Mycobacterium setense sp. nov., a *Mycobacterium fortuitum*-group organism isolated from a patient with soft tissue infection and osteitis

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A Gram-positive, rod-shaped acid-fast bacterium was isolated from a patient with a post-traumatic chronic skin abscess associated with osteitis. Morphological analysis, 16S rRNA, *hsp65*, *sodA* and *rpoB* gene sequence analysis, cell-wall fatty acid and mycolic acid composition analyses and biochemical tests showed that the isolate, designated ABO-M06^T, belonged to the genus *Mycobacterium*. Its phenotype was unique and genetic and phylogenetic findings suggest that strain ABO-M06^T represents a novel species within the *Mycobacterium fortuitum* group. The name *Mycobacterium setense* sp. nov. is proposed for this novel species, with the type strain ABO-M06^T (=CIP 109395^T=DSM 45070^T).

The *Mycobacterium fortuitum* group traditionally included three taxa, *Mycobacterium fortuitum*, *Mycobacterium peregrinum* and the unnamed third biovariant complex. It is defined by growth in less than 7 days, absence of pigmentation, 3 day arylsulfatase production, nitrate reduction, iron uptake and growth in the presence of 5 % NaCl. With the combination of molecular methods and phenotypic criteria, reports of novel species within the group have steadily increased during the past decade and the subject has become complicated. To date, the *M. fortuitum* 16S rRNA gene clade is composed of *Mycobacterium fortuitum*, *Mycobacterium peregrinum*, *Mycobacterium senegalense*, *Mycobacterium porcinum*, *Mycobacterium houstonense*, *Mycobacterium neworleansense*, *Mycobacterium boenickei*, *Mycobacterium conceptionense*, *Mycobacterium septicum* and *Mycobacterium alvei* (Schinsky *et al.*, 2000, 2004; Brown-Elliott & Wallace, 2002; Adékambi *et al.*, 2006). Debate still persists on whether *Mycobacterium mageritense* belongs to the *M. fortuitum* group (Brown-Elliott & Wallace, 2002; Adékambi & Drancourt, 2004).

M. fortuitum-group members cause a variety of human infections (Brown-Elliott & Wallace, 2002, 2005). Accurate bacterial identification, rising from a relevant classification, is crucial as antimicrobial resistance is species-dependent (Wallace *et al.*, 1991).

Here we describe a new member of the *M. fortuitum* group. The isolate, designated ABO-M06^T, was obtained from a patient with post-traumatic soft tissue infection and osteitis. A polyphasic study showed a unique genotype and phenotype, suggesting that this strain is representative of a novel species.

Strain ABO-M06^T was isolated from a 52-year-old patient admitted to Bassin de Thau Hospital, Sète, France, for a chronic soft tissue infection of the left foot that occurred after he stepped on a nail. The local infection was later complicated with osteitis and tenosynovitis. The organism was initially isolated after a 21 day culture on Löwenstein–Jensen slants incubated at 37 and 30 °C. It was subcultured on Löwenstein–Jensen slants and blood agar plates, and formed colonies in less than 7 days at 20, 25 and 37 °C. Strain ABO-M06^T was a Gram-positive, acid-fast bacterium initially identified as *M. peregrinum* with the INNO-LiPA method (Innogenetics) and the patient was treated

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *hsp65*, *sodA* and *rpoB* gene sequences of strain ABO-M06^T are EF138818, EF138819, EF138820 and EF414447.

Supplementary figures showing the cell wall fatty acid analysis and phylogenetic trees are available with the online version of this paper.

with clarithromycin (1 g per day for 30 days). However, treatment failure led us to go further into bacterial identification. The initial isolate was maintained in glycerol suspension (10 % v/v) at −80 °C.

The isolate was examined for pigmentation and morphological characteristics as described previously (Schinsky *et al.*, 2004). Arylsulfatase (3 day) and catalase activities, iron uptake and growth on Löwenstein–Jensen medium containing 5 % NaCl were tested as described by Vincent *et al.* (2003). We also inoculated API Coryne, API 20E and API 20NE strips (bioMérieux) as recommended by the manufacturer, and incubated them for 5 days at 30 and 37 °C in a highly humidified atmosphere (Adékambi *et al.*, 2006). Standard fatty acid and mycolic acid analyses were performed by the Deutsche Sammlung von Mikroorganismen und Zellkulturen by means of gas chromatography and HPLC, respectively, as previously described (Butler *et al.*, 1992), using standard Microbial Identification System software (MIDI). The broth microdilution method was used to test susceptibility to amikacin, clarithromycin, cefoxitin, doxycycline, gatifloxacin, moxifloxacin, sulfamethoxazole and tobramycin, as recommended by the Clinical Laboratory Standards Institute (NCCLS, 2002, 2003). Imipenem susceptibility was tested with the disc diffusion method on 5 % sheep blood agar plates incubated for 3 days at 30 °C.

A multigene sequencing approach was used, according to Adékambi & Drancourt (2004) and Devulder *et al.* (2005). Double-strand partial sequences were obtained for four genes amplified as described elsewhere, namely (i) 16S rRNA gene [near-complete sequence of 1361 nt, corresponding to positions 46–1400 of the *Escherichia coli* numbering system (Rodríguez-Nava *et al.*, 2004)], (ii) *rpoB* gene [encoding the β-subunit of bacterial RNA polymerase; 764 nt corresponding to positions 2573–3337 of *Mycobacterium smegmatis* ATCC 14468 (Adékambi *et al.*, 2003)], (iii) *hsp65* gene [65 kDa heat-shock protein; 441 nt corresponding to positions 396–836 of *Mycobacterium tuberculosis* CIP 105795 (Ringuet *et al.*, 1999)], and (iv) *sodA* gene [superoxide dismutase, 442 nt corresponding to positions 82–523 of *Nocardia farcinica* IFM 10152 (Zolg & Philippi-Schulz, 1994)]. For phylogenetic analysis, these sequences (excluding the primer sequences used for amplification) were compared with sequences in the GenBank and EMBL databases using the BLAST (Altschul *et al.*, 1997), LALIGN (www.expasy.org) and leBIBI programs (Devulder *et al.*, 2003). Gene sequences were selected from GenBank for the type strains of the 13 or 14 most closely related *Mycobacterium* species and aligned with CLUSTAL_X 1.83 (Thompson *et al.*, 1997). The alignment was checked and corrected manually before reconstruction of phylogenetic trees. The phylogenetic trees were constructed with the software packages MEGA (Kumar *et al.*, 2004) and PHYLO_WIN (Galtier *et al.*, 1996), using the maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Kluge & Farris, 1969) and neighbour-joining (Saitou & Nei, 1987) treeing algorithms. Tree robustness was determined by using 1000 bootstrapped datasets.

The morphological and chemotaxonomic characteristics of strain ABO-M06^T corresponded to those of the genus *Mycobacterium* and are detailed in the species description. Phenotype characteristics that differ from closely related species are shown in Table 1. Fatty acid analysis showed straight-chain saturated and unsaturated fatty acids, as expected for a member of the genus *Mycobacterium*. Major fatty acids included $C_{16:0}$ (37.8 %), $C_{18:1}\omega9c$ (24.1 %), tuberculostearic acid (10-methyl $C_{18:0}$) (9.7 %), $C_{16:1}$ (7.6 %) and $C_{14:0}$ (7.5 %). Cell wall analysis showed mycolic acids typically found in mycobacteria (long-chain mycolic acids *sensu stricto*), with three major groups of peaks (Supplementary Fig. S1; available in IJSEM Online).

Table 1. Differential phenotypic characteristics of *Mycobacterium setense* ABO-M06^T and related rapidly growing species

Strains: 1, *M. setense* ABO-M06^T; 2, *M. fortuitum* CIP 104534^T; 3, *M. peregrinum* CIP 105382^T; 4, *M. houstonense* ATCC 49403^T; 5, *M. conceptionense* CIP 108544^T; 6, *M. porcinum* CIP 105392^T; 7, *M. septicum* ATCC 700731^T; 8, *M. senegalense* CIP 104941^T; 9, *M. neworleansense* ATCC 49404^T; 10, *M. boenickei* CIP 107829^T; 11, *M. alvei* CIP 103464^T; 12, *M. wolinskyi* ATCC 700010^T; 13, *M. mageritense* CIP 104973^T. Data from Adékambi *et al.* (2006), Brown *et al.* (1999), Schinsky *et al.* (2000, 2004) and Wallace *et al.* (2002). −, Negative; +, positive; ND, no data available.

Test	1	2	3	4	5	6	7	8	9	10	11	12	13
Growth on Löwenstein–Jensen slant at 42 °C	−	+	−	+	−	−	−	+	−	−	−	+	+
Arylsulfatase activity at 3 days	+	+	+	+	+	+	−*	+	+	+	+	−	+
Growth on carbon source:													
D-Mannitol	+	−	+	+	−	−	+	+	+	+	−	+	+
myo-Inositol	−	−	−	+	+	+	+	−	+	+	−	+	+
Sorbitol	−	−	−	+	−	−	−	−	−	−	ND	+	+
D-Glucose	+	+	ND	+	−	−	ND	ND	ND	ND	+	ND	ND
Citrate utilization	−	−	−	−	−	−	−	+	−	−	−	+	−
Gelatinase activity	+	−	ND	ND	−	−	ND	ND	ND	ND	ND	ND	ND

*Positive for arylsulfatase activity at 14 days.

The fatty acid and mycolic acid elution profile did not correspond to a specific *Mycobacterium* species stored in the MIDI databases and was clearly distinct from *M. fortuitum* (Butler & Kilburn, 1990; Butler & Guthertz, 2001). Although it showed some similarities with patterns from members of the *M. fortuitum* group, the mycolic acid elution profile is characteristic and strain ABO-M06^T can be differentiated from these species by quantitative differences in the mycolic acid composition.

The isolate was susceptible to imipenem, moxifloxacin, gatifloxacin, cefoxitin and amikacin, intermediate to tobramycin and clarithromycin, and resistant to doxycycline, erythromycin and sulfamethoxazole. These results were in keeping with the clinical data, as clarithromycin was ineffective while levofloxacin–amikacin (1 g each per day) therapy for 30 days eradicated the infection.

The gene sequences of strain ABO-M06^T differed markedly from those of *Mycobacterium* species with validly published names. These differences were 0.68 % in the 16S rRNA gene sequence (corresponding to 9 differences among 1336 nucleotides) with *M. houstonense* ATCC 49403^T and *M. senegalense* CIP 104534^T, 4.64 % in *rpoB* gene sequence (32 differences among 690 nucleotides) with *M. conceptionense* CIP 108544^T, 1.56 % in *hsp65* gene sequence (6 differences among 387 nucleotides) with *M. houstonense* ATCC 49403^T and 3.8 % in *sodA* gene sequence (15 differences among 395 nucleotides) with *M. septicum* ATCC 700731^T. As previously described in the genus *Mycobacterium*, the *sodA* and *rpoB* genes were found to be more discriminatory than the 16S rRNA and *hsp65* genes for differentiation of strain ABO-M06^T from closely related species. Moreover, partial *rpoB* gene sequence analysis supported the affiliation of strain ABO-M06^T to a novel species according to Adékambi *et al.* (2003), who have shown that intraspecies and interspecies variabilities in partial *rpoB* gene sequences are <1.7 % and >3 %, respectively, for rapidly growing mycobacteria. This was confirmed by partial *sodA* gene sequence analysis. Although data on intraspecies variability are lacking, interspecies variability on this gene fragment was found to be $\geqslant 0.75$ % for rapidly growing mycobacteria.

The evolutionary trees inferred from the three treeing algorithms gave congruent results (data not shown) and the neighbour-joining trees are shown in Figs 1 and 2 and Supplementary Figs S2 and S3. Phylogenetic analysis of the near-complete 16S rRNA gene sequence indicated that strain ABO-M06^T belonged to the *M. fortuitum* group (Fig. 1). A similar result was observed after phylogenetic analysis based on *rpoB* (Fig. 2), *hsp65* (Supplementary Fig. S2) and *sodA* (Supplementary Fig. S3) gene sequences. Independent lineage was observed in all the trees reconstructed for the strain ABO-M06^T. The species most closely related to strain ABO-M06^T differed with the phylogenetic marker, but the bootstrap values were too low (below 50 %) to induce much confidence on any phylogenetic relatedness within the *M. fortuitum* group. The percentages of gene sequence difference, together with the marked evolutionary distances and lack of congruence observed in four-gene sequence analysis, showed that isolate ABO-M06^T was clearly distinct from the nearest species. Together with the unique phenotype, these results warranted its classification as a novel *Mycobacterium* species belonging to the *M. fortuitum* group, for which we propose the name *Mycobacterium setense* sp. nov.

Description of *Mycobacterium setense* sp. nov.

Mycobacterium setense [se.ten′se N.L. neut. adj. *setense* pertaining to Sète (France), the city from which the infected patient originated].

Cells are acid-fast, Gram-positive and pleomorphic rods. Colonies are smooth, convex, round, entire-edged, non-pigmented (beige) and small (approx. 1 mm in diameter). They do not produce aerial hyphae. The cells grow on 5 % sheep blood agar and Löwenstein–Jensen agar within 2 to 4 days at 25 °C, 30 °C (optimum) and 37 °C when

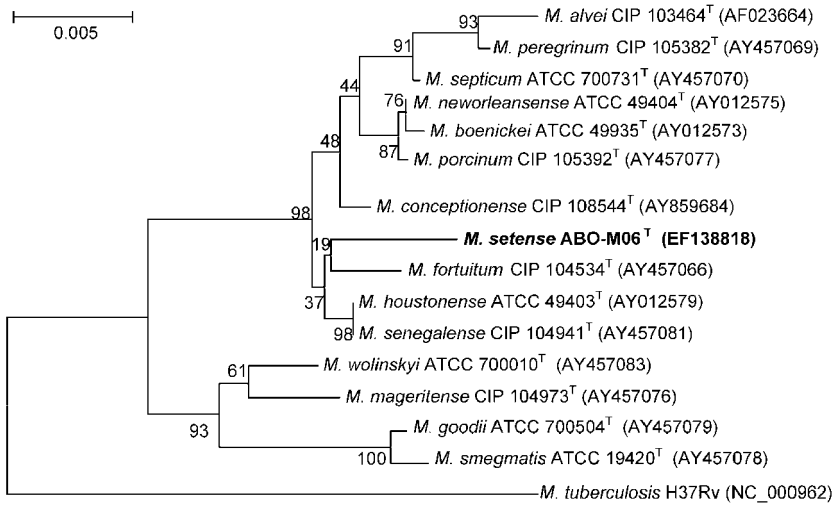

Fig. 1. Phylogenetic tree based on the 16S rRNA gene sequence showing the relationship of *Mycobacterium setense* ABO-M06^T with the 14 most closely related *Mycobacterium* species. This tree was constructed by using the neighbour-joining method and was based on a comparison of a stretch of 1336 nt. The tree was rooted using *M. tuberculosis* H37Rv as the outgroup. Bootstrap values are indicated by the value at each node as a percentage of 1000 replications. The different branches were supported by the results of three different algorithms. Bar, 0.5 % difference in nucleotide sequence.

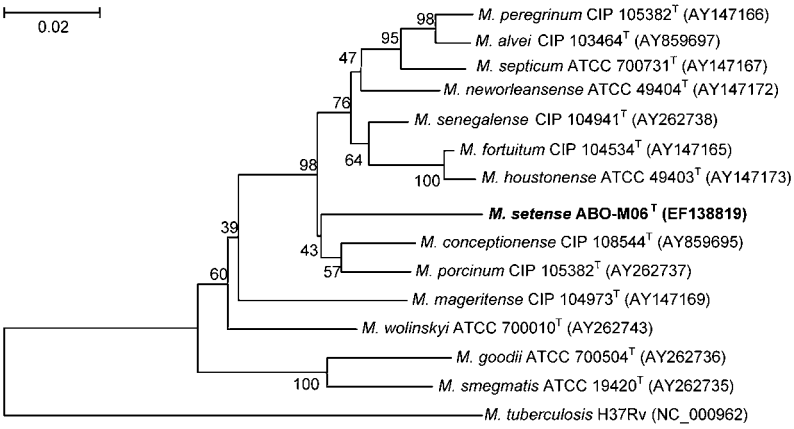

Fig. 2. Phylogenetic tree based on a partial *rpoB* gene sequence, showing the relationship of *M. setense* ABO-M06^T with the 13 most closely related *Mycobacterium* species. See Fig. 1 for details. *M. boneickei* ATCC 49935^T was not included because its *rpoB* gene sequence is absent from the GenBank database. Bar, 2 % difference in nucleotide sequence.

subculturing. They also grow in the presence of 5 % NaCl. No growth occurs at 42 °C. Positive for 3 day arylsulfatase production, iron uptake and thermostable catalase activity. It utilizes D-mannitol and D-glucose, but not D-inositol, L-rhamnose or L-arabinose, or citrate as the sole carbon source. It is positive for pyrazinamidase, alkaline phosphatase, nitrate reductase, urease and gelatinase activities. *M. setense* ABO-M06^T sp. nov. belongs to the *M. fortuitum* group and can be differentiated phenotypically from other species of this group as follows. It differs from *M. conceptionense* CIP 108544^T by D-glucose and D-mannitol utilization, lack of inositol degradation and gelatinase activity. It differs from *M. porcinum* CIP 105392^T by D-glucose utilization and lack of inositol degradation, from *M. houstonense* ATCC 49403^T by the lack of inositol degradation and failure to grow at 42 °C, from *M. fortuitum* CIP 105534^T by failure to grow at 42 °C and by D-mannitol utilization, and from *M. septicum* ATCC 700731^T by lack of inositol degradation. It exhibits unique cellular fatty acid and mycolic acid patterns. Strain ABO-M06^T shows 99.32 % 16S rRNA gene similarity with *M. houstonense* ATCC 49403^T and 95.36 % *rpoB* gene similarity with *M. conceptionense* CIP 108544^T, the phylogenetically closest type strains.

The type strain, ABO-M06^T (=CIP 109395^T=DSM 45070^T), was recovered from an excised skin and soft tissue specimen in a context of osteitis.

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References

Adékambi, T. & Drancourt, M. (2004). Dissection of phylogenetic relationships among 19 rapidly growing *Mycobacterium* species by 16S rRNA, *hsp65*, *sodA* and *rpoB* gene sequencing. *Int J Syst Evol Microbiol* **54**, 2095–2105.

Adékambi, T., Colson, P. & Drancourt, M. (2003). *rpoB*-based identification of nonpigmented and late-pigmented rapidly growing mycobacteria. *J Clin Microbiol* **41**, 5699–5708.

Adékambi, T., Stein, A., Carvajal, J., Raoult, D. & Drancourt, M. (2006). Description of *Mycobacterium conceptionense* sp. nov., a *Mycobacterium fortuitum* group organism isolated from a posttraumatic osteitis inflammation. *J Clin Microbiol* **44**, 1268–1273.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.

Brown, B. A., Springer, B., Steingrube, V. A. & other authors (1999). *Mycobacterium wolinskyi* sp. nov. and *Mycobacterium goodii* sp. nov., two new rapidly growing species related to *Mycobacterium smegmatis* and associated with human wound infection: a cooperative study from the International Working Group on mycobacterial taxonomy. *Int J Syst Evol Microbiol* **49**, 1493–1511.

Brown-Elliott, B. A. & Wallace, R. J., Jr (2002). Clinical and taxonomic status of pathogenic nonpigmented or late-pigmented rapidly growing mycobacteria. *Clin Microbiol Rev* **15**, 716–746.

Brown-Elliott, B. A. & Wallace, R. J., Jr (2005). Infections caused by nontuberculous *Mycobacteria*. In *Principles and Practice of Infectious Diseases*, 6th edn, pp. 2909–2916. Edited by G. L. Mandell, J. E. Bennett & R. Dolin. Philadelphia: Elsevier.

Butler, W. R. & Guthertz, L. S. (2001). Mycolic acid analysis by high performance liquid chromatography for identification of *Mycobacterium* species. *Clin Microbiol Rev* **14**, 704–726.

Butler, W. R. & Kilburn, J. O. (1990). High performance liquid chromatography patterns of mycolic acids as criteria for identification of *Mycobacterium chelonae*, *Mycobacterium fortuitum* and *Mycobacterium smegmatis*. *J Clin Microbiol* **28**, 2094–2098.

Butler, W. R., Thibert, L. & Kilburn, J. O. (1992). Identification of *Mycobacterium avium complex* strains and some similar species by high performance liquid chromatography. *J Clin Microbiol* **30**, 2698–2704.

Devulder, G., Perrière, G., Baty, F. & Flandrois, J. P. (2003). BIBI, a bioinformatics bacterial identification tool. *J Clin Microbiol* **41**, 1785–1787.

Devulder, G., Pérouse de Montclos, M. & Flandrois, J. P. (2005). A multigene approach to phylogenetic analysis using the genus *Mycobacterium* as a model. *Int J Syst Evol Microbiol* **55**, 293–302.

Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* **17**, 368–376.

Galtier, N., Gouy, M. & Gautier, C. (1996). SEAVIEW and PHYLO_WIN, two graphic tools for sequence alignment and molecular phylogeny. *Comput Appl Biosci* **12**, 543–548.

Kluge, A. G. & Farris, F. S. (1969). Quantitative phyletics and the evolution of anurans. *Syst Zool* **18**, 1–32.

Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.

NCCLS (2002). Performance standards for antimicrobial susceptibility testing. Twelfth informational supplement. M100–S12. Wayne, PA: NCCLS.

NCCLS (2003). Susceptibility testing of *Mycobacteria*, *Nocardia*, and other aerobic actinomycetes. Approved standard M24-A. Wayne, PA: NCCLS.

Ringuet, H., Akoua-Koffi, C., Honore, S., Varnerot, A., Vincent, V., Berche, P., Gaillard, J. L. & Pierre-Audigier, C. (1999). *hsp65* sequencing for identification of rapidly growing mycobacteria. *J Clin Microbiol* **37**, 852–857.

Rodríguez-Nava, V., Couble, A., Molinard, C., Sandoval, H., Boiron, P. & Laurent, F. (2004). *Nocardia mexicana* sp. nov., a new pathogen isolated from human mycetomas. *J Clin Microbiol* **42**, 4530–4535.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

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.

Schinsky, M. F., Morey, R. E., Steigerwalt, A. G. & other authors (2004). Taxonomic variation in the *Mycobacterium fortuitum* third biovariant complex: description of *Mycobacterium boenickei* sp. nov., *Mycobacterium houstonense* sp. nov., *Mycobacterium neworleansense* sp. nov. and *Mycobacterium brisbanense* sp.nov. and recognition of *Mycobacterium porcinum* from human clinical isolates. *Int J Sys Evol Microbiol* **54**, 1653–1667.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

Vincent, V., Brown-Elliott, B. A., Jost, K. C., Jr & Wallace, R. J., Jr (2003). *Mycobacterium*: phenotypic and genotypic identification. In *Manual of Clinical Microbiology*, 8th edn, pp. 560–584. Edited by P. M. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller & R. H. Yolken. Washington, DC: American Society for Microbiology.

Wallace, R. J., Jr, Brown, B. A., Silcox, V. A., Tsukamura, M., Nash, D. R., Steele, L. C., Steingrube, V. A., Smith, J., Sumter, G. & other authors (1991). Clinical disease, drug susceptibility, and biochemical patterns of the unnamed third biovariant complex of *Mycobacterium fortuitum*. *J Infect Dis* **163**, 598–603.

Wallace, R. J., Jr, Brown-Elliott, B. A., Hall, L., Roberts, G., Wilson, R. W., MannL. B., Crist, C. J., Chiu, S. H., Dunlap, R. & other authors (2002). Clinical and laboratory features of *Mycobacterium mageritense*. *J Clin Microbiol* **40**, 2930–2935.

Zolg, J. W. & Philippi-Schulz, S. (1994). The superoxide dismutase gene, a target for detection and identification of mycobacteria by PCR. *J Clin Microbiol* **32**, 2801–2812.