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Mycobacterium riyadhense sp. nov., a non-tuberculous species identified as *Mycobacterium tuberculosis* complex by a commercial line-probe assay

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A non-chromogenic, slowly growing *Mycobacterium* strain was isolated from a maxillary sinus lavage from a symptomatic patient in Riyadh, Saudi Arabia. It was initially identified as a member of the *Mycobacterium tuberculosis* complex by a commercial line-probe assay. Its 16S rRNA, *hsp65* and *rpoB* gene and 16S–23S internal transcribed spacer sequences were unique; phylogenetic analysis based on the 16S rRNA gene sequence groups this organism close to *Mycobacterium szulgai* and *Mycobacterium malmoense*. Its unique biochemical properties and mycolic acid profile support separate species status. We propose the name *Mycobacterium riyadhense* sp. nov. to accommodate this strain. The type strain is NLA000201958^T (=CIP 109808^T =DSM 45176^T).

A 19-year-old male reported to the otolaryngology department of the King Faisal Hospital with pain and swelling of the left side of his face with protrusion of left eye after blunt trauma. A computed tomography scan of the sinuses was performed, which revealed a tumour in the left maxillary sinus. The tumour involved the nasal septum, extended into the left orbit and infiltrated the medial and inferior rectus as well as the optic nerve. A chest radiograph revealed no abnormalities. A lavage of both maxillary sinuses was performed for diagnosis. Mycobacterial cultures were performed on Lowenstein–Jensen medium and yielded an isolate with unusual colony morphology after 3 weeks of incubation at 36 °C.

The patient was presumed to have bone tuberculosis and started a 9-month anti-tuberculosis regimen. The patient improved both clinically and radiologically. He has not suffered a relapse since. The isolated strain was sent to the Dutch National Mycobacteria Reference Laboratory (RIVM, Bilthoven, Netherlands) for identification, as part of a second-line quality control programme. Identification of the strain was first attempted using three commercial line-probe assays, Hain GenoType MTBC and GenoType AS/CM (Hain Lifescience GmbH) and INNO-LiPA MYCOBACTERIA v2 (Innogenetics NV), all used according to the manufacturers' instructions.

To obtain identification to the species level, we sequenced the 16S rRNA gene, 16S–23S internal transcribed spacer (ITS) and *rpoB* and *hsp65* genes, using previously described approaches (Springer *et al.*, 1996; Roth *et al.*, 1998; Kim

Abbreviation: ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the sequences of the 16S rRNA, 16S–23S ITS, *rpoB*, *hsp65*, *esat-6* and *cfp-10* genes of strain NLA000201958^T are EU274642, EU274643, EU274644, EU921671, EU552926 and EU552927.

Phylogenetic trees based on 16S–23S internal transcribed spacer and *hsp65* sequences of selected mycobacterial species are available as supplementary material with the online version of this paper.

et al., 1999; Telenti *et al.*, 1993). The DNA sequences obtained were compared with the RIDOM (Ribosomal Differentiation of Medical Microorganisms; http://rdna.ridom.de) and GenBank (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov) databases.

To establish the presence of a region of difference 1 (RD1)-like element and the DNA sequences of the *esat-6* and *cfp-10* genes, we used primers from a previous study (Arend *et al.*, 2005).

The most commonly investigated biochemical and phenotypic features [colony morphology, ability to grow at temperatures ranging from 24 to 45 °C, niacin accumulation, nitrate reduction, β-glucosidase, Tween 80 hydrolysis, 3 day arylsulfatase, urease, tellurite reduction, 68 °C and semiquantitative catalase, growth rate, pigmentation, growth on MacConkey agar and tolerance to NaCl, thiophene-2-carboxylic hydrazide (TCH), oleate and *p*-nitrobenzoic acid and to 10 μg thiacetazone ml^{-1}, 1 μg hydroxylamine ml^{-1} and 500 μg isoniazid ml^{-1}, all three in Middlebrook 7H10 agar] as well as HPLC analysis of cell-wall mycolic acids were tested by the Regional Reference Center for Mycobacteria (Careggi Hospital, Florence, Italy) using standard procedures described previously (Kent & Kubica, 1985; CDC, 1996).

Drug susceptibility testing was performed using the 25-well agar-dilution method (van Klingeren *et al.*, 2007). We included isoniazid, rifampicin, rifabutin, ethambutol, clarithromycin, ciprofloxacin, cycloserine, prothionamide, amikacin, clofazimine and streptomycin in the test panel.

Applying the Hain GenoType MTBC assay, a non-specific reaction was noted with hybridization of the *M. tuberculosis* complex band only (banding pattern 1, 2, 3). The InnoLipa MYCOBACTERIA v2 line-probe assay yielded a *Mycobacterium* genus probe reaction, though no species-specific result. The Hain GenoType CM (Common Mycobacteria) kit identified the strain as *Mycobacterium tuberculosis* complex based on a band 1, 2, 3, 10, 16 pattern. The supplementary AS kit identified the strain as a non-specified *Mycobacterium* species, with banding pattern 1, 2, 3, 12. Sequencing of the full 23S rRNA gene, the assay's target, established its identity as a non-tuberculous mycobacterium (Table 1). Based on these findings, IS*6110* and IS*1081* RFLP were performed to confirm the identity of the strain as *M. tuberculosis* (van Soolingen *et al.*, 1993). No IS*6110* or IS*1081* element copies could be demonstrated (results not shown).

The sequencing results are listed in Table 1; the 16S rRNA gene, ITS and *rpoB* and *hsp65* gene sequences were all unique. Sequencing of the full 16S rRNA gene identified the bacterium as a *Mycobacterium szulgai*-like species (Table 1). The 16S rRNA gene sequence 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 MEGA software package (Tamura *et al.*, 2007), were evaluated by bootstrap analyses based on 1000 resamplings (Fig. 1). The tree was rooted with *Nocardia abscessus* ATCC BAA-279^T as an outgroup. Similar multisequence alignments and trees were created based on the ITS and *hsp65* sequences (Supplementary Fig. S1, available in IJSEM Online).

We were able to amplify an RD1 region, including *esat-6* (270 bp) and *cfp-10* (251 bp) genes. Results of sequence comparisons for both genes are recorded in Table 1.

Table 1. Sequence comparisons between strain NLA000201958^T and its closest relatives

Where no strain is indicated, multiple highly similar sequences are deposited in GenBank.

Gene/region	Most similar sequences	
	BLAST	RIDOM
16S rRNA gene (full)	*M. malmoense* (99 %), *M. szulgai* (99 %), *M. bohemicum* (98 %)	*M. szulgai* DSM 44166^T (99.1 %), *M. intracellulare* ATCC 35770 (98.4 %), *M. intracellulare* ATCC 35772 (98.4 %), *M. haemophilum* ATCC 29548^T (98.4 %)
16S–23S ITS (273 bp)	*M. szulgai* (91 %), *M. kansasii* (91 %), *M. marinum* (91 %)	*M. kansasii* DSM 44162^T (92 %), *M. gastri* DSM 43505^T (91 %), *M. marinum* DSM 44344^T (90 %)
23S rRNA gene (full)	*M. kansasii* (97 %), *M. avium* (97 %), *M. ulcerans* agy99 (97 %)	
rpoB (472 bp)	*M. avium* 104 (93 %), *M. paratuberculosis* k10 (93 %), *M. tuberculosis* H37Rv^T (91 %)	
hsp65 (421 bp)	*M. genavense* DSM 44424^T (95 %), *M. bohemicum* CIP 105811^T (95 %), *M. malmoense* CIP 105775^T (95 %)	
esat-6 (270 bp)	*M. kansasii* (89 %), *M. tuberculosis* H37Rv^T (87 %), *M. szulgai* (85 %)	
cfp-10 (251 bp)	*M. tuberculosis* H37Rv^T (88 %), *M. marinum* M (85 %), *M. ulcerans* (84 %)	

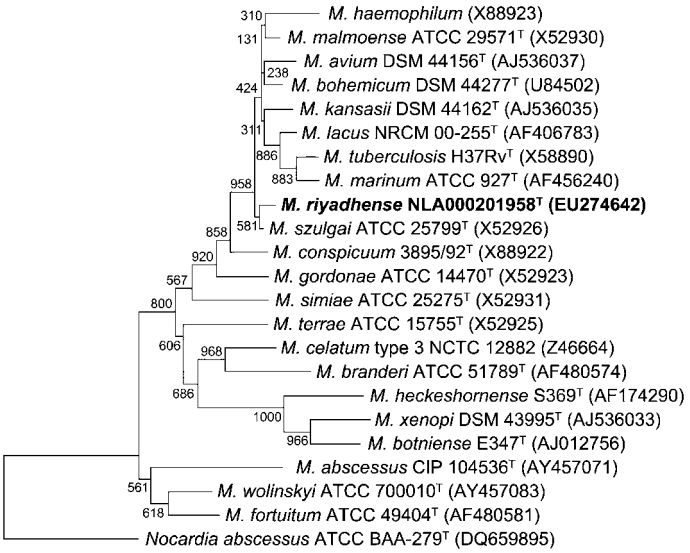

Fig. 1. Phylogenetic relationships of strain NLA000201958^T (*Mycobacterium riyadhense* sp. nov.) and related species of *Mycobacterium*, based on 16S rRNA gene sequences. Neighbour-joining tree created and bootstrapped 1000 times with CLUSTAL_X (Thompson *et al.*, 1997) and visualized with MEGA (Tamura *et al.*, 2007). Bootstrap values are indicated at nodes.

On Middlebrook 7H10, Ogawa and Stonebrink media, the strain produced small, rough, non-pigmented colonies after 28 days of incubation at 36 °C. After 3 days of exposure to light at ambient temperature, no pigmentation was observed. Growth on Middlebrook 7H10 agar was only observed at 24, 30 and 36 °C. Optimal growth occurred at 36 °C. Colony morphology on Middlebrook 7H10 agar was similar at all temperatures.

Generally, the biochemical profile of the isolate is unique, although it shares characteristics with *M. szulgai* and *Mycobacterium malmoense* (Marks *et al.*, 1972). The isolate was negative for niacin accumulation, heat-stable catalase (pH 7, 68 °C), β-glucosidase, tellurite reduction, growth on MacConkey agar and tolerance to *p*-nitrobenzoic acid, hydroxylamine and oleic acid, but positive for nitrate reduction, semiquantitative catalase, Tween 80 hydrolysis, 3 day arylsulfatase and urease activity and tolerance to TCH, thiacetazone and isoniazid. A comparison with profiles of *M. szulgai* and *M. malmoense* is recorded in Table 2.

HPLC revealed a pattern characterized by a single, narrow, late-emerging cluster of peaks. A similar profile is presented by a limited number of mycobacteria (*Mycobacterium brumae*, *M. fallax*, *M. triviale* and *M. tuberculosis* complex), with none of them fully overlapping the pattern of the strain characterized here (Fig. 2). We

Table 2. Biochemical identification results of strain NLA000201958^T and related species

+/−, Strain-dependent reaction.

Test	Strain NLA000201958^T	*M. szulgai*	*M. malmoense*
Nitrate reduction	+	+	−
68 °C Catalase	−	+	+/−
Catalase >45 mm	+	+	−
β-Glucosidase	−	+/−	−
Tween 80 hydrolysis	+	+/−	+
Tellurite reduction	−	+/−	+
3 day Arylsulfatase	+	+	−
Urease	+	+	−
Pigmentation	Absent	Photochromogen	Absent
Colony morphology	Rough	Smooth/rough	Smooth
Growth at 25 °C	+	+	+/−
Tolerance to:			
p-Nitrobenzoic acid	−	+	+
Isoniazid (500 μg ml^{-1})*	+	+/−	+
Hydroxylamine (1 μg ml^{-1})*	−	−	+/−

*In Middlebrook 7H10 agar.

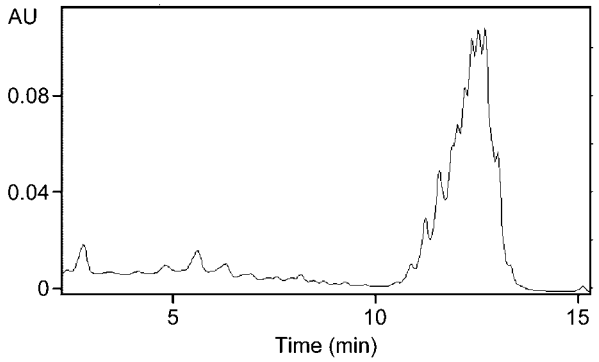

Fig. 2. Mycolic acid pattern of strain NLA000201958^T (*M. riyadhense* sp. nov.) obtained by HPLC analysis. The single, narrow, late-emerging cluster of peaks is similar, though not identical, to results for *M. brumae*, *M. fallax*, *M. triviale* and *M. tuberculosis* complex.

used the HPLC mycobacterium library (http://www.mycobactoscana.it/English.htm) for this comparison.

Drug susceptibility testing (minimal inhibitory concentrations given in μg ml^{-1}) revealed *in vitro* resistance to amikacin (10) and *p*-aminosalicylate (>1), intermediate susceptibility to isoniazid (1) and susceptibility to rifampicin (0.2), rifabutin (<0.2), ethambutol (5), clarithromycin (<2), ciprofloxacin (2), cycloserine (20), prothionamide (<1), clofazimine (<0.5) and streptomycin (5).

Although identification of *M. tuberculosis* complex is not the main use of the GenoType CM test, false-positive results may lead to incorrect diagnoses of tuberculosis and unwarranted treatment. Aside from the identification as *M. tuberculosis* by a line-probe assay, which sparked this study, both the molecular and HPLC analyses of the novel strain suggest a phylogenetic relationship with *M. tuberculosis*; the presence of an RD1 region strengthens this assumption. Its identity as a non-tuberculous mycobacterium is easily proven by sequencing of the 16S rRNA, *rpoB*, *hsp65* or RD1 genes and the absence of IS*6110* and IS*1081* elements. The novel strain seems closely related to *M. szulgai*, based on 16S rRNA gene and ITS sequences (Fig. 1 and Supplementary Fig. S1a), although it is related more distantly to *M. szulgai* based on *rpoB* and *hsp65* sequences (Table 1 and Supplementary Fig. S1b). Its non-chromogenicity, however, is distinct from the photochromogenic *M. szulgai*.

Retrospectively, this isolate seems clinically relevant, based on its isolation from a normally sterile body site and the symptomatic improvement of the patient after 9 months of tuberculosis therapy (Griffith *et al.*, 2007). This suggests that the novel strain was the causative agent of this patient's disease.

Both the close phylogenetic relationship with species such as *M. szulgai* and *Mycobacterium kansasii*, which are among the most pathogenic non-tuberculous mycobacteria (Griffith *et al.*, 2007; van Ingen *et al.*, 2008), and the presence of an RD1 region with *esat-6* and *cfp-10* genes, which is a virulence factor in *M. tuberculosis* (Lewis *et al.*, 2003), add to our view that this strain is a human pathogen. On the basis of the data presented here, we describe a novel species to accommodate strain NLA000201958^T, for which we propose the name *Mycobacterium riyadhense* sp. nov.

Description of *Mycobacterium riyadhense* sp. nov.

Mycobacterium riyadhense (ri.ya.dhen′se. N.L. neut. adj. *riyadhense* after Riyadh, capital of the Kingdom of Saudi Arabia and origin of the patient from whom the type strain was isolated).

Slowly growing, non-tuberculous mycobacterium that produces rough, white colonies after 28 days of incubation at 36 °C; growth is slower at 25 and 30 °C and no growth occurs at 42 °C. Incorrectly identified as *M. tuberculosis* complex by the Hain GenoType CM assay. Negative for niacin accumulation, heat-stable catalase, β-glucosidase, tellurite reduction, growth on MacConkey agar and tolerance to *p*-nitrobenzoic acid, hydroxylamine and oleic acid, but positive for nitrate reduction, semiquantitative catalase, Tween 80 hydrolysis, arylsulfatase and urease activity and tolerance to TCH, thiacetazone and isoniazid. Readily identifiable by its unique rRNA gene sequences.

The type strain is NLA000201958^T (=CIP 109808^T =DSM 45176^T), recovered from maxillary sinus lavage fluid.

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