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Mycobacterium noviomagense sp. nov.; clinical relevance evaluated in 17 patients

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Eighteen isolates of a nonchromogenic, slowly growing, non-tuberculous species of the genus *Mycobacterium* were cultured from respiratory specimens obtained over the last eight years from 17 patients in the Netherlands. These isolates were grouped because they revealed a unique 16S rRNA gene sequence and were related to *Mycobacterium xenopi*. None of the 17 patients met the American Thoracic Society diagnostic criteria for non-tuberculous mycobacterial disease, which distinguishes the novel isolates from the related species, *M. xenopi*. A polyphasic taxonomic approach, including identification by biochemical and phenotypical analysis, *hsp65* gene sequencing and PCR restriction enzyme pattern analysis, and sequence analyses of the *rpoB* gene and 16S–23S internal transcribed spacer supported the separate species status of the novel isolates. The name *Mycobacterium noviomagense* sp. nov. is proposed for the novel strains. The type strain is NLA000500338^T (=DSM 45145^T=CIP 109766^T). A more distinctive taxonomy of NTM is a prerequisite for the assessment of their clinical relevance.

Improved detection and identification techniques have triggered renewed interest in non-tuberculous mycobacteria (NTM) and their role as opportunistic pathogens. PCR techniques and 16S rRNA gene sequence analysis have brought to light a series of novel NTM species, however, the clinical relevance of these species is not always clear (Tortoli, 2003; Tortoli *et al.*, 2001; Griffith *et al.*, 2007).

NTM infections present predominantly as chronic pulmonary disease, although extrapulmonary and disseminated infections have also been described (Griffith *et al.*, 2007). Local immunosuppression due to pre-existing pulmonary disease and systemic immunosuppression, e.g. in haematological malignancy, immunosuppressive medication and HIV/AIDS, have been identified as predisposing factors (Griffith *et al.*, 2007) for NTM infections. Infection has to be differentiated from contamination and pseudo-infection, characterized by the recovery of single NTM isolates from the respiratory or digestive tract without signs of disease (Griffith *et al.*, 2007; Portaels, 1995). Their ubiquitous presence in the environment, survival in flowing water systems and resistance to disinfectants implies that NTM often represent laboratory or medical equipment contamination (Griffith *et al.*, 2007; Portaels, 1995; van Klingeren & Pullen, 1993). The diagnostic criteria proposed in a Statement by the American Thoracic Society (ATS) are designed to differentiate between true infection and pseudo-infection or contamination, based on clinical, radiological and microbiological features (Griffith *et al.*, 2007).

This study describes the grouping of 18 previously unknown *Mycobacterium* isolates with identical 16S rRNA gene sequences and with a high degree of gene sequence similarity to strains of *Mycobacterium xenopi*. As other features of these novel strains were highly distinct

Abbreviations: ATS, American Thoracic Society; ITS, internal transcribed spacer; NTM, non-tuberculous mycobacteria.

The GenBank/EMBL/DDBJ accession numbers 16S rRNA, 16S–23S ITS 1 and 2, *hsp65* and *rpoB* gene sequences of strain NLA000500338^T are EU239955, EU439248, EU439249, EU600390 and EU810775, respectively

Phylogenetic trees based on *hsp65* and *rpoB* gene sequences of selected mycobacterial species are available as supplementary material with the online version of this paper.

from *M. xenopi* and the clinical relevance differed significantly between the new isolates and *M. xenopi*, the 18 isolates are proposed to represent a novel species of the genus *Mycobacterium*.

The 18 novel isolates were acquired from pulmonary samples (13 from sputum, 4 from broncho-alveolar lavage fluid and 1 from a post-mortem lung biopsy) of 17 patients in the Netherlands between January 1999 and January 2007. To determine clinical relevance, we examined the medical records of all 17 patients; their baseline characteristics are displayed in Table 1. The predominance of male patients, mean age and history of chronic pulmonary disease are comparable with previous NTM studies (Griffith *et al.*, 2007; Henry *et al.*, 2004). None of the patients had clinical and radiographic features suggestive of mycobacterial lung disease; one was systemically immunocompromised due to HIV co-infection. The post-mortem lung biopsy sample showed histological features of bronchopneumonia and invading bacteria, without features of mycobacterial disease such as granuloma formation. All patient samples were negative for acid-fast bacilli on direct microscopy which suggested the presence of a low number of NTM in the original sample or contamination after sample division for culture and microscopy. Although follow-up sputum cultures were performed for 16 patients, only one patient produced another culture that was positive for the novel strains. All others had a single positive culture. Based on these findings, none of the patients from whom the novel strains were isolated met the ATS criteria for pulmonary NTM disease (Griffith *et al.*, 2007). Good clinical response to non-antimycobacterial regimes, the subsequent establishment of alternative diagnoses and the observed spontaneous conversion to negative cultures suggested that the novel strains were not the causative agent of these patients' symptoms. The novel strains therefore seem to have no clinical relevance, which distinguishes them from phylogenetically related, but more pathogenic species such as *M. xenopi* and *Mycobacterium heckeshornense* (Griffith *et al.*, 2007; Roth *et al.*, 2000; van Ingen *et al.*, 2008).

Two patients received antituberculosis treatment for an average period of 10 weeks, after a presumptive diagnosis of TB. Treatment of patients not meeting the ATS diagnostic criteria is potentially harmful to patients in terms of adverse effects and costs (van Crevel *et al.*, 2001).

Geographical clustering of the patients was observed, favouring the south-east of the Netherlands, in adjacent areas of the Limburg (7 cases), Gelderland (5 cases) and Noord-Brabant (2 cases) provinces. This clustering suggested the presence of the novel strains in specific local environments or tap water. Since the clustering was seen geographically, but not over time, contamination from medical machinery or the laboratories involved seemed less likely.

All of the novel isolates were subjected to laboratory diagnosis by the National Mycobacteria Reference Laboratory of the National Institute for Public Health and the Environment (RIVM). The RIVM provides molecular identification, drug susceptibility testing and epidemiological typing of mycobacterial isolates for all healthcare institutions in the Netherlands.

Biochemical identification and HPLC analysis of cell-wall mycolic acid content were performed using previously described approaches (Lévy-Frébault & Portaels, 1992; Centers for Disease Control and Prevention, 1996). Eight isolates (4 *M. xenopi*, 4 of the novel strains) were subjected to biochemical testing. The results are detailed in Table 2. Morphologically, two patterns were clearly discernible. On Middlebrook 7H10 media, the *M. xenopi* isolates were scotochromogenic, showing yellow pigmentation, whereas

Table 1. Characteristics of the patients in the study group

The total number of patients was 17. COPD, Chronic obstructive pulmonary disease.

Characteristic	No. of patients
Male	13 (77 %)
Mean age (yr) (range)	53 (29–86)
Dutch origin	16 (94 %)
Pre-existing pulmonary disease	15 (88 %)
COPD	8 (47 %)
Lung cancer	4 (24 %)
Healed tuberculosis	2 (12 %)
Recurrent pulmonary infection*	3 (18 %)
Bronchiectasis	2 (12 %)
Current smoker	5 (29 %)
Past smoker	3 (18 %)
Alcohol abuse	3 (18 %)
HIV infection	1 (6 %)

*>3 In 6 months prior to sampling.

Table 2. Biochemical identification results for the novel isolates and closely related species

Taxa: 1, *M. noviomagense* sp. nov.; 2, *M. xenopi*; 3, *M. heckeshornense* (data from Roth *et al.*, 2000); 4, *M. botniense* (Torkko *et al.*, 2000). None of the taxa were able to tolerate 5 % NaCl in Lowenstein–Jensen (LJ) medium. +, Positive; −, negative; V, variable; NC, nonchromogenic; SC, scotochromogenic; NP, not published.

Characteristic	1	2	3	4
Growth at 45 °C	−	+	+	+
Morphology	NC	SC	SC	SC
Colony size	Small	Large	NP	Small
Tolerance to (in LJ medium):				
Isoniazid 10 μg ml^{-1}	−	−	−	NP
Thiophene 2-carboxylic acid	+	+	NP	NP
Hydroxylamine 250 μg ml^{-1}	V	+	NP	NP
Para-nitrobenzoate 500 μg ml^{-1}	+	V	NP	NP

colonies of the novel species were smaller and nonchromogenic. All *M. xenopi* isolates grew at 45 °C, as previously reported (Torkko *et al.*, 2000), but the novel strains were unable to grow at this temperature (Table 2). Biochemically, the novel isolates were indistinguishable from the cluster comprising *M. xenopi*, *Mycobacterium botniense* and *M. heckeshornense*, with negative results for urease, Tween 80 hydrolysis, niacin production, nitrate reductase, acid phosphatase and semi-quantitative catalase. HPLC of the novel isolates revealed a pattern characterized by one early and one late cluster of peaks, a profile similar to that of *M. xenopi*, *M. heckeshornense* and *M. botniense* (Fig. 1). The HPLC mycobacterium library (available online at http://www.MycobacToscana.it) was used for this comparison.

Susceptibility testing was performed for eleven of the novel isolates from eleven patients using the agar dilution method (van Klingeren *et al.*, 2007). For the novel isolates, we recorded *in vitro* resistance to rifampicin (MIC 2 mg l^{-1}), resistance or intermediate susceptibility to ethambutol (MIC 10–20 mg l^{-1}) and intermediate susceptibility to isoniazid (MIC 0.5–1 mg l^{-1}). The novel species proved susceptible *in vitro* to streptomycin, cycloserine, prothionamide, amikacin, ciprofloxacin, clofazimine, clarithromycin and rifabutin. The drug susceptibility pattern for the novel isolates differed slightly from clinical isolates of *M. xenopi* tested at RIVM which were mostly susceptible, *in vitro*, to rifampicin (MIC 0.5–1 mg l^{-1}). These results for *M. xenopi* are in accordance with previous reports (Dauendorffer *et al.*, 2002).

For molecular identification, sequencing of the full 16S rRNA gene and 16S–23S internal transcribed spacer (ITS), partial *rpoB* and *hsp65* genes, and PCR restriction enzyme pattern analysis (PRA) of the *hsp65* gene were performed using previously described methods (Kim *et al.*, 1999; Roth *et al.*, 1998; Springer *et al.*, 1996; Telenti *et al.*, 1993). The sequences obtained were compared with the GenBank/EMBL (NCBI, http://www.ncbi.nlm.nih.gov) gene sequence databases.

The full 16S rRNA gene sequence for the novel species showed 96 % sequence similarity with that of *M. xenopi* and was 97 % similar to those of *M. heckeshornense* and *Mycobacterium shimoidei*. A sequence difference of 1 % has been proposed in the literature as the threshold for the designation of a novel species (Hall *et al.*, 2003; Tortoli, 2003).

The full 16S rRNA gene sequence of the novel strains was aligned with those of reference strains of the closest related mycobacteria using CLUSTAL_X software (Thompson *et al.*, 1997). The resulting topology and tree, inferred by neighbour-joining and visualized using the LOFT software package (van der Heijden *et al.*, 2007) were evaluated by bootstrap analyses based on 1000 resamplings (Fig. 2).

Analysing only the hypervariable region A of the 16S rRNA gene (151 bp), we found a 100 % match in the GenBank/EMBL database to a strain designated 'most closely resembling *M. xenopi*', as reported by Hall *et al.* (2003). Analysis of the phenotypic and genetic characteristics of the novel species (Table 2, Figs 1, 2 and 3) demonstrates that very subtle 16S rRNA gene sequence differences can be associated with extensive divergence from the closest related species. This brings into question the use of monophasic identification methods.

Analysis of the 16S–23S ITS region revealed mixed sequence patterns for all 18 novel isolates, even from single colony cultures, and necessitated cloning. For cloning of the ITS, amplicons were generated using primers provided with the INNO LiPA Mycobacteria v2 kit (Innogenetics), and cloned in the PGEM-T Easy vector (Promega) according to the manufacturer's instructions. Thirty colonies of transformed *Escherichia coli* strain DH5F were picked for each sample, cultured and used to prepare plasmid DNA using the QIAprep 96 Turbo BioRobot kit (Qiagen). For sequencing of the ITS region cloned into the pGEM-T vector, the universal vector primers M13(−21) and M13rev were used on the plasmid preparation as target. Cloning resulted in the recognition of two distinct copies of the ITS, both with <68 % sequence similarity to *M. xenopi*. A 93 % sequence similarity was noted between the two ITS copies. The presence of multiple copies of the 16S–23S ITS region, thus possibly multiple rRNA operons,

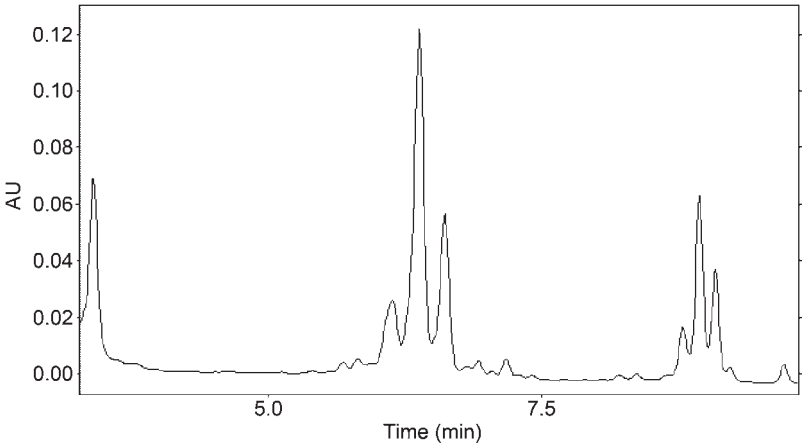

Fig. 1. Mycolic acid patterns of *Mycobacterium noviomagense* sp. nov. obtained by HPLC analysis. One early and one late cluster of peaks are present, similar to *M. xenopi*. The first (left) and last (right) peaks are the low and high weight molecular standards.

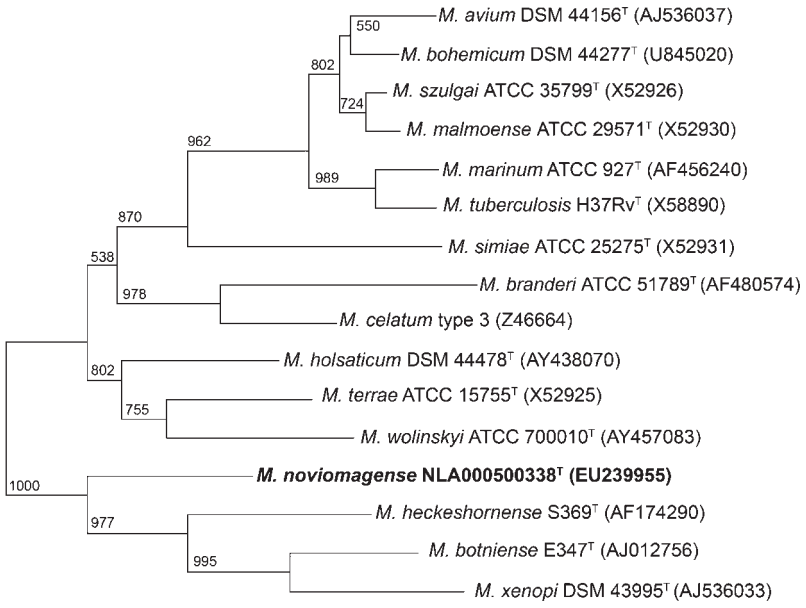

Fig. 2. Phylogenetic relationship of the type strain of the novel species, *M. noviomagense* sp. nov., and related species of the genus *Mycobacterium*, based on 16S rRNA gene sequences. The neighbour-joining tree was created and bootstrapped 1000 times with CLUSTAL_X (Thompson *et al.*, 1997) and visualized with LOFT (Levels of Orthology through Phylogenetic Trees; van der Heijden *et al.*, 2007). Bootstrap values are indicated at the nodes.

is unexpected. This phenomenon has not been described for *M. xenopi* or for closely related slow-growing NTM species and thus supports the separate species status of the novel isolates.

The *rpoB* gene sequence of the novel isolates was 95 % similar to that of the recently described species *Mycobacterium seoulense* and only 92 % similar to that of *M. xenopi*. For the *hsp65* gene sequence, the most closely related sequences (95 %) were found among members of the *Mycobacterium avium* complex and *Mycobacterium branderi*, with <93 % similarity to *M. xenopi*. The considerable divergence in these two targets from the otherwise related cluster comprising *M. xenopi*, *M. botniense* and *M. heckeshornense* supports the separate species status of the novel isolates. The *hsp65* and *rpoB* gene sequences were aligned with those of related mycobacterial species, as for the 16S rRNA gene sequence. The resulting topologies and trees are available as Supplementary Figs S1 and S2 (in IJSEM Online).

The *hsp65* gene PRA results for the novel isolates, *M. xenopi*, *Mycobacterium tuberculosis* H37Rv^T and *M. botniense* ATCC 700701^T are shown in Fig. 3. For the novel isolates, digestion with *BstE*II resulted in fragments of 240/120/100 bp, digestion with *Hae*III gave fragments of 130/10/70/45 bp. A PRAsite (http://app.chuv.ch/prasite/index.html) comparison showed this to be a unique fragment length combination. Isolates of *M. xenopi* and *M. tuberculosis* tested simultaneously were correctly identified using the PRAsite database; no entry was found for *M. botniense*.

Description of *Mycobacterium noviomagense* sp. nov.

Mycobacterium noviomagense (no.vi.o.ma.gen′se. N.L. neut. adj. pertaining to Noviomagus, the Roman name of

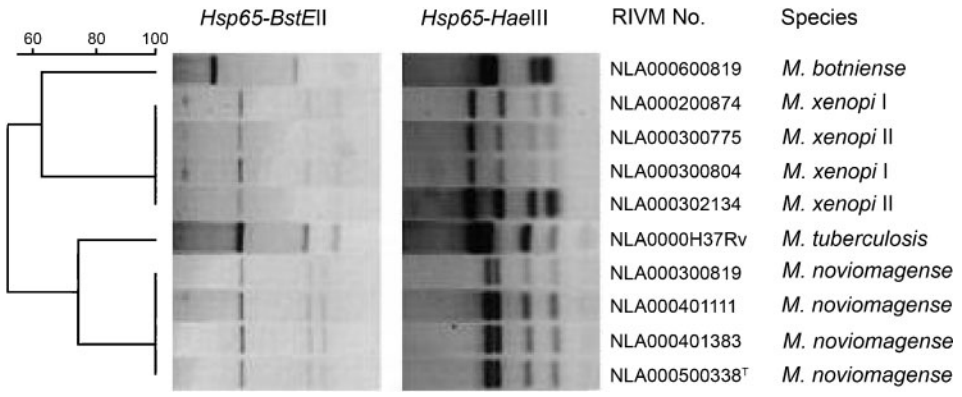

Fig. 3. PRA typing results for the *hsp*65 gene. Different fragment length patterns are observed for *M. botniense* (lane 1), *M. xenopi* (lanes 2–5), *M. tuberculosis* H37Rv^T (lane 6) and strain NLA000500338^T (*M. noviomagense* sp. nov.; lanes 7–10).

the major city in the endemic region in the Netherlands and the location of the reference hospital; current name: Nijmegen).

Acid-fast and Gram-positive rods. Colonies are nonchromogenic and appear after 4 weeks of culture at 37 °C, no growth occurs at 45 °C. Negative in tests for urease, Tween 80 hydrolysis, niacin production, nitrate reductase, acid phosphatase and semi-quantitative catalase. Can be readily identified by its unique rRNA gene sequences.

The type strain, NLA000500338^T (=DSM 45145^T=CIP 109766^T), was recovered from sputum.

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