Mycobacterium parascrofulaceum sp. nov., novel slowly growing, scotochromogenic clinical isolates related to *Mycobacterium simiae*

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A group of pigmented, slowly growing mycobacteria identified by 16S rRNA gene sequencing as 'MCRO 33' (GenBank accession no. AF152559) have been isolated from several clinical specimens in various laboratories across Canada. Genotypically, the organism is most closely related to *Mycobacterium simiae*. However, it presents with a similar phenotypic profile to *Mycobacterium scrofulaceum*. Several reference strains obtained from ATCC and TMC culture collections, previously identified as *M. scrofulaceum* or *M. simiae*, have also been found to possess the MCRO 33 16S rRNA gene sequence. Biochemical testing, susceptibility testing, HPLC, *hsp65* gene and 16S–23S spacer (ITS1) sequencing were performed on clinical and reference strains to characterize further this unique species. Of the clinical strains, one was isolated from a cervix biopsy whereas all other clinical isolates were obtained from respiratory samples. In one patient, symptoms, imaging and repeat clinical specimens positive on culture for this organism were suggestive of active clinical disease. The description of this species, for which the name *Mycobacterium parascrofulaceum* sp. nov. is proposed, follows the present trend of a large number of novel *Mycobacterium* species identified due in great part to sequence-based methods. The type strain is HSC68^T (= ATCC BAA-614^T = DSM 44648^T).

INTRODUCTION

Sequence-based identification of non-tuberculous mycobacteria (NTM) has become a method of choice in several mycobacteriology laboratories and is accomplished by analysis of various targets, such as the 16S rRNA gene,

16S–23S spacer region (ITS1) and *hsp65* gene. As a result, we are faced with the discovery of many novel species previously undifferentiable using other methods of identification. These cannot be ignored, since current studies show that the detection of novel species is a common occurrence that will only be compounded with an increase in use of automated sequencers in clinical laboratories, with some of these unusual isolates deemed clinically significant (Tortoli *et al.*, 2001). The purpose of this study was to establish as a novel species a group of representative mycobacterial strains commonly found, not only in human clinical isolates obtained from our laboratory, but also in several past publications where evidence of its existence has been found. This species has shown similarities to several known NTM species using single identification methods, which may have been the cause for the delay in its establishment, but it is clearly a unique species as determined from a polyphasic approach. In one symptomatic patient, this organism was isolated in multiple respiratory

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Abbreviations: AFB, acid-fast bacilli; BAL, bronchoalveolar lavage; CT, computed tomography; NTM, non-tuberculous mycobacteria; PRA, PCR-restriction enzyme analysis.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence of strain HSC-68^T and the ITS1 and partial *hsp65* gene representing each sequevar are AY337273–AY337283.

Sequence identity and Hamming distances between *M. parascrofulaceum* and its closest established relatives, ITS1 sequence analysis and sequence alignment of sequevars I–V are available as supplementary material in IJSEM Online.

samples, and associated computed tomography (CT) scan and chest X-ray showed evidence of respiratory disease, as described below.

METHODS

Case history. A 41-year-old woman sought medical attention for post-landing immigration surveillance, having arrived in Canada from India in June 1999. She was asymptomatic and admitted to a history of previous pulmonary tuberculosis (TB) that was treated for 1 year in 1988 while she was in India. She denied previous BCG vaccination and TB skin testing. Her initial post-landing chest X-ray showed minimal right upper lobe scarring suggestive of previous TB. Three sputum samples collected for acid-fast bacilli (AFB) smear and culture were negative. Her medical follow-up included yearly chest X-rays that remained unchanged for 2 years post-immigration.

In February 2002, her annual screening chest X-ray showed a small peripheral cavitory lesion in the right mid-lung region. She denied symptoms of TB except for a dry cough. Sputum samples were repeated for AFB smear and culture. A CT scan of the chest showed multiple calcified granulomas with scarring in the right upper lobe and calcified pre-tracheal lymph nodes. An 18 mm thick-walled cavity in the superior segment of the right lower lobe associated with smaller satellite nodules (the largest of which measured 12 mm) was also visible. No air–fluid level was visible. Fibre-optic bronchoscopy and bronchoalveolar lavage (BAL) was performed. Samples were negative for AFB on smear. However, both sputum and bronchoscopy specimens grew an atypical mycobacterium, later identified by 16S rRNA gene sequencing as *Mycobacterium* sp. strain MCRO 33 (described herein as *Mycobacterium parascrofulaceum* sp. nov.). Bronchoscopy specimens were negative for malignancy.

Repeat chest X-rays in October 2002 confirmed an enlarging cavity in the right lower lobe. Sputum samples were obtained and empirical anti-TB treatment was instituted: daily isoniazid 300 mg, rifampicin 600 mg, ethambutol 800 mg, pyrazinamide 1 g, levofloxacin 500 mg

and vitamin B₆ 25 mg, a regimen chosen due to concerns for atypical mycobacteria and known associated drug resistance. Her human immunodeficiency virus status was negative. After 2 months of treatment, the cavity continued to increase in size and wall thickness. Medication side-effects included oral dryness and persistent arthralgias. Liver tests remained within normal limits. Discontinuation of pyrazinamide led to resolution of her joint symptoms. The scotochromogenic mycobacterium was again isolated from her sputum and, in January 2003, the final identification and sensitivities of the organism became available. The organism was resistant to streptomycin, isoniazid, low-dose ethambutol and levofloxacin and was sensitive to rifampicin, high-dose ethambutol, clarithromycin, kanamycin, ethionamide and amikacin. On this basis, the treatment regimen was adjusted to include clarithromycin 500 mg twice daily and high-dose ethambutol 1200 mg and rifampicin 600 mg once daily, a regimen that was well tolerated and which resulted in resolution of her cough.

A follow-up CT scan of the chest was performed after 3 months and showed a decrease in the thickness of the cavity wall although the overall size of the cavity had increased from 18 to 22 mm in maximal dimension. The dominant satellite nodule had reduced in size from 12 mm to less than 5 mm. The other surrounding satellite nodules measuring less than 5 mm were still present and remained unchanged. Sputum cultures are now negative for *M. parascrofulaceum*.

Strain selection. Strains used in this study were obtained in three ways. Table 1 lists 12 strains obtained from patient samples. Five of these, designated ‘HSC’, were identified by 16S rRNA gene sequencing as part of a retrospective analysis of Accuprobe-negative slowly growing NTM strains isolated at the Health Sciences Centre Mycobacteriology Laboratory, Winnipeg, Manitoba, from 1981 until 1999 (Pauls *et al.*, 2003). These five strains had been previously identified as *Mycobacterium scrofulaceum* by conventional phenotypic testing and morphology. The other seven strains, designated ‘U’, were submitted to the National Reference Centre for Mycobacteriology for identification by other provincial public health laboratories from 2001 to 2003. Table 2 lists reference strains from the ATCC and TMC at our disposal, designated *M. scrofulaceum* or

Table 1. Demographics of clinical strains of *Mycobacterium parascrofulaceum* sp. nov.

Isolates from Manitoba were initially identified as *M. scrofulaceum* based on biochemical testing. Various sequevars for the 441 bp Telenti fragment of the *hsp65* gene and the ITS1 spacer region are indicated for each strain. Location reflects Canadian province from which strains were isolated (BC, British Columbia). CIN, Cervical intraepithelial neoplasia.

Strain	<i>hsp65</i>	ITS1	Location	Year	Source	Comments
HSC68	I	I	Manitoba	1981	Cervix biopsy	Female, 21-year-old; diagnosed with CIN I–II
HSC87	I	I	Manitoba	1982	Sputum	Female, 59-year-old
HSC90	I	I	Manitoba	1982	Sputum	Female
HSC273	I	I	Manitoba	1983	Sputum	Male, 77-year-old; squamous cell carcinoma in right main bronchus
HSC2054	I	I	Manitoba	1996	Sputum	Female, 78-year-old
U01260	IV	IV	BC	2001	Sputum	64-year-old
U02127	V	I	Alberta	2002	Sputum	Female, 70-year-old
U02307	II	IIa	BC	2002 (Feb)	Bronchial washing	Refer to case report
U02505	II	IIa		2002 (Oct)	Sputum	Refer to case report
U02532	III	III	BC	2002	Bronchial washing	Female, 72-year-old
U03053	I	I	BC	2003	Sputum	Male, 70-year-old
U03081	II	IIb	BC	2003	Bronchial washing (right upper lobe)	Male, 71-year-old

Table 2. Sequence-based identification of non-type reference strains initially identified as *M. scrofulaceum* or *M. simiae*

Strains were identified by sequencing of the 16S rRNA gene, *hsp65* and ITS1 region. Results indicate 100% sequence identity with the designated type strain of either *M. scrofulaceum sensu stricto* (1) or *M. parascrofulaceum* sp. nov. (2), unless stated otherwise. ND, Not determined.

Strain	16S rRNA	<i>hsp65</i>	ITS1
<i>M. scrofulaceum</i> TMC 1302 (=ATCC 35785)	2	2	2
<i>M. scrofulaceum</i> ATCC 35786	1	1	1 (with 1 nt difference)
<i>M. scrofulaceum</i> TMC 1306 (=ATCC 35787)	2	2	2
<i>M. scrofulaceum</i> TMC 1307 (=ATCC 35788)	2	2	2
<i>M. scrofulaceum</i> TMC 1309 (=ATCC 35789)	1	ND	1 (with 1 nt difference)
<i>M. scrofulaceum</i> TMC 1312 (=ATCC 35790)	1	ND	1
<i>M. scrofulaceum</i> TMC 1314 (=ATCC 35791)	1	1	1
<i>M. scrofulaceum</i> TMC 1315 (=ATCC 35792)	1	1	1
<i>M. scrofulaceum</i> TMC 1316 (=ATCC 35793)	2	2	2
<i>M. scrofulaceum</i> TMC 1320 (=ATCC 35794)	2	2	ND
<i>M. simiae</i> ATCC 15080	2	2	2

Mycobacterium simiae, and deemed potential candidates for belonging to this novel species group due to phenotypic similarities. Strains *M. scrofulaceum* ATCC 19981^T and *M. simiae* ATCC 25275^T were also included for comparative purposes. All strains had been stored at -80 °C in skimmed milk with glycerol and were subcultured to Bactec12B liquid medium and Middlebrook 7H10 agar at 37 °C for testing.

Phenotypic properties. A standard series of biochemical tests, growth temperatures and morphological examination for mycobacterium characterization were performed as described by Kent & Kubica (1985); these characteristics are listed in Table 3.

Susceptibility testing was performed using serial twofold broth microdilution as described by Brown *et al.* (1992), Brown-Elliott *et al.* (2003), NCCLS (2003) and Wallace *et al.* (1986). Microdilution plates were prepared in-house using Middlebrook 7H9 broth (Difco Laboratories) supplemented with 5% oleic acid/albumin/glucose enrichment. Seven or eight concentrations were tested for each antimicrobial agent. Stock concentrations of the following antimicrobial agents were prepared from manufacturer's powders:

rifampicin, ethambutol, isoniazid, amikacin and sulfamethoxazole (Sigma), ciprofloxacin and moxifloxacin (Bayer), rifabutin and linezolid (Pharmacia), streptomycin (ICN), clarithromycin (Abbott) and levofloxacin (Jansen-Othro). The isolates were tested at a final inoculum of 10³–10⁴ c.f.u. ml⁻¹. Microdilution plates were sealed in plastic bags and incubated at 37 °C until macroscopic growth was sufficient for interpretation (7–14 days). Minimum inhibitory concentration (MIC) was defined as the lowest concentration of drug to inhibit visible growth with the exception of sulfamethoxazole, for which the MIC is determined at 80% inhibition of growth compared to the growth control well. Quality control was performed using *Mycobacterium avium* ATCC 700898, *Mycobacterium marinum* ATCC 927^T (Wallace *et al.*, 1986) and *M. avium* NJ-9141 (Heifets *et al.*, 1993). *Staphylococcus aureus* ATCC 29213 was used as a quality control strain in cation-supplemented Mueller–Hinton broth when MIC ranges for mycobacteria were not available.

HPLC. Analysis of the cell wall mycolic acids was performed by HPLC with UV detection according to standard methods (Butler *et al.*, 1996). Briefly, the mycolic acids were cleaved from the cells by saponification, extracted with chloroform and then derivatized to

Table 3. Phenotypic characteristics of *M. parascrofulaceum* sp. nov. clinical and reference strains (*n* = 18 unless otherwise indicated) in comparison with other slowly growing species

For the biochemical tests not listed in this table, *M. avium*, *M. simiae* and *M. scrofulaceum* present with the same results as for *M. parascrofulaceum* sp. nov. (see text). Data for reference species were taken from Goodfellow & Magee (2001), Kent & Kubica (1985) and Metchock *et al.* (1999). NG, No growth; V, variable.

Test	<i>M. parascrofulaceum</i> sp. nov.	<i>M. avium</i>	<i>M. simiae</i>	<i>M. scrofulaceum</i>
Growth at 42 °C	NG (100%)	+	NG	NG
Pigmentation*	S (100%)	N (85–87%)	P (90–91%)	S (97–99%)
Niacin	– (14/14; 100%)	– (99–100%)	+	– (99–100%)
Semi-quantitative catalase >45 mm	V (56%)	– (98%)	+	+
Arylsulfatase activity (14 days)	+	+	– (84%)	– (64%)
Tellurite reduction	– (10/10; 100%)	+	+	+
Urease activity	+	–	+	+

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

UV-absorbing bromophenacyl esters. The esters were separated in a column, where they were eluted using a graded series of methanol and methylene chloride. For identification of the species, visual comparison of patterns with those of recognized species, in-house and published data, is still the most reliable procedure. The reference strain of *Mycobacterium intracellulare* ATCC 13950^T was used as a control as well as a comparative strain.

Sequencing of the 16S rRNA gene and ITS1 region. A loopful of cells from pure culture was suspended in sterile distilled water, boiled for 10 min and then subjected to mechanical lysis using the Mini Bead-Beater (Biospec Products). The lysates were then centrifuged for 2 min at 12 000 g and the supernatant was used for PCR. Amplification of the 16S rRNA gene (Rogall *et al.*, 1990; Turenne *et al.*, 2001) and the ITS1 region (Harmsen *et al.*, 2002; Turenne *et al.*, 2002) was performed as previously described and the PCR products were directly sequenced using forward and reverse primers and loaded on an ABI PRISM 310 Genetic Analyzer capillary sequencer (Applied Biosystems). Sequence editing and phylogenetic analyses by CLUSTAL V (neighbour-joining method) were performed using the SEQMAN and MEGALIGN components of LASERGENE 5 (DNASTAR).

Sequencing and PCR-restriction enzyme analysis (PRA) of the *hsp65* gene. A 441 bp fragment of the *hsp65* gene was amplified from the DNA lysates prepared above using the protocol of Telenti *et al.* (1993). The resulting PCR product was sequenced using both the forward and the reverse PCR primers TB11 and TB12. Computer-generated PRA profiles of 441 bp sequences with enzymes *Hae*III and *Bst*EII were determined using MAPDRAW (DNASTAR) and were compared with available PRA profiles from PRASITE (<http://www.hospvd.ch/prasite>).

RESULTS AND DISCUSSION

Phenotypic properties

Acid-fast microscopy revealed pleomorphic forms of AFB with moderate beading. Growth was observed from 25 to 37 °C and was optimum at 37 °C, with colonies appearing after 2–4 weeks. Dysgonic growth was observed at 25 °C, with no growth at 42 °C. On Middlebrook 7H10 agar medium, all strains were scotochromogenic with yellow to orange pigmentation. Colony morphology varied among strains, with most appearing smooth and domed, one appearing dry and wrinkled (HSC2054), and one showing a combination of smooth and dry (ATCC 15080). Strain U01260 was mucoid on solid media. All isolates were positive for urease, 68 °C catalase and pyrazinamidase and negative for Tween 80 hydrolysis, nitrate reductase, iron uptake, tolerance to 5 % NaCl, growth on MacConkey agar, niacin, β -glucosidase, acid phosphatase and 3-day arylsul-fatase tests. Results were variable for the semi-quantitative catalase test and 14-day arylsulfatase test and were not attributed to any specific *hsp65* or ITS1 sequvars.

Susceptibility testing

According to current NCCLS guidelines for susceptibility testing of *M. avium* complex and resistance breakpoints for *Mycobacterium kansasii* (NCCLS, 2003), all *M. parascrofulaceum* isolates were sensitive to rifampicin, clarithromycin, amikacin and, with the exception of one isolate

Table 4. MIC₅₀ and MIC₉₀ values ($\mu\text{g ml}^{-1}$) of 17 isolates of *M. parascrofulaceum* sp. nov. as determined by broth microdilution

Of the two isolates from the case study, only U02307 was included.

Antimicrobial agent	Concentration range tested ($\mu\text{g ml}^{-1}$)	MIC ₅₀	MIC ₉₀
Rifampicin	0.06–8.0	≤ 0.06	≤ 0.06
Isoniazid	0.31–40.0	40.0	> 40.0
Streptomycin	0.63–80.0	2.5	20.0
Clarithromycin	1.0–128.0	≤ 1.0	≤ 1.0
Amikacin	0.63–80.0	5.0	10.0
Ciprofloxacin	0.125–16.0	8.0	16.0
Sulfamethoxazole	2.0–256.0	8.0	32.0
Linezolid	0.5–64.0	≤ 2.0	4.0
Moxifloxacin	0.03–4.0	0.5	> 4.0

(U02307), sulfamethoxazole (Table 4). Seventy-six per cent were susceptible to a streptomycin breakpoint of 10 $\mu\text{g ml}^{-1}$. All isolates were resistant to isoniazid. Eighty-eight per cent of isolates were resistant to an ethambutol breakpoint of 5 $\mu\text{g ml}^{-1}$ and 70 % were resistant to the ciprofloxacin breakpoint of 2 $\mu\text{g ml}^{-1}$. If proposed breakpoints for linezolid and slowly growing mycobacteria are followed, all isolates are susceptible to this antimicrobial (Brown-Elliott *et al.*, 2003). For moxifloxacin, 71 % of isolates had an MIC $\leq 1.0 \mu\text{g ml}^{-1}$. *M. scrofulaceum* ATCC 19981^T revealed a susceptibility pattern identical to the consensus profile for *M. parascrofulaceum* sp. nov. (data not shown).

Mycolic acid pattern by HPLC

The HPLC-generated pattern for isolate HSC-68^T revealed a clearly separated double cluster with prominent peaks in the early cluster. As illustrated in Fig. 1 with the reference control strain *M. intracellulare*, the pattern produced by this particular isolate of *M. parascrofulaceum* overlaps with and is indistinguishable from patterns seen with isolates of *M. avium* complex and some *M. scrofulaceum* isolates (Butler *et al.*, 1999).

Sequencing of the 16S rRNA gene

The nearly complete 16S rRNA gene sequence, from positions 28 to 1483 of the *Escherichia coli* sequence, was determined for strain HSC-68^T as well as the 5' region, from positions 54 to 510, of all subsequent isolates. All isolates were identical to strain HSC-68^T in the 5' region. Strains that showed some variation in either the ITS1 or *hsp65* sequence from the type strain were subsequently fully sequenced for the 16S rRNA gene, although no other mismatches from HSC-68^T were present. The sequence of *M. parascrofulaceum* contains the characteristic 12 bp omission (short helix) in the hypervariable region 18 (V3) observed in rapidly growing mycobacteria, *M. simiae* and other slowly growing species related to *M. simiae*, but which

Fig. 1. Mycolic acid patterns of *M. parascrofulaceum* sp. nov. strain HSC68^T (a) and *M. intracellulare* ATCC 13950^T (b) obtained by HPLC analysis. Peak designation is as previously suggested (Glickman *et al.*, 1994). HMM, High molecular mass internal standard.

is not found in *M. scrofulaceum*. Analysis of the nearly complete 16S rRNA gene sequence using BLAST against sequences from the GenBank, EMBL or DDBJ databases revealed an identical match with *Mycobacterium* sp. MCRO 33 (AF152559), followed by *Mycobacterium* sp. MCRO 19

(X93030) with 6 nt differences, and *Mycobacterium palustre* BAA-377^T (AJ308603) and *Mycobacterium* sp. MCRO 19 (X93030) both with 14 nt differences. The closest match using the RIDOM database (Harmsen *et al.*, 2002), from *E. coli* bp 54–510, was [*M. simiae*] ATCC 15080, with 100 % identity. This strain is not the type strain of *M. simiae* and the RIDOM results prompted us to include this strain among the misidentified reference strains suspected of belonging to *M. parascrofulaceum* sp. nov. The second closest match by RIDOM was *M. simiae* DSM 44165^T with seven mismatches. Complete 16S rRNA gene sequence comparisons with *M. simiae* revealed a 19 bp mismatch (98.6 % identity).

Final sequence analysis was done using our in-house sequence database (in combination with GenBank sequences) and evaluating from bases 54 to 1470 on the *E. coli* 16S rRNA gene (Fig. 2). Sequence identity and Hamming distances against the closest established species are available as Supplementary Table A in IJSEM Online. An alignment of hypervariable regions A and B reveals signature sequences in the A region (Fig. 3), with the B region being identical to that of *M. simiae*.

Sequencing of the ITS1

The ITS1 sequences determined for all strains identified as *M. parascrofulaceum* on the basis of 16S rRNA gene sequence analysis revealed five sequevars, designated sqv. I, IIa, IIb, III and IV. Sequevars I, IIa, IIb and III differ from each other by 1–2 bases only (Supplementary Fig. A in IJSEM Online). Sequevar IV is the most distant from sqv. I by 13 bp. Neither of the sequences corresponded to ITS1 sequences in public databases or RIDOM.

BLAST search analysis of sqv. I revealed closest similarity with various sequences designated *M. scrofulaceum*. Highest scores were for *M. scrofulaceum* KPM 2027 (AB026702) and *M. scrofulaceum* ATCC 19981^T (= TMC 1323^T) (L15622), both with five mismatches over ~350 bases, and identical to *M. scrofulaceum* ATCC 19981^T determined in-house. Shorter GenBank entries (280 bases) resulting in a lower score were in fact 100 % identical to sqv. I: *M. scrofulaceum* C58 (AJ314885) and *M. scrofulaceum* ATCC 19073 (AJ314884). As these are not representative of the type strain, we assume that these also belong to *M. parascrofulaceum*. RIDOM analysis results indicated 100 % identity over 280 bases with [*M. simiae*] ATCC 15080, followed by *M. scrofulaceum* S343 (four mismatches) and *M. scrofulaceum* DSM 43992^T (five mismatches) and finally sequences of *M. intracellulare*, with 28 nt differences and above.

Analysis of sqv. IV revealed a closest match with *M. scrofulaceum* DSM 43992^T (11 mismatches) using RIDOM and the same *M. scrofulaceum* sequences as determined for sqv. I using BLAST, but with 12–15 mismatches from entries corresponding to the type strain of *M. scrofulaceum* or 6–7 mismatches from entries corresponding to sqv. I.

Fig. 2. Phylogenetic tree of *Mycobacterium parascrofulaceum* sp. nov. with its closest genotypic and phenotypic relatives. CLUSTAL W analysis was performed using the MEGALIGN component of the LASERGENE software (DNASTAR). End gaps were removed to include positions 54 to 1470 of the *E. coli* 16S rRNA gene. *Nocardia asteroides* served as the outgroup sequence. Sequences were determined in our laboratory unless indicated by a GenBank accession number in parentheses.

80	129	177	
GTCTCTTCGGAGATA	CAATCTGCCCTGCACTTC	CGGATAGGACCACTTGGCGCATGCCTTGT	<i>M. parascrofulaceum</i>
. C. C. G. G. .			MCRO 19 (X93030)
. C. C. G. G. .			<i>M. scrofulaceum</i>
. C. C. G. G. .			<i>M. simiae</i>
. C. G.		 TT.	<i>M. lentiflavum</i>
.	 A.	 T. GA.	<i>M. heidelbergense</i>
. C. C. G. G. .		 TT. A.	<i>M. palustre</i> (AJ308603)
. C. C. G. G. .	TG. A.	 TGA. AT. T. A.	<i>M. kubicae</i>

Fig. 3. Alignment of hypervariable region A (V2) of the 16S rRNA gene. Nucleotide positions indicated are according to the *E. coli* sequence. With the exception of one base difference in strain MCRO 19, all strains listed in the alignment have an identical hypervariable region B sequence and this is therefore not included. Species are represented by the corresponding type strain. Sequences were determined in our laboratory unless indicated by a GenBank accession number.

Sequencing and PRA of the *hsp65* gene

Sequences of the *hsp65* 441 bp region of strains of *M. parascrofulaceum* revealed five distinct sequevars, sqv. I–V (Table 5; Supplementary Fig. B), with one or two base variations from each other, with the exception of strains U02532 (sqv. II) and U02127 (sqv. V), differing by 17 and 23 bases from sqv. I, respectively. Sequevar I was the most prevalent, followed by sqv. II, which differed from sqv. I by

one base at position 129 of the 441 bp fragment. Sequevar IV, represented by strain U01260 alone, differed from sqv. I by two bases at positions 189 and 327 of the 441 bp fragment. Sequevars I, II and IV are most closely related to *M. scrofulaceum* ATCC 19981^T, which differs from sqv. I by 16 bases. The partial protein sequences of the 441 bp fragments reveal two groups, the first consisting of sqv. I, II and IV and the other consisting of sqv. III and V. They differ from each other by three amino acids.

Table 5. Computer-generated PRA patterns using *hsp65* gene sequences of the various *M. parascrofulaceum* sequevars and its closest relatives by HPLC, 16S rRNA, ITS1 and biochemical analysis

Results are given as fragment sizes in bp; fragments below 50 bp are omitted. The score obtained from PRASITE reflects the distance between the result and query, i.e. the smaller the distance, the greater the resemblance. In general, computer-generated PRA data will differ slightly from RFLP data, giving a score of approximately 10 for known identical strains (data not shown). Results above 10 generally suggest a different pattern.

<i>hsp65</i> sequevar	<i>Bst</i> EI	<i>Hae</i> III	Best results using PRASITE (score)
I (12 strains)	231/116/79	139/94	1. <i>M. lentiflavum</i> type 4: 240/130/85–130/95 (20)
II (3 strains)	231/116/79	127/94	2. <i>M. lentiflavum</i> type 4: 240/130/85–130/95 (18)
III (1 strain)	231/210	127/94/81/78	3. <i>M. kansasii</i> type 1: 240/210–130/105/80 (15)
IV (1 strain)	231/131/79	127/94	4. <i>M. lentiflavum</i> type 4: 240/130/85–130/95 (11) (probable match)
V (1 strain)	231/210	145/139/75	5. <i>M. scrofulaceum</i> type 1: 240/210–145/130/95 (24)

BLAST analysis of the partial *hsp65* sequence was performed with sqv. I, III and V, using from bp 46 to 405 of the 441 bp fragment to obtain the most accurate highest score with mycobacterial *hsp65* sequences in the public database, most being either 401 or 360 bp in length. BLAST results for sqv. I revealed a highest score with [*Mycobacterium gordonae*] SN 601 (AF434735) of 100 % identity; this is believed to be misidentified, as it shows 30 nt differences from the type strain, *M. gordonae* ATCC 14470^T. The next closest matches were *M. scrofulaceum* (AF547871 and AF434733), with 96 % similarity (16 bp variation), corresponding to that determined with our in-house sequence of the type strain. BLAST results of strain U02532 (sqv. III) revealed a highest score and 12 mismatches (in 360 bp) with *M. avium* complex strain 121B-2 (AF241211), which differs from the type strain of *M. avium* by 16 bp, followed by [*M. gordonae*] SN 601 (AF434735), also with 16 bp variations. BLAST results with strain U02127 (sqv. V) revealed a highest score with *Mycobacterium bohemicum* CIP 105811^T (AF547811), 95 % similarity (15 mismatches), followed by both *Mycobacterium interjectum* DSM 44064^T (AF547846) and *M. avium* complex strain 121B-2 (AF241211), with 94 % similarity or 19 nt differences.

Computerized restriction maps of the 441 bp *hsp65* fragment as determined by MAPDRAW (LaserGene) using enzymes *Bst*EII and *Hae*III revealed a unique pattern for each sequevar, with the exception of sqv. IV, whose pattern is close to that of *Mycobacterium lentiflavum* type 4 as determined by PRASITE. These patterns are listed in Table 5; fragments smaller than 50 bp were not taken into account.

M. scrofulaceum was first described by Prissick & Masson (1956) and was often grouped with *M. avium*, *M. intracellulare* and related organisms to form the so-called MAIS complex. It has long since been established as a distinct species, with its main characteristics being orange or yellow pigmentation, positive reaction for urease, negative for Tween, semi-quantitative catalase >45 mm and negative nitrate results. *M. scrofulaceum* is found in the environment and is an opportunistic pathogen associated with childhood lymphadenitis, disseminated infections, pulmonary disease and skin infection (Aikin & Johnston, 1978; Corbett *et al.*, 1999; Dustin *et al.*, 1980; Hsueh *et al.*, 1996; Joshi *et al.*, 1989; Murray-Leisure *et al.*, 1987; Prissick & Masson, 1956; Sanders *et al.*, 1995; Wolinsky, 1979, 1995). However, in most of these clinical cases, identification of the organism was obtained via conventional methods, such as pigmentation and biochemical testing. A recent increase in novel mycobacterial species due to molecular-based techniques, including scotochromogenic slowly growing species, suggests that these species probably always existed in clinical samples but were unidentifiable by conventional methodology. This idea was built upon by Tortoli (2003), who stated a possible link between a decrease in cases of *M. scrofulaceum* lymphadenitis and the recent discovery of several novel species of scotochromogenic slowly growing mycobacterial species.

In our study, most isolates of *M. parascrofulaceum* were obtained from respiratory samples. Determining the role of the organism in the disease process was difficult owing to a lack of medical records. Medical charts were reviewed for two of the Winnipeg isolates, but the isolates could not indisputably be associated with disease (Pauls *et al.*, 2003). However, the clinical case described here represents a clear example of the pathogenic potential of this organism.

Similar species reported in other studies

We found that a great proportion of reference strains from ATCC and TMC, and also clinical isolates identified as *M. scrofulaceum* prior to sequence-based testing, were deemed to belong to the novel species *M. parascrofulaceum*. This is not surprising given the phenotypic similarities between the two species. However, molecular data, such as from sequencing of the 16S rRNA gene, *hsp65* and ITS1, clearly distinguish this species from *M. scrofulaceum*. Springer *et al.* (1996) first documented this organism (which they designated MCRO 33) and described it as a unique slowly growing species related to *M. simiae* based on 16S rRNA gene analysis but most closely resembling *M. avium*–*M. intracellulare*–*M. scrofulaceum* (or MAIS complex) based on biochemical testing. Since then, other laboratories that use sequence-based methods for identification of mycobacterial species have shown the presence of this previously unidentified species in clinical samples, either by recognition of a 16S rRNA gene sequence similarity with MCRO 33 or through our own analyses of their sequences (published or deposited in GenBank).

A BLAST analysis using only the first 500 bases of the 16S rRNA gene revealed additional GenBank submissions with identical sequences to that of 'MCRO 33'. These consisted of unspciated strains *Mycobacterium* sp. N228 (AY215339) (Hall *et al.*, 2003) and *Mycobacterium* sp. N625 (AY215248) (Hall *et al.*, 2003), assumed to be derived from clinical specimens. Although using only the partial gene for analysis is satisfactory for clinical strains in a routine laboratory, it is important to bear in mind that analysis using the nearly complete 16S rRNA gene remains the best strategy for accurate eubacterial speciation and is essential for taxonomic studies.

Holberg-Petersen *et al.* (1999) identified a clinical isolate (strain M.16/89) as *M. scrofulaceum* by conventional testing, but with a clearly distinct 16S rRNA gene sequence closer to *M. simiae* and a lipid profile of MAIS. They suggested the strain may not belong to either *M. simiae* or *M. scrofulaceum* (Holberg-Petersen *et al.*, 1999). Our evaluation of their 16S rRNA gene hypervariable regions A and B indicated perfect identity with *M. parascrofulaceum*. Tortoli *et al.* (2001), using a polyphasic approach to decipher the taxonomic status of unusual mycobacteria, described two strains (FI-12100 and FI-25197) identified by conventional testing as *M. lentiflavum*, by HPLC as MAC and by 16S rRNA gene sequencing as MCRO 33. Strain FI-25197 was

obtained from blood of an AIDS patient and was therefore deemed of probable clinical significance.

Swanson *et al.* (1996) recognized five isolates of *M. scrofulaceum* as having a distinct *hsp65* gene sequence (corresponding to our sqv. 1) along with a 16S rRNA gene sequence corresponding to MCRO 33, and suggested that this group probably represented a novel species. One of these strains, Lane 3081, was also part of a past study that identified this strain as having the 'MAC-G' ITS1 sequevar (corresponding to our ITS1 sqv. I) (Frothingham & Wilson, 1993) and has been described as 'exhibiting cultural, biochemical and serological properties characteristic of *M. scrofulaceum*' (Saito *et al.*, 1990; Tomioka *et al.*, 1993). Four of five clinical isolates in Brazil identified as *M. scrofulaceum* by da Silva Rocha *et al.* (2002) revealed a 'new' pattern similar to our sqv. II: 235/115/85-125/95, as determined by gel electrophoresis.

Roth *et al.* (2000), using the ITS1 spacer for mycobacterial identification, revealed five taxonomically uncertain strains closest to *M. scrofulaceum*. Based on a partial ITS1 alignment of these strains, we determined that one corresponds to our ITS1 sqv. III and the other four to our sqv. IIB. Roth *et al.* (2000) also determined that these strains had a distinctive 16S rRNA gene sequence that was identical to that of MCRO 33. Because many researchers have already detected this organism in past studies and with sequence-based methods being commonplace in laboratories, it seems inevitable that this organism be described as a distinct species. We propose the name *Mycobacterium parascrofulaceum* sp. nov. for this unique *Mycobacterium* species.

Description of *Mycobacterium parascrofulaceum* sp. nov.

Mycobacterium parascrofulaceum (pa.ra.scro.fu.la'ce.um. Gr. prep. *para* like; N.L. neut. adj. *scrofulaceum* specific epithet of a bacterial species; N.L. neut. adj. *parascrofulaceum* referring to the phenotypic resemblance of the isolate to *M. scrofulaceum*).

Microscopy shows pleomorphic bacillary forms of AFB with moderate beading. Strains are slowly growing and scotochromogenic and grow on Löwenstein–Jensen medium at 25–37 °C, optimally at 37 °C. Isolates are positive for urease, 68 °C catalase and pyrazinamidase; negative for Tween 80 hydrolysis, nitrate reductase, iron uptake, tolerance to 5 % NaCl, growth on MacConkey agar, niacin, β -glucosidase, acid phosphatase and 3-day arylsulfatase; variable results seen for the semi-quantitative catalase test and 14-day arylsulfatase test. Morphology and biochemical profile indistinguishable from *M. scrofulaceum*. Isolates show *in vitro* susceptibility to rifampicin, clarithromycin, amikacin and sulfamethoxazole and resistance to isoniazid. The organism is almost identical to MAC and some *M. scrofulaceum* by HPLC. Various molecular targets confirm this organism as a distinct species. 16S rRNA gene sequence analysis reveals that the organism contains

the characteristic short helix 18 of the *M. simiae* group and whose closest established species is the recently described *M. palustre*. Five sequevars each of *hsp65* and ITS1 have been observed among isolates of *M. parascrofulaceum*, none corresponding to established strains of known species in public sequence databases.

The type strain, HSC-68^T (=ATCC BAA-614^T=DSM 44648^T), was the first isolate studied in our laboratory and was representative of the most common *hsp65* or ITS1 sequevar (I) of *M. parascrofulaceum*. Most isolates were from human respiratory samples and, in at least one case, was felt to be clinically significant, requiring antimycobacterial therapy.

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