C. E., Villas-Bôas da Silva, M., Fonseca, L., Saad, M. H., Degraive, W. M. & Suffys, P. N. (2002). Novel allelic variants of mycobacteria isolated in Brazil as determined by PCR-restriction enzyme analysis of *hsp65*. *J Clin Microbiol* **40**, 4191–4196.
- Fandinho, F. C., Grinsztejn, B., Veloso, V. G., Lourenço, M. C. S., Werneck-Barroso, E., João, E., Nogueira, A. S. & de Souza Fonseca, L. (1997). Diagnosis of disseminated mycobacterial infection: testing a simple and inexpensive method for use in developing countries. *Bull World Health Organ* **75**, 361–366.
- Galarraga, M. C., Torreblanca, A. & Jimenez, M. S. (2002). Isolation of *Mycobacterium lentiflavum* in a case of suspected lung cancer. *Enferm Infecc Microbiol Clin* **20**, 93–94 (in Spanish).
- Guerrero, C., Bernasconi, C., Burki, D., Bodmer, T. & Telenti, A. (1995). A novel insertion element from *Mycobacterium avium*, IS1245, is a specific target for analysis of strain relatedness. *J Clin Microbiol* **33**, 304–307.
- Haase, G., Kentrup, H., Skopnik, H., Springer, B. & Bottger, E. C. (1997). *Mycobacterium lentiflavum*: an etiologic agent of cervical lymphadenitis. *Clin Infect Dis* **25**, 1245–1246.
- Ibanez, R., Serrano Heranz, R., Jimenez-Palop, M., Roman, C., Corteguera, M. & Jimenez, S. (2002). Disseminated infection caused by slow-growing *Mycobacterium lentiflavum*. *Eur J Clin Microbiol Infect Dis* **21**, 691–692.
- Idigoras, P., Beristain, X. & Jimenez-Pajares, M. S. (2004). Clinically insignificant *Mycobacterium lentiflavum* isolation in 30 patients. *Enferm Infecc Microbiol Clin* **22**, 199–200 (in Spanish).
- Kent, P. T. & Kubica, G. P. (1985). *Public Health Mycobacteriology. A Guide for the Level III Laboratory*. Atlanta: Centers for Disease Control.
- Leão, S. C., Bernardelli, A., Cataldi, A. & 23 other authors (2005). Multicenter evaluation of mycobacteria identification by PCR restriction enzyme analysis in laboratories from Latin America and the Caribbean. *J Microbiol Methods* **61**, 193–199.
- Mendum, T. A., Chilima, B. Z. & Hirsch, P. R. (2000). The PCR amplification of non-tuberculous mycobacterial 16S rRNA sequences from soil. *FEMS Microbiol Lett* **185**, 189–192.
- Miller, N., Infante, S. & Cleary, T. (2000). Evaluation of the LiPA MYCOBACTERIA assay for identification of mycobacterial species from BACTEC 12B bottles. *J Clin Microbiol* **38**, 1915–1919.

- Molteni, C., Gazzola, L., Cesari, M., Lombardi, A., Salerno, F., Tortoli, E., Codecasa, F., Penati, V., Franzetti, F. & Gori, A. (2005). *Mycobacterium lentiflavum* infection in immunocompetent patients. *Emerg Infect Dis* 11, 119–122.
- Niobe, S. N., Bebear, C. M., Clerc, M., Pellegrin, J.-L., Bebear, C. & Maugein, J. (2001). Disseminated *Mycobacterium lentiflavum* infection in a human immunodeficiency virus-infected patient. *J Clin Microbiol* 39, 2030–2032.
- Oliveira, R. S., Sircili, M. P., Ueki, S. Y. M., Telles, M. A. S., Schnabel, B., Briones, M. R. S. & Leão, S. C. (2000). PCR-restriction enzyme analysis of a bone marrow isolate from a human immunodeficiency virus-positive patient discloses polyclonal infection with two *Mycobacterium avium* strains. *J Clin Microbiol* 38, 4643–4645.
- Saad, M. H., Telles, M. A., Porfirio, F., Ferrazoli, L., de Souza Fonseca, L., Johnson, W., Jr & Riley, L. W. (2000). Multiple isolates from aids patients: aspects of an analysis by a genotypic marker and microbial susceptibilities variations. *Mem Inst Oswaldo Cruz* 95, 729–732.
- Saito, H., Murakami, K., Yajima, M., Ishii, N. & Kwon, H. H. (2000). The virulence for mice of newly described mycobacterial species. *Kekkaku* 75, 65–69 (in Japanese).
- Springer, B., Wu, W.-K., Bodmer, T. & 10 other authors (1996). Isolation and characterization of a unique group of slowly growing mycobacteria: description of *Mycobacterium lentiflavum* sp. nov. *J Clin Microbiol* 34, 1100–1107.
- Suffys, P. N., da Silva Rocha, A., de Oliveira, M. & 9 other authors (2001). Rapid identification of mycobacteria to the species level using INNO-LiPA Mycobacteria, a reverse hybridization assay. *J Clin Microbiol* 39, 4477–4482.
- Telenti, A., Marchesi, F., Balz, M., Bally, F., Bottger, E. & Bodmer, T. (1993). Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. *J Clin Microbiol* 31, 175–178.
- Thierry, D., Vincent, V., Clement, F. & Guesdon, J. L. (1993). Isolation of specific DNA fragments of *Mycobacterium avium* and their possible use in diagnosis. *J Clin Microbiol* 31, 1048–1054.
- Tortoli, E., Piersimoni, C., Kirschner, P. & 10 other authors (1997). Characterization of mycobacterial isolates phylogenetically related to, but different from *Mycobacterium simiae*. *J Clin Microbiol* 35, 697–702.
- Tortoli, E., Bartoloni, A., Erba, M. L., Levrè, E., Lombardi, N., Mantella, A. & Mecocci, L. (2002). Human infections due to *Mycobacterium lentiflavum*. *J Clin Microbiol* 40, 728–729.
- Torvinen, E., Suomalainen, S., Lehtola, M. J., Miettinen, I. T., Zacheus, O., Paulin, L., Katila, I. T. & Martikainen, P. J. (2004). Mycobacteria in water and loose deposits of drinking water distribution systems in Finland. *Appl Environ Microbiol* 70, 1973–1981.