NOTE

***Mycobacterium lacus* sp. nov., a novel slowly growing, non-chromogenic clinical isolate**Christine Turenne,¹ Pamela Chedore,² Joyce Wolfe,¹ Frances Jamieson,² George Broukhanski,² Kevin May² and Amin Kabani^{1,3}¹ National Reference Centre for Mycobacteriology, National Microbiology Laboratory, Population and Public Health Branch, Health Canada, Winnipeg, Manitoba, Canada R3E 3R2² Tuberculosis and Mycobacteriology Laboratory, Central Public Health Laboratory, Ontario Ministry of Health and Long Term Care, Etobicoke, Ontario, Canada M9P 3T1³ Dept of Medical Microbiology, University of Manitoba, Winnipeg, Manitoba, Canada

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A strain of a novel non-chromogenic mycobacterium was isolated from synovial tissue from a 68-year-old female with bursitis of her right elbow. The slowly growing strain had a unique PCR-restriction enzyme analysis (PRA) profile of the *hsp65* gene and 16S rRNA gene sequence in comparison with other mycobacterium species. The most closely related species, as determined by 16S rRNA gene sequence analysis, are *Mycobacterium malmoeense*, *Mycobacterium marinum*, *Mycobacterium ulcerans* and members of the *Mycobacterium tuberculosis* complex. The HPLC and biochemical profiles resembled those of *Mycobacterium gastri*, although differences were noted in the peak-height ratio of the HPLC pattern and the nitrate and pyrazinamidase tests. On the basis of PRA, HPLC, biochemical and 16S rRNA gene sequence analyses, the name *Mycobacterium lacus* sp. nov. is proposed for this potential pathogen. The type strain is strain NRCM 00-255^T (= ATCC BAA-323^T = DSM 44577^T).

Keywords: *Mycobacterium lacus* sp. nov., 16S rRNA, HPLC, *hsp65*

Case history and strain isolation

In May 2000, a 68-year-old female developed bursitis of her right elbow. She reported that she had bumped her elbow on a rock while swimming in a lake in Ontario, Canada. No break in the skin was noticed at the time of trauma. Soon afterwards, her elbow became sore and swollen. She sought medical advice and was treated with surgical excision alone and, at 6 months, the bursitis had not recurred. The excised synovial tissue revealed caseating granulomas on histopathological stains. A portion of unfixed tissue was submitted for routine bacteriology and mycology and another portion was decontaminated and processed for mycobacteriology. A fluorescent stain of the specimen after decontamination revealed scarce acid-fast bacilli. The Gen-Probe AMPLIFIED Mycobacterium Tuberculosis Direct test was negative. After 30 days, an acid-fast organism was isolated on Lowenstein–Jensen (LJ) medium. No other microorganisms were isolated. The Gen-Probe AccuProbe

tests performed on the culture for the detection of *Mycobacterium tuberculosis* complex, *Mycobacterium avium* complex and *Mycobacterium kansasii* were all negative. HPLC results revealed an unknown pattern resembling that of *M. tuberculosis* complex and analysis of peak-height ratios gave a pattern close to that of *Mycobacterium gastri*. PCR-restriction enzyme analysis (PRA) of the *hsp65* gene revealed an unknown pattern. The isolate, strain NRCM 00-255^T, was subsequently evaluated using phenotypic and genotypic methods [16S rRNA gene and 16S–23S spacer region (ITS1)].

We have identified a unique strain of mycobacterium that did not correspond to any known *Mycobacterium* species by 16S rRNA gene and ITS1 sequence analysis, HPLC, PRA or biochemical tests, for which the name *Mycobacterium lacus* sp. nov. is proposed. Several examples in the literature describe a single strain or strains from one patient that have been characterized and established as novel species (Reischl *et al.*, 1998; Roth *et al.*, 2000; Schinsky *et al.*, 2000; Schröder *et al.*, 1997; Shojaei *et al.*, 1997, 2000; Springer *et al.*, 1993). These data allow other laboratories to recognize novel species easily among their own patient collections and encourage the publication of case studies, furthering our understanding of non-tuberculous mycobacterium

Abbreviations: LJ, Lowenstein–Jensen; PRA, PCR-restriction enzyme analysis.

The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain NRCM 00-255^T is AF406783.

Table 1. Phenotypic characteristics of *M. lacus* sp. nov. in comparison with other slowly growing *Mycobacterium* species

Species are indicated as: 1, *M. lacus* sp. nov. NRCM 00-255^T; 2, *M. gastri*; 3, *M. malmoense*; 4, *M. marinum*; 5, *M. bohemicum* (data from Reischl *et al.*, 1998); 6, *M. tuberculosis* (Goodfellow & Magee, 2001). All species were positive for growth at 37 °C and negative for tolerance of 5 % NaCl. Strain NRCM 00-255^T was also positive for β -glucosidase. w, Weak positive result; v, variable; ns, not stated.

Test	1	2	3	4	5	6
Growth at:						
25 °C	w	+	v	v	+	—
31 °C	+	+	+	+	+	NS
42 °C	+	—	—	—	NS*	—
Pigmentation†	N	N	N	P	S	N
Niacin	—	—	—	—	—	+
Nitrate reductase	+	—	—	—	—	+
Semi-quantitative catalase	< 45	< 45	< 45	< 45	—	< 45
68 °C Catalase	—	—	+	—	+	—
Arylsulfatase activity:						
3 days	w	—	—	—	NS	—
14 days	w	+	—	+	— (10 days)	—
Tween 80 hydrolysis	w	+	+	+	—	v
Tellurite reduction	+	v	+	v	NS	v
Iron uptake	—	—	—	—	NS	—
MacConkey agar without crystal violet	—	NS	NS	—	NS	—
Urease activity	+	+	—	+	w	+
Pyrazinamidase (4 and 7 days)	+	— (4 days)	+	+	—	+
Acid phosphatase	w	+	—	+	—	—

* Growth was tested at both 40 (+) and 45 (—) °C.

† N, Non-chromogen; P, photochromogen; S, scotochromogen.

pathogenicity (De Baere *et al.*, 2001; Torkko *et al.*, 2001; Tortoli *et al.*, 1998, 2000).

Morphological and biochemical properties of strain NRCM 00-255^T

Acid-fast microscopy showed large, dispersed acid-fast bacilli with prominent beading. No spores or hyphae were seen. Upon subculture, growth was observed on LJ medium at 2 weeks at both 37 and 42 °C and at 3 weeks at 30 °C. At 4 weeks, very little growth was observed at 25 °C, and no growth was observed at 52 °C. Colonies on LJ medium were non-chromogenic, small, with a dry appearance. On Middlebrook 7H10 agar, colonies are small, non-pigmented and smooth to rough, with a slightly irregular edge. Younger colonies appeared slightly transparent. Biochemical tests were performed in duplicate and as described in Kent & Kubica (1985), with the exception of β -glucosidase activity (David & Jahan, 1977). Results are shown in comparison with the closest species in Table 1.

Mycolic acid pattern by HPLC

Mycolic acids were extracted and analysed according to the standardized method (Butler *et al.*, 1996). The

chromatographic data were compared with an extensive in-house library of mycobacterial species HPLC profiles as well as with published data (Butler *et al.*, 1999). The HPLC chromatogram of strain NRCM 00-255^T showed a mycolic acid pattern characterized by a single cluster of 10 peaks. The pattern closely resembled that of *M. gastri*, with the same retention times but with variation in the peak-height ratios (Fig. 1).

PRA of the *hsp65* gene

PRA was performed as described previously (Telenti *et al.*, 1993). Briefly, a 439 bp fragment of the 65 kDa heat-shock protein (*hsp65*) was amplified by PCR using conserved mycobacterium primers and digested with *Bst*EII and *Hae*III. The restriction fragments were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. The restriction pattern was compared to the published algorithm (Telenti *et al.*, 1993). PRA analysis showed a pattern distinct from previously published patterns as well as from those in an in-house library of PRA patterns. *Bst*EII digestion showed two fragments, of 325 and 135 bp, and *Hae*III digestion showed three fragments, of 125, 95 and 70 bp.

Fig. 1. Comparison of mycolic acid patterns of (a) *M. lacus* sp. nov. strain NRCM 00-255^T and (b) *M. gastri* ATCC 15754^T, obtained by HPLC analysis. The relative retention time is indicated for each peak. INT, High molecular mass internal standard.

Sequence analysis of the nearly complete 16S rRNA gene and ITS1

DNA lysate preparation, amplification of the partial 16S rRNA gene (from positions 28 to 1509) and sequencing of the PCR product using several primers spanning both strands of the gene were performed as described previously (Turenne *et al.*, 2001). The same

DNA lysate was used for amplification of the ITS1 spacer. The PCR for ITS1 contained approximately 10 ng DNA, 1.5 mM MgCl₂, 1 × PCR buffer, 200 μM each of dCTP, dGTP, dATP and TTP, 500 nmol each of forward and reverse primers and 2.5 U *Taq* DNA polymerase in a final volume of 50 μl. Primers used were 1511f (5'-AAGTCGTAACAAGG-TARCCG-3') and 23r (5'-TCGCCAAGGCATCCACC-3') (Harmsen *et al.*, 2002). The PCR was performed using the Perkin-Elmer GeneAmp PCR system 2400 with a cycle of 94 °C for 5 min, 28 cycles of 94, 55 and 72 °C for 45 s, 1 min and 90 s, respectively, with a final extension at 72 °C for 10 min, and the reaction mixture was held indefinitely at 4 °C. Sequencing reactions of the PCR product were prepared with the same primers used for the PCR. Sequencing template preparation, running of samples on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) and sequence output analysis were performed as described previously (Turenne *et al.*, 2001). The Lasergene program version 4.01 (DNASTAR, Inc.) was used for sequence assembly (Seqman), sequence alignment and phylogenetic analyses (MEALIGN). Multiple sequence alignments were determined using the CLUSTAL method algorithm. Analysis of the 16S rRNA gene was performed by comparison against the sequences of reference strains determined in our laboratory (Turenne *et al.*, 2001), GenBank (BLAST, NCBI) and RIDOM (<http://www.ridom.de>), a publicly available, quality-controlled 5'-16S rRNA gene (positions 54–510) sequence database, with a comprehensive collection of mycobacterial sequences. The ITS1 spacer sequence was evaluated using the RIDOM database and BLAST (NCBI).

The 16S rRNA gene sequence of strain NRCM 00-255^T was characteristic of slowly growing species, with a long helix 18 (positions 451–482 of the *Escherichia coli* numbering system; Brosius *et al.*, 1978). For this strain, distinguishing nucleotide sequences from all other mycobacteria species, including the genotypically closest relatives, were found in hypervariable regions A and B (Kirschner *et al.*, 1993) (Fig. 2). Sequence analysis revealed that *Mycobacterium malmoeense* and *Mycobacterium marinum* were related most closely to strain NRCM 00-255^T, both with 99 % similarity and a Hamming distance of 14 (base variations and insertions/deletions weighted equally) (Table 2). Phylogenetic relationships of NRCM 00-255^T to its closest relatives and other members of the genus are shown in Fig. 3. Although the HPLC and biochemical profiles resembled those of *M. gastri* most closely, there were 18 base variations between the two 16S sequences. RIDOM analysis revealed a top score with *M. marinum* DSM 44344^T and *Mycobacterium ulcerans* ATCC 19423^T, with 98.18 % identity, corresponding to eight mismatches. *M. marinum* and *M. ulcerans* cannot be differentiated from each other in the 5' end of the 16S rRNA gene.

Analysis of the complete ITS1 spacer region of strain NRCM 00-255^T (282 bp) using BLAST revealed that,

The region covering positions 28–1506 of the *E. coli* sequence was analysed using the MEGALIGN component of the Lasergene software. NRCM, National Reference Centre for Mycobacteriology, National Microbiology Laboratory, Health Canada. Hamming distances are pairwise distances calculated by weighting nucleotide differences and insertions/deletions equally (value of 1).

Strain	Source of sequence data	Identity (%)	Divergence (%)	Hamming distance
<i>M. malmoense</i> ATCC 29571 ^T	NRCM	99·0	0·9	14
<i>M. marinum</i> ATCC 927 ^T	NRCM	99·0	0·9	14
<i>M. bohemicum</i> DSM 44277 ^T	NRCM	99·0	1·0	15
<i>M. bohemicum</i> E590	GenBank (AJ277283)	99·0	1·0	15
<i>M. bohemicum</i> E743	GenBank (AJ277284)	98·9	1·1	16
<i>M. ulcerans</i> ATCC 19423 ^T	NRCM	98·9	1·0	16
<i>M. tuberculosis</i> ATCC 27294 ^T	NRCM	98·8	0·9	17
<i>M. gastri</i> ATCC 15754 ^T ,	NRCM	98·4	1·2	18
<i>M. kansasii</i> ATCC 12478 ^{T*}				

* *M. gastr*i and *M. kansa*sii cannot be differentiated from each other based on the 16S rRNA gene.

based on the ITS1 sequences for mycobacterium species available from GenBank, the top score was *M. marinum* (AB026701), with a distant sequence identity of 88 %. An ITS1 alignment of the top-scoring species is shown in Fig. 4. Analysis of the ITS1 sequence using RIDOM revealed the highest identity to *M. gastri* DSM 43505^T (88.19 %), followed by both *M. marinum* DSM 44344^T and *M. ulcerans* ATCC 19423^T (87.41 %) and *M. kansasii* Borste 10492/98 (87.29 %), respectively corresponding to 34, 35 and 37 mismatches.

Bactec 12B radiometric broth macrodilution susceptibility testing was performed on isolate NRCM 00-255^T according to the method used for *M. avium* complex (Heifets *et al.*, 1993; Siddiqi *et al.*, 1993).

Minimum inhibitory concentration (MIC) results ($\mu\text{g ml}^{-1}$) were: amikacin (1.0), capreomycin (5.0), clarithromycin (≤ 2.0), ciprofloxacin (2.0), ethambutol (2.0), ethionamide (2.5), isoniazid (0.1), kanamycin (2.5), levofloxacin (≤ 0.5), ofloxacin (1.0), rifabutin (≤ 0.12), rifampin (≤ 0.5) and streptomycin (4.0).

Mycobacterium lacus (la'cus. L. gen. n. *lacus* of the lake, where the organism was acquired).

Microscopy shows large acid-fast bacilli with prominent beading. Colonies on LJ medium are non-chromogenic and can grow at temperatures between 25 and 42 °C, optimally at 37 °C. A slowly growing organism with features similar to *M. gastri* by HPLC and biochemical tests, with the exception of positive

Fig. 3. Phylogenetic relationships of *M. lacus* sp. nov. NRCM 00-255^T to its closest genotypic relatives. Multiple sequence alignments were determined from positions 54 to 1470 of the *E. coli* 16S rRNA gene using the CLUSTAL method algorithm in the MEGALIGN component of the Lasergene program version 4.01. The tree was rooted using *Nocardia asteroides* as the outgroup sequence. Sequences were determined in our laboratory unless indicated by a GenBank accession number. Bar, 1.0% sequence divergence.

nitrate and pyrazinamidase test results and the ability to grow at 42 °C. Positive test reactions are also observed for urease, β -glucosidase activity and tellurite reduction. Does not grow on 5% NaCl or MacConkey

without crystal violet. Arylsulfatase, Tween 80 hydrolysis and acid-phosphatase tests are weak. Negative results for the niacin test, semi-quantitative catalase, heat-stable catalase, iron uptake, acetamidase, nicotinamidase and succinamidase. Genetic testing confirms the isolate as a unique species, based on a unique PRA pattern and 16S rRNA gene sequence. The 16S rRNA gene shows signature sequences characteristic of slowly growing mycobacteria and unique sequence characteristics in the hypervariable regions A and B of the gene. Phylogenetically closest to *M. malmoense*, *M. marinum*, *Mycobacterium bohemicum*, *M. ulcerans* and *M. tuberculosis* complex. Susceptible *in vitro* to all antimicrobial drugs tested [amikacin (MIC 0.1 $\mu\text{g ml}^{-1}$), capreomycin (5.0), clarithromycin (≤ 2.0), ciprofloxacin (2.0), ethambutol (2.0), ethionamide (2.5), isoniazid (0.1), kanamycin (2.5), levofloxacin (≤ 0.5), ofloxacin (1.0), rifabutin (≤ 0.12), rifampin (≤ 0.5)], except streptomycin, with intermediate susceptibility (4.0 $\mu\text{g ml}^{-1}$). The type strain is strain NRCM 00-255^T (= ATCC BAA-323^T = DSM 44577^T).

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References

- Brosius, J., Palmer, M. L., Kennedy, P. J. & Noller, H. F. (1978). Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *Proc Natl Acad Sci U S A* **75**, 4801–4805.

Fig. 4. Alignment of the ITS1 regions of *M. lacus* sp. nov. and genotypically related species. Nucleotides that differ from *M. tuberculosis* are shown; dashes in the *M. tuberculosis* sequence indicate deletions. Sequences were determined in our laboratory unless indicated by a GenBank accession number.

- Butler, W. R., Floyd, M. M., Silcox, V. A. & 9 other authors (1996). *Standardized Method for HPLC Identification of Mycobacteria*. Atlanta, GA: Centers for Disease Control and Prevention, US Dept of Health and Human Services.
- Butler, W. R., Floyd, M. M., Silcox, V. A. & 9 other authors (1999). *Mycolic Acid Pattern Standard for HPLC Identification of Mycobacteria*. Atlanta, GA: Centers for Disease Control and Prevention, US Dept of Health and Human Services.
- David, H. L. & Jahan, M. T. (1977). β -Glucosidase activity in mycobacteria. *J Clin Microbiol* **5**, 383–384.
- De Baere, T., Moerman, M., Rigouts, L., Dhooge, C., Vermeersch, H., Verschraegen, G. & Vanechoutte, M. (2001). *Mycobacterium interjectum* as causative agent of cervical lymphadenitis. *J Clin Microbiol* **39**, 725–727.
- Goodfellow, M. & Magee, J. G. (2001). Taxonomy of *Mycobacteria*. In *Mycobacteria. I: Basic Aspects*, pp. 1–71. Edited by P. R. Gangadharam & P. A. Jenkin. New York: International Thomson Publishing.
- Harmsen, D., Rothgänger, J., Frosch, M. & Albert, J. (2002). RIDOM: ribosomal differentiation of medical micro-organisms database. *Nucleic Acids Res* **30**, 416–417.
- Heifets, L., Lindholm-Levy, P., Libonati, J. P., Hooper, N., Laszlo, A., Cynamon, M. & Siddiqi, S. (1993). *Radiometric Broth Macrodilution Method for the Determination of Minimal Inhibitory Concentrations (MIC) with Mycobacterium avium Complex Isolates*. Denver, CO: National Jewish Centre for Immunology and Respiratory Medicine.
- Kent, P. T. & Kubica, G. P. (1985). *Public Health Mycobacteriology: a Guide for the Level III Laboratory*. Atlanta, GA: Centers for Disease Control, US Dept of Health and Human Services.
- Kirschner, P., Meier, A. & Bottger, E. C. (1993). Genotypic identification and detection of mycobacteria – facing novel and uncultured pathogens. In *Diagnostic Molecular Microbiology: Principles and Applications*, pp. 173–190. Edited by D. H. Persing, T. F. Smith, F. C. Tenover & T. J. White. Washington, DC: American Society for Microbiology.
- Reischl, U., Emler, S., Horak, Z., Kaustova, J., Kroppenstedt, R. M., Lehn, N. & Naumann, L. (1998). *Mycobacterium bohemicum* sp. nov., a new slow-growing scotochromogenic mycobacterium. *Int J Syst Bacteriol* **48**, 1349–1355.
- Roth, A., Reischl, U., Schonfeld, N., Naumann, L., Emler, S., Fischer, M., Mauch, H., Loddenkemper, R. & Kroppenstedt, R. M. (2000). *Mycobacterium heckeshornense* sp. nov., a new pathogenic slowly growing *Mycobacterium* sp. causing cavitary lung disease in an immunocompetent patient. *J Clin Microbiol* **38**, 4102–4107.
- Schinsky, M. F., McNeil, M. M., Whitney, A. M., Steigerwalt, A. G., Lasker, B. A., Floyd, M. M., Hogg, G. G., Brenner, D. J. & Brown, J. M. (2000). *Mycobacterium septicum* sp. nov., a new rapidly growing species associated with catheter-related bacteraemia. *Int J Syst Evol Microbiol* **50**, 575–581.
- Schröder, K.-H., Naumann, L., Kroppenstedt, R. M. & Reischl, U. (1997). *Mycobacterium hassiacum* sp. nov., a new rapidly growing thermophilic mycobacterium. *Int J Syst Bacteriol* **47**, 86–91.
- Shojaei, H., Goodfellow, M., Magee, J. G., Freeman, R., Gould, F. K. & Brignall, C. G. (1997). *Mycobacterium novocastrense* sp. nov., a rapidly growing photochromogenic mycobacterium. *Int J Syst Bacteriol* **47**, 1205–1207.
- Shojaei, H., Magee, J. G., Freeman, R., Yates, M., Horadagoda, N. U. & Goodfellow, M. (2000). *Mycobacterium elephantis* sp. nov., a rapidly growing non-chromogenic *Mycobacterium* isolated from an elephant. *Int J Syst Evol Microbiol* **50**, 1817–1820.
- Siddiqi, S. H., Heifets, L. B., Cynamon, M. H., Hooper, N. M., Laszlo, A., Libonati, J. P., Lindholm-Levy, P. J. & Pearson, N. (1993). Rapid broth macrodilution method for determination of MICs for *Mycobacterium avium* isolates. *J Clin Microbiol* **31**, 2332–2338.
- Springer, B., Kirschner, P., Rost-Meyer, G., Schröder, K. H., Kroppenstedt, R. M. & Bottger, E. C. (1993). *Mycobacterium interjectum*, a new species isolated from a patient with chronic lymphadenitis. *J Clin Microbiol* **31**, 3083–3089.
- Telenti, A., Marchesi, F., Balz, M., Bally, F., Bottger, E. C. & 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.
- Torkko, P., Suomalainen, S., Iivanainen, E. & 7 other authors (2001). Characterization of *Mycobacterium bohemicum* isolated from human, veterinary, and environmental sources. *J Clin Microbiol* **39**, 207–211.
- Tortoli, E., Reischl, U., Besozzi, G. & Emler, S. (1998). Characterization of an isolate belonging to the newly described species *Mycobacterium hassiacum*. *Diagn Microbiol Infect Dis* **30**, 193–196.
- Tortoli, E., Bartoloni, A., Manfrin, V., Mantella, A., Scarparo, C. & Bottger, E. (2000). Cervical lymphadenitis due to *Mycobacterium bohemicum*. *Clin Infect Dis* **30**, 210–211.
- Turenne, C. Y., Tschetter, L., Wolfe, J. & Kabani, A. (2001). Necessity of quality-controlled 16S rRNA gene sequence databases: identifying nontuberculous *Mycobacterium* species. *J Clin Microbiol* **39**, 3637–3648.