Genotyping of *Mycobacterium avium* complex organisms using multispacer sequence typing

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Mycobacterium avium complex (MAC) currently comprises eight species of environmental and animal-associated, slowly-growing mycobacteria: *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium chimaera*, *Mycobacterium colombiense*, *Mycobacterium arosiense*, *Mycobacterium bouchardurhonense*, *Mycobacterium marseillense* and *Mycobacterium timonense*. In humans, MAC organisms are responsible for opportunistic infections whose unique epidemiology remains poorly understood, in part due to the lack of a genotyping method applicable to all eight MAC species. In this study we developed multispacer sequence typing (MST), a sequencing-based method, for the genotyping of MAC organisms. An alignment of the genome sequence of *M. avium* subsp. *hominissuis* strain 104 and *M. avium* subsp. *paratuberculosis* strain K-10 revealed 621 intergenic spacers <1000 bp. From these, 16 spacers were selected that ranged from 300 to 800 bp and contained a number of variable bases, <50 within each of the 16 spacers. Four spacers were successfully PCR-amplified and sequenced in 11 reference strains. Combining the sequence of these four spacers in 106 MAC organisms, including 83 *M. avium*, 11 *M. intracellulare*, six *M. chimaera*, two *M. colombiense* and one each of *M. arosiense*, *M. bouchardurhonense*, *M. marseillense* and *M. timonense*, yielded a total of 45 spacer types, with an index of discrimination of 0.94. Each spacer type was specific for a species and certain spacer types were specific for subspecies of *M. avium*. MST is a new method for genotyping of organisms belonging to any one of the eight MAC species tested in this study.

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INTRODUCTION

Mycobacterium avium complex (MAC) comprises a growing number of species of slowly growing environmental and animal-associated mycobacteria responsible for opportunistic human infections (Turenne *et al.*, 2006). *Mycobacterium avium* comprises *M. avium* subsp. *hominissuis*, an environmental species responsible for opportunistic infections in pigs and humans including HIV-infected, immunocompromised patients (Komijn *et al.*,

1999; Mijs *et al.*, 2002), and clones evolved from this subspecies, namely *M. avium* subsp. *avium*, *M. avium* subsp. *silvaticum* and *M. avium* subsp. *paratuberculosis* (Harris & Barletta, 2001; Thorel *et al.*, 1990; Turenne *et al.*, 2008). *Mycobacterium intracellulare* is an environmental organism responsible for enlarged lymph nodes in children and progressive pulmonary disease in elderly women (Kyriakopoulos *et al.*, 1997). The analysis of the intergenic transcribed spacer 16S–23S rDNA region (ITS-1) has aided in the recognition of the species *Mycobacterium chimaera*, incorporating sequevar MAC-A organisms isolated from patients with pulmonary cavitations, pulmonary abscess, chronic obstructive pulmonary disease and bronchiectasis (Tortoli *et al.*, 2004; Bills *et al.*, 2009), and of the species *Mycobacterium colombiense*, incorporating sequevar MAC-X organisms isolated from the blood and sputum of HIV-infected patients in Colombia (Murcia *et al.*, 2006) and from diseased lymph nodes in children (Esparcia *et al.*, 2008; Vuorenmaa *et al.*, 2009). *Mycobacterium arosiense* was recently described in an immunocompromised child with disseminated osteomyelitic lesions (Bang *et al.*, 2008).

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Abbreviations: ITS-1, intergenic transcribed spacer 16S–23S rDNA region; MAC, *Mycobacterium avium* complex; MST, multispacer sequence typing; ST, spacer type.

The GenBank/EMBL/DDBJ accession numbers for the genotype sequences of the isolates under study are FJ417966–FJ417968, FJ417971–FJ417998, FJ418000–FJ418007, FJ418009–FJ418034, FJ418037–FJ418044 and FJ892702–FJ892715.

A supplementary table and two supplementary figures are available with the online version of this paper.

The analysis of the partial *rpoB* gene sequence further distinguished *Mycobacterium bouchedurhonense*, *Mycobacterium marseillense* and *Mycobacterium timonense* organisms isolated from patients with pulmonary disease (Ben Salah *et al.*, 2009).

MAC organisms have been widely isolated from inanimate environments such as soil and water, including drinking water (Covert *et al.*, 1999; Falkinham *et al.*, 2001; Le Dantec *et al.*, 2002; Santos *et al.*, 2005; Hilborn *et al.*, 2008) and water from a hospital water distribution system (Du Moulin *et al.*, 1988). Several animals, including birds, pigs, cattle and sheep, could be also colonized or infected by MAC organisms (Biet *et al.*, 2005). Despite the fact that several investigations found a statistically significant correlation between the exposure of patients to fresh water and MAC infection, demonstration of a microbiological link was lacking (Nishiuchi *et al.*, 2007; Reed *et al.*, 2006) or yielded only partial evidence of direct transmission (Hilborn *et al.*, 2008). Therefore, the source of MAC organisms responsible for infections in most patients remains poorly understood.

One limitation in understanding the source of exposure and risk of disease due to MAC species has been in part a lack of a genotyping method applicable to all organisms belonging to the MAC. Sequencing the 16S rRNA gene is only of value for the identification of MAC at the species level. Partial *rpoB* gene sequencing identified all species and *M. avium* subspecies but was not able to genotype MAC isolates (Ben Salah *et al.*, 2008). In fact, all methods have been applied to only a few MAC species (Blackwood *et al.*, 2000; Cilliers *et al.*, 1997; Kasai *et al.*, 2000; Morita *et al.*, 2004; Thierry *et al.*, 1993; Tortoli *et al.*, 2004; Turenne *et al.*, 2006, 2007, 2008) including time-consuming RFLP analysis (Guerrero *et al.*, 1995) and PFGE (Mazurek *et al.*, 1993; Bruijnesteijn van Coppenraet *et al.*, 2008; Pestel-Caron *et al.*, 1999).

In order to gain further knowledge of MAC species populations, in this study we developed multispacer sequence typing (MST), a new method for the genotyping of MAC organisms, based on the sequence analysis of four variable intergenic spacers. MST combines the advantages of PCR and sequencing, and allows the limitations of previous genotyping methods to be circumvented.

METHODS

***Mycobacterium* strains.** Reference strains (see Supplementary Table S1, available with the online version of this paper) *M. avium* subsp. *avium* ATCC 25291^T, *M. avium* subsp. *silvaticum* ATCC 49884^T, *M. avium* subsp. *paratuberculosis* ATCC 19698^T and *M. intracellulare* ATCC 15985 were purchased from the American Tissue Culture Collection (ATCC; Manassas, VA, USA). *M. chimaera* DSM 44623^T and *M. arosiense* DSM 45069^T were purchased from the German Collection of Micro-organisms (DSMZ, Braunschweig, Germany). *M. avium* subsp. *hominissuis* IWGMT 49, a pig isolate, was kindly provided by Dick van Soolingen (National Institute of Public Health and the Environment, Bilthoven, The Netherlands).

M. colombiense CIP 108962^T was purchased from the Institut Pasteur Collection, Paris, France. *M. bouchedurhonense* CSUR P34^T (Collection de Souches de l'Unité des Rickettsies), *M. marseillense* CSUR P30^T and *M. timonense* CSUR P32^T were previously isolated and characterized in our laboratory (Ben Salah *et al.*, 2009). We further studied 64 MAC clinical isolates preserved on beads at -20 °C in our clinical microbiology laboratory in 1997–2008 (Supplementary Table S1). These isolates were previously identified by a polyphasic approach based on PCR amplification and sequencing of *rpoB*, 16S rRNA and *hsp65* genes and ITS-1. For this study, each isolate was inoculated into Middlebrook 7H9 liquid medium in the BACTEC 9000MB apparatus (Becton Dickinson) and subcultured onto Middlebrook 7H10 agar (Becton Dickinson) at 30 °C. Purity was confirmed by microscopic examination after Ziehl–Neelsen staining. DNA was extracted from colonies using the QIAamp mini kit (Qiagen) according to the manufacturer's instructions, after inactivation by Chelex and heating at 96 °C for 1 h. We further analysed the DNA extracted from 31 MAC organisms in the M. A. Behr laboratory, McGill University, Canada, including 15 MAC isolates provided by Des Collins (AgResearch, NCBID Wallaceville, Upper Hutt, New Zealand) (Supplementary Table S1). These isolates were previously characterized by *hsp65* gene sequencing, insertion-sequence typing, RFLP and large-sequence polymorphism (LSP) regions analysis (Turenne *et al.*, 2008).

Selection of intergenic spacers and primer design. The sequences of intergenic spacers were obtained after alignment of the *M. avium* subsp. *hominissuis* strain 104 (GenBank accession number NC_008595) and *M. avium* subsp. *paratuberculosis* strain K-10 (GenBank accession number NC_002944) genomes. Intergenic spacer sequences were then aligned by using the ssaha2 software (Ning *et al.*, 2001) and were ranked by size from 100 bp to 1000 bp and by percentage of sequence similarity. The selection of spacers was done according to the following criteria: (i) a spacer sequence size comprised between 300 bp and 800 bp, in line with the capabilities of a single capillary sequencer run, and (ii) a number of variable bases <50 within each of the 16 spacers in order to increase the probability that primers designed for the *M. avium* subspecies would also target other MAC species. PCR primers were designed using Primer3 software (<http://fokker.wi.mit.edu>). The specificity of PCR primers was predicted by comparing their sequences with those deposited in GenBank using BLASTN software (<http://www.ncbi.nlm.nih.gov>).

PCR amplification and sequencing of spacers. PCRs were carried out in a Biometra thermocycler (LABGENE Scientific Instruments). The PCR mixture (50 µl) contained 5 µl 10 × Taq buffer, 200 µM of each dNTP, 2.5 µl 25 mM MgCl₂, 1.25 U HotStarTaq polymerase (Qiagen), 1 µl 10 pM each primer (Eurogentec) and 5 µl purified DNA. Amplification was carried out using the following conditions: an initial 15 min denaturation at 95 °C was followed by 40 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 60 °C and extension for 1 min at 72 °C; amplification was completed by holding the reaction mixture for 5 min at 72 °C. Sterile water was used as a negative control in each PCR assay. PCR products were visualized under UV illumination after electrophoresis on a 1.5% agarose gel stained with ethidium bromide. PCR products were purified by using a MultiScreen PCR filter plate as recommended by the manufacturer (Millipore). Forward and reverse sequencing mixtures contained 3 µl buffer (BigDye V1, Applied Biosystems), 2 µl BigDye Terminator reaction mix, 5 µl distilled H₂O and 1 µl of 10 pmol primer µl⁻¹, in a final volume of 15 µl. The sequencing reaction comprised an initial 1 min denaturation step at 95 °C followed by 25 cycles of denaturation at 96 °C for 10s, annealing at 50 °C for 5 s and elongation at 60 °C for 3 min. Sequencing products were purified using Sephadex plates (Amersham Biosciences), centrifuged at 910 g for 6 min, and purified sequencing products were deposited on a MicroAmp Optical 96-well reaction plate (Applied Biosystems).

Sequencing electrophoresis was performed on a 3130 Genetic Analyzer (Applied Biosystems).

Multiple spacer sequence analysis. Nucleotide sequences were edited using the AutoAssembler 2.1 software (Applied Biosystems). We determined the Hunter–Gaston index (Hunter & Gaston, 1988) for *M. avium*, *M. intracellulare*, *M. chimaera* and *M. colombiense* isolates (this was not done for the other species comprising only one isolate). Multiple alignments of sequences were carried out using the CLUSTAL_X software (Thompson *et al.*, 1997). Phylogeny reconstruction of all sequence alignments was performed in MEGA 3.1 using the neighbour-joining and bootstrapping methods (Kumar *et al.*, 2004). Each sequence was translated *in silico* with the web software ExPASy translate tool (<http://www.expasy.ch>) in order to verify the presence of potential transcripts. The *Mycobacterium tuberculosis* H37Rv genome sequence (GenBank accession number NC 000962) was used as an out-group root.

Stability assay. In order to check the stability of intergenic spacer sequences, we repeated MST genotyping as described above after two subcultures for isolate numbers 7, 10, 13/00, 59 and 71, *M. intracellulare* ATCC 15985, *M. avium* subsp. *paratuberculosis* strain K-10 and *M. avium* subsp. *paratuberculosis* ATCC 19698^T, randomly chosen for this purpose.

Reference IS1245-RFLP typing. Two *M. avium* subsp. *hominissuis* isolates were randomly selected in each one of the MST subclusters determined in this study in order to analyse them by reference IS1245-RFLP typing performed as previously described (Turenne *et al.*, 2008) and to compare IS1245-RFLP typing with MST genotyping.

Discrimination power. The discrimination power of the MST and IS1245-RFLP genotyping methods was calculated using the Hunter–Gaston index, which was estimated as

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^S n_j(n_j - 1)$$

where *N* was the total number of isolates in the sample population, *S* was the total number of types described, and *n_j* is the number of isolates belonging to the *j*th type (Hunter & Gaston, 1988).

Nucleotide sequence accession numbers. Each genotype sequence was deposited in the GenBank database under accession numbers FJ417966–FJ417968, FJ417971–FJ417998, FJ418000–FJ418007, FJ418009–FJ418034, FJ418037–FJ418044 and FJ892702–FJ892715 and in our local database (<http://ifr48.timone.univ-mrs.fr/portail2/>).

RESULTS

Spacer sequencing

Of 621 intergenic spacers (<800 bp) detected via alignment of the genomic sequence of *M. avium* subsp. *hominissuis* strain 104 with that of *M. avium* subsp. *paratuberculosis* strain K-10, 16 spacers fulfilled our selection criteria. PCR primers could be designed for only 11 spacers as the sequence of five spacers was not conserved enough to allow for PCR primer design. After tentative PCR amplification of the spacers on seven reference strains including the *M. avium* subspecies, *M. intracellulare*, *M. chimaera* and *M. colombiense*, seven spacers could not be amplified in all reference strains. Further analyses were therefore undertaken using the four spacers herein referred to as MST2, MST4, MST15 and MST16 (Table 1). Spacer MST16, a 595–674 bp spacer, was found to be the most variable spacer, classifying the 106 isolates under study into 24 genotypes. PCR amplification of MST4 yielded a product size between 360 bp for *M. colombiense* CIP 108962^T and 473 bp for clinical isolate no. 75 and classified the 106 isolates into 23 genotypes. Spacer MST2 classified the 106 isolates into 23 genotypes, with a product size between 611 bp for *M. avium* subsp. *hominissuis* IWGMT 49 and 646 bp for clinical isolate no. 13/00. PCR amplification of MST15 yielded a product size of between 338 bp for isolate no. 43 and 405 bp for isolate no. 47 and classified the 106 MAC isolates into 17 genotypes. For any spacer, the differences in sequence between isolates were due to mutations, deletions and insertions.

Table 1. Spacer characteristics and the primers used in this study

Intergenic spacer	Theoretical size (bp)*	Position in <i>M. avium</i> subsp. <i>hominissuis</i> strain 104 genome	Primers	Framing genes in <i>M. avium</i> subsp. <i>hominissuis</i> strain 104
MST2	646	1503–2148	F: ACG TCA AGG AGC TCA CCA CT R: GCG AAA GAC TCC CGC ACT	5': <i>dnaA</i> 3': <i>dnaN</i>
MST4	463	2895321–2895783	F: CTT CCT GCG GCT CTG ACA C R: CAC AGG TGG TCC ATG ACG	5': MAV-2868 (hypothetical protein) 3': MAV-2869 (transcriptional repressor copy family protein)
MST15	404	2328269–2328662	F: GGA GCA GAT CGA TGA GGA AT R: CTG CTC ATG ATC GGA GAG TG	5': MAV-2324 (acetyltransferase, gnat family protein) 3': MAV-2325 (hypothetical protein)
MST16	659	3337905–3338522	F: TCG TAG AAA GCT GCG GTC A R: CTG GAC ATG GTC GAG GAG TT	5': MAV-3236 (hypothetical protein) 3': MAV-3237 (2-nitropropanedioxy-genase, NPD)

*Theoretical size of PCR products was determined according to the position primers within *M. avium* subsp. *hominissuis* strain 104 (GenBank accession number NC_008595) genome.

MST genotyping

Combining the four spacer sequences obtained in 106 MAC isolates, including the reference strains, yielded 45 spacer types (STs), with an index of discrimination of 0.94 (Table 2). Thirty-five of the 45 types (STs 1–5, 9, 12, 14–19, 21–23, 25–32, 36, 38–45) contained one isolate each; three STs (nos 24, 34 and 37) contained two isolates each; ST-8 contained three isolates; STs 7, 33 and 35 contained four isolates; ST-20 contained five isolates; STs 11 and 13 contained seven isolates each; ST-6 contained 12 isolates; and ST-10 contained 21 isolates. Each ST was species-specific: ST-31 and ST-32 were specific for *M. colombiense*; ST-33 and ST-34 were specific for *M. chimaera*; ST-35 to ST-41 were specific for *M. intracellulare*; and ST-1 to ST-30 were specific for *M. avium*. In the latter species, each ST was subspecies specific: ST-1 was specific for *M. avium* subsp. *silvaticum*; ST-2 to ST-5 were specific for *M. avium* subsp. *avium*; ST-6 to ST-9 were specific for *M. avium* subsp. *paratuberculosis*; ST-10 to ST-30 were specific for *M. avium* subsp. *hominissuis*. ST-42 to ST-45 were specific for *M. bouchardurhonense*, *M. marseillense*, *M. timonense* and *M. arosiense*, respectively. The Hunter–Gaston index was 1 in *M. colombiense* (2 strains), 0.90 in *M. avium* (83 strains), 0.87 in *M. intracellulare* (11 strains) and 0.53 in *M. chimaera* (6 strains). As for the *M. avium* subspecies, the Hunter–Gaston index was 1 in *M. avium* subsp. *avium* (4 strains), 0.84 in *M. avium* subsp. *hominissuis* (58 strains) and 0.60 in *M. avium* subsp. *paratuberculosis* (20 strains).

Stability assay

After two subcultures, spacer sequences determined were: ST-17 for isolate no. 10; ST-18 for isolate no. 13/00; ST-14 for isolate no. 59; ST-21 for isolate no. 71; ST-16 for isolate no. 7; ST-35 for *M. intracellulare*; and ST-6 for the two strains of *M. avium* subsp. *paratuberculosis*. The spacer sequences determined for these eight isolates after two subcultures were identical to those determined before subculturing.

Phylogenetic tree

The phylogenetic tree based on the concatenation of the four intergenic spacer sequences separated the MAC species into two principal clusters. One cluster comprised all the *M. avium* isolates and a second cluster comprised *M. intracellulare*, *M. chimaera*, *M. timonense*, *M. bouchardurhonense*, *M. marseillense*, *M. arosiense* and *M. colombiense* isolates (Fig. 1). The latter cluster was divided into one subcluster comprising *M. colombiense* and *M. timonense* isolates with a 100 % bootstrap value and other subclusters comprising *M. chimaera*, *M. intracellulare*, *M. bouchardurhonense* and *M. marseillense* isolates with a 100 % bootstrap value. The *M. avium* cluster comprised six subclusters.

Comparison between reference RFLP and MST genotyping

We compared MST and IS1245 genotyping for 15 strains: three *M. avium* subsp. *avium* (ATCC 25291, ATCC 35713 and ATCC 15769) and 12 *M. avium* subsp. *hominissuis* (ATCC 700898, DV, 104, 76102, 28132, MB 63764, MB 64490, 26/05, 10, 33, 59 and 71). As for *M. avium* subsp. *hominissuis*, we observed that strains with different STs had different RFLP profiles (Supplementary Figs S1 and S2). Therefore, strains with STs 11, 10, 29, 20, 25, 17, 18, 14 and 21 had distinct RFLP profiles. Strains DV and MB 63764 had the same ST-10 but exhibited different RFLP profiles (>3 bands difference). Likewise, strains 700898 and 104 (ST no. 11; 4 bands difference), and 28132 and MB64490 (ST no. 20; 5 bands difference), had similar but distinct RFLP profiles. For STs 29, 25, 17, 18, 14 and 21 only one strain was tested.

As has previously been described, IS1245 genotyping on *M. avium* subsp. *avium* (Turenne *et al.*, 2007) showed the expected three-band pattern with the same position for each band.

DISCUSSION

Selection bias of MAC isolates was minimized as we included clinical isolates from eight distinct countries, i.e. France, Canada, New Zealand, Australia, UK, Faroe Islands, USA and Colombia. However, we analysed only clinical isolates from human or animal sources and the application of MST to isolates collected from the inanimate environment remains to be studied. We observed that spacer sequence variability was due to mutations, deletions and insertions. We did not observe repeat variation or variable number tandem repeats, contrary to what is observed in *Mycobacterium tuberculosis* complex species (Djelouadji *et al.*, 2008a, b) and *Rickettsia* species (Fournier & Raoult, 2007).

The identification of recently described MAC species relied on 16S rRNA gene sequencing (Tortoli *et al.*, 2004; Murcia *et al.*, 2006; Ben Salah *et al.*, 2009; Bang *et al.*, 2008). However, only one base (position 403) discriminates between *M. intracellulare* and *M. chimaera*, with 97.8–99.7 % 16S rRNA gene sequence identity among the different MAC species. We observed that each MAC isolate contained one or more STs whereas each ST was found in only one species. Despite the fact that MST discriminated most of the *M. chimaera* isolates from the *M. intracellulare* isolates, nevertheless MST provided limited discrimination among the *M. chimaera* isolates. In addition, *hsp65*, *rpoB* and ITS-1 sequencing were further used as a basis for the description of *M. chimaera* and *M. colombiense* and to complete that of *M. arosiense*, *M. bouchardurhonense*, *M. marseillense* and *M. timonense* (Murcia *et al.*, 2006; Tortoli *et al.*, 2004; Bang *et al.*, 2008; Ben Salah *et al.*, 2009). In particular, the description of *M. chimaera* mainly relied on ITS-1 sequencing. Unsurprisingly, the percentage of ITS-1

Table 2. List of MST types identified

Each MST allele was determined by sequencing the corresponding spacer. The combination of spacer genotypes, strains and species included are presented for each MST type. M.a.a., *M. avium* subsp. *avium*; M.a.s., *M. avium* subsp. *silvaticum*; M.a.h., *M. avium* subsp. *hominissuis*; M.a.p., *M. avium* subsp. *paratuberculosis*; C, cattle genotype; S, sheep genotype; M.i., *M. intracellulare*; M.ch., *M. chimaera*; M.co., *M. colombiense*; M.m., *M. marseillense*; M.t., *M. timonense*; M.b., *M. bouchardurhonense*; M.a., *M. arosiense*.

ST	Genotypes				Strains	Species	Total no. of strains
	MST2	MST4	MST15	MST16			
1	1	1	1	1	ATCC 49884 ^T	M.a.s.	1
2	1	2	1	2	ATCC 25291 ^T	M.a.a.	1
3	2	3	1	2	ATCC 35718	M.a.a.	1
4	1	1	1	2	ATCC 15769	M.a.a.	1
5	3	3	1	2	ATCC 35713	M.a.a.	1
6	4	4	2	3	ATCC 19698 ^T ; K-10; 04-4531; 119; ATCC 43015; TMC 1613; 5979; 7428; 87/8880; 6601; 316F; 7296	M.a.p. C	12
7	5	4	2	3	6758; 6756; P133/79; 6759	M.a.p. S	4
8	6	4	2	4	86-45; LN20; 85/14	M.a.p. S	3
9	6	5	2	4	P465	M.a.p. S	1
10	1	8	1	7	IWGMT 49; 45; 46; 14/00; 251544; 251545; 251548; 52/07; DV; MB 63764; 5; 9; 12; 13; 23; 26; 52; 63; 81; 86/03; 49	M.a.h.	21
11	1	8	1	8	104; 251538; 60/03; 1; 76; 18; ATCC 700898	M.a.h.	7
12	8	8	5	6	47	M.a.h.	1
13	1	9	1	8	43/00; 48/04; 55/06; 31; 60; 75; 6	M.a.h.	7
14	1	10	1	9	59	M.a.h.	1
15	1	11	6	10	43	M.a.h.	1
16	9	11	6	10		M.a.h.	1
17	1	12	7	8		M.a.h.	1
18	10	13	4	6	13/00	M.a.h.	2
19	11	12	4	11	42	M.a.h.	1
20	1	13	1	7	39; 50; 61; 28132; MB64490	M.a.h.	5
21	1	14	8	6	71	M.a.h.	1
22	1	9	1	12	5/06	M.a.h.	1
23	1	8	1	13	6/05	M.a.h.	1
24	1	9	1	7	7/01	M.a.h.	1
25	10	13	9	6	26/05, 4	M.a.h.	1
26	1	10	1	7	37/03	M.a.h.	1
27	1	15	1	7	49/03	M.a.h.	1
28	1	8	1	12	69/01	M.a.h.	1
29	1	12	4	8	76102	M.a.h.	1
30	11	12	1	8	56/01	M.a.h.	1
31	12	16	10	14	CIP 108962 ^T	M.co.	1
32	13	17	11	15	98/02	M.co.	1
33	14	18	12	16	DSM 44623 ^T ; 29/06; 3/08; 73/05	M.ch.	4
34	15	19	12	17	251556; 251550	M.ch.	2
35	16	20	13	18	ATCC 15985; 251542; 17/03; ATCC 13950 ^T	M.i.	4
36	17	20	13	19	251540	M.i.	1
37	8	20	13	20	251541; 55/03	M.i.	2
38	18	21	13	17	251554	M.i.	1
39	14	20	14	16	79	M.i.	1
40	19	22	15	21	27/04	M.i.	1
41	20	20	16	17	ATCC 35761	M.i.	1
42	21	23	17	22	CIP 109828 ^T	M.m.	1
43	22	24	18	23	CIP 109830 ^T	M.t.	1
44	23	25	19	24	CIP 109827 ^T	M.b.	1
45	24	0	0	25	DSM 45069 ^T	M.a.	1

Fig. 1. Tree based on concatenated sequences of four intergenic spacers for each MST type. The topology was calculated using bootstrap values derived from 1000 repetitions (see Methods). Only bootstrap values >90% are indicated. *Mycobacterium tuberculosis* H37Rv sequences were used as root. The Arabic numbers refer to the ST number as indicated in Table 2. Roman numbers refer to clusters formed by the bracketed STs. 'Other MAC species' are *M. intracellulare*, *M. chimaera*, *M. colombiense*, *M. bouchodurhonense*, *M. timonense*, *M. arosiense* and *M. marseillense*.

sequence similarity values was in the same range as that we observed for MST, both systems being based on spacer sequencing. Because of the possible difficulty in resolving *M. intracellulare* and *M. chimaera* isolates and the fact that ITS-1, *hsp65* and *rpoB* gene sequencing can identify all MAC species, MST may not be the most efficient tool for the accurate identification of any MAC isolate.

We further observed that some STs were found in only one of the four *M. avium* subspecies, suggesting that MST could be used for the identification of *M. avium* subspecies. In particular, MST analysis found one ST specific for *M. avium* subsp. *silvaticum*; however, whether a single spacer sequence, including a unique ST, is sufficient to support naming an organism as a unique subspecies requires further study (Turenne *et al.*, 2008). Indeed, multi-locus sequence analysis (MLSA) found that the number of single nucleotide polymorphisms (SNP) separating *M. avium* subsp. *silvaticum* from *M. avium* subsp. *avium* ($n=4$) was similar to the number of SNPs found within *M. avium* subsp. *avium* ($n=6$). In our study, we analysed only one *M. avium* subsp.

silvaticum by MST; nevertheless, this isolate exhibited a unique MST profile, as was also found using MLSA (Turenne *et al.*, 2008). MST analysis, being based on spacer sequencing, may indeed reveal a higher diversity than MLSA, which is based on gene sequencing, because less evolutionary pressure on spacers could yield higher sequence diversity. This point warrants further analysis. We further observed a high degree of diversity in *M. avium* subsp. *hominissuis* clinical isolates; sequence identity varied from 96.3% to 99.9%. Some spacers exhibited a higher degree of sequence diversity among the *M. avium* subsp. *hominissuis* isolates than among isolates belonging to other *M. avium* subspecies. This observation agrees with previous observations of heterogeneity among *M. avium* subsp. *hominissuis* isolates (Turenne *et al.*, 2008), and suggests that this group of organisms warrants further taxonomic investigation.

In this study, 106 MAC isolates belonging to eight MAC species were separated into 45 STs with an index of discrimination of 0.94. Moreover, we observed that sequences were stable after two subcultures; indeed, we

Fig. 2. Guidelines for selection of spacers for MST genotyping of MAC isolates.

obtained the same genotype for the isolates tested. This is of interest, as no method has been published for the genotyping of all organisms belonging to any one of the eight MAC species. Indeed, sequencing of the *hsp65* gene or ITS-1, and the MLSA method were restricted to only some of the MAC species (Murcia *et al.*, 2006; Turenne *et al.*, 2006, 2008). No genotyping method has been published for the recently described *M. colombiense*, *M. chimera*, *M. timonense*, *M. bouchardurhonense*, *M. marseillense* and *M. arosiense* species. MST is therefore the first method published for the genotyping of any MAC isolate, regardless of the species it belongs to. The analysis of RFLP associated with IS1245 and PFGE, two reference methods for MAC genotyping, are time-consuming methods that require a large amount of mycobacterial DNA and provide comparative, rather than stand-alone results. MST, being based on PCR amplification as a first step, circumvents this limitation and allows genotyping starting from a single colony. Consistent with the different applications of classification methods (such as MST) and strain-tracking methods (such as RFLP), we observed that different MST types had different RFLP profiles but that inside a MST type the strains could have different RFLP profiles. These findings indicate that these methods are complementary. For identifying the species/subspecies of an organism, MST is simple and practical. When comparing two isolates, for instance looking for transmission or testing for clinical relapse, a method such as RFLP is likely to be more discriminatory. Although sequencing all spacers was necessary to identify all MST types, STs 17, 19, 29 and 30 could be defined by three spacers only, for STs 1, 3–8, 10–16, 18, 20, 22–26, 28, 35–37, 39 and 41 only two spacers were necessary, and for STs 2, 9, 21, 27, 31–34, 38 and 40–45 only one spacer was sufficient. Therefore, the MST genotyping could be simplified by first determining the sequence of spacer MST4, followed by the analysis of other spacers depending on the initial result (Fig. 2).

In conclusion, MST was unique in being able to classify MAC isolates into species, *M. avium* subspecies and strains. We propose MST as a suitable method to genotype any MAC organism after species and subspecies identification.

REFERENCES

- Bang, D., Herlin, T., Stegger, M., Andersen, A. B., Torkko, P., Tortoli, E. & Thomsen, V. O. (2008). *Mycobacterium arosiense* sp. nov., a slowly growing, scotochromogenic species causing osteomyelitis in an immunocompromised child. *Int J Syst Evol Microbiol* **58**, 2398–2402.
- Ben Salah, I., Adekambi, T., Raoult, D. & Drancourt, M. (2008). *rpoB* sequence-based identification of *Mycobacterium avium* complex species. *Microbiology* **154**, 3715–3723.
- Ben Salah, I., Cayrou, C., Raoult, D. & Drancourt, M. (2009). *Mycobacterium marseillense* sp. nov., *Mycobacterium timonense* sp. nov. and *Mycobacterium bouchardurhonense* sp. nov., novel species in the *Mycobacterium avium* complex. *Int J Syst Evol Microbiol* **59**, 2803–2808.
- Biet, F., Boschioli, M. L., Thorel, M. F. & Guilloteau, L. A. (2005). Zoonotic aspects of *Mycobacterium bovis* and *Mycobacterium avium-intracellulare* complex (MAC). *Vet Res* **36**, 411–436.
- Bills, N. D., Hinrichs, S. H., Aden, T. A., Wickert, R. S. & Iwen, P. C. (2009). Molecular identification of *Mycobacterium chimera* as a cause of infection in a patient with chronic obstructive pulmonary disease. *Diagn Microbiol Infect Dis* **63**, 292–295.
- Blackwood, K. S., He, C., Gunton, J., Turenne, C. Y., Wolfe, J. & Kabani, A. M. (2000). Evaluation of *recA* sequences for identification of *Mycobacterium* species. *J Clin Microbiol* **38**, 2846–2852.
- Bruijnesteijn van Coppenraet, L. E., de Haas, P. E., Lindeboom, J. A., Kuijper, E. J. & van Soolingen, D. (2008). Lymphadenitis in children is caused by *Mycobacterium avium hominissuis* and not related to 'bird tuberculosis'. *Eur J Clin Microbiol Infect Dis* **27**, 293–299.
- Cilliers, F. J., Warren, R. M., Hauman, J. H., Wiid, I. J. & van Helden, P. D. (1997). Oligonucleotide (GTG)₅ as an epidemiological tool in the study of nontuberculous mycobacteria. *J Clin Microbiol* **35**, 1545–1549.
- Covert, T. C., Rodgers, M. R., Reyes, A. L. & Stelma, G. N., Jr (1999). Occurrence of nontuberculous mycobacteria in environmental samples. *Appl Environ Microbiol* **65**, 2492–2496.

- Djelouadji, Z., Arnold, C., Gharbia, S., Raoult, D. & Drancourt, M. (2008a). Multispacer sequence typing for *Mycobacterium tuberculosis* genotyping. *PLoS One* 3, e2433.
- Djelouadji, Z., Raoult, D., Daffé, M. & Drancourt, M. (2008b). A single step sequencing method for identification of *Mycobacterium tuberculosis* complex species. *PLoS Negl Trop Dis* 2, e253.
- Du Moulin, G. C., Stottmeier, K. D., Pelletier, P. A., Tsang, A. Y. & Hedley-Whyte, J. (1988). Concentration of *Mycobacterium avium* by hospital hot water systems. *JAMA* 260, 1599–1601.
- Esparcia, O., Navarro, F., Quer, M. & Coll, P. (2008). Lymphadenopathy caused by *Mycobacterium colombiense*. *J Clin Microbiol* 46, 1885–1887.
- Falkinham, J. O., III, Norton, C. D. & LeChevallier, M. W. (2001). Factors influencing numbers of *Mycobacterium avium*, *Mycobacterium intracellulare*, and other mycobacteria in drinking water distribution systems. *Appl Environ Microbiol* 67, 1225–1231.
- Fournier, P. E. & Raoult, D. (2007). Identification of rickettsial isolates at the species level using multi-spacer typing. *BMC Microbiol* 7, 72.
- Guerrero, C., Bernasconi, C., Burki, D., Bodmer, T. & Telenti, A. (1995). A novel insertion element from *Mycobacterium avium*, IS1245, is a specific target for analysis of strain relatedness. *J Clin Microbiol* 33, 304–307.
- Harris, N. B. & Barletta, R. G. (2001). *Mycobacterium avium* subsp. *paratuberculosis* in veterinary medicine. *Clin Microbiol Rev* 14, 489–512.
- Hilborn, E. D., Yakus, M. A., Covert, T. C., Harris, S. I., Donnelly, S. F., Schmitt, M. T., Toney, S., Bailey, S. A. & Stelma, G. N., Jr (2008). Molecular comparison of *Mycobacterium avium* isolates from clinical and environmental sources. *Appl Environ Microbiol* 74, 4966–4968.
- Hunter, P. R. & Gaston, M. A. (1988). Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *J Clin Microbiol* 26, 2465–2466.
- Kasai, H., Ezaki, T. & Harayama, S. (2000). Differentiation of phylogenetically related slowly growing mycobacteria by their *gyrB* sequences. *J Clin Microbiol* 38, 301–308.
- Komijn, R. E., de Haas, P. E., Schneider, M. M., Eger, T., Nieuwenhuijs, J. H., van den Hoek, R. J., Bakker, D., van Zijl Erveld, F. G. & van Soolingen, D. (1999). Prevalence of *Mycobacterium avium* in slaughter pigs in The Netherlands and comparison of IS1245 restriction fragment length polymorphism patterns of porcine and human isolates. *J Clin Microbiol* 37, 1254–1259.
- Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinform* 5, 150–163.
- Kyriakopoulos, A. M., Tassios, P. T., Matsiota-Bernard, P., Marinis, E., Tsaousidou, S. & Legakis, N. J. (1997). Characterization to species level of *Mycobacterium avium* complex strains from human immunodeficiency virus-positive and -negative patients. *J Clin Microbiol* 35, 3001–3003.
- Le Dantec, C., Duguet, J. P., Montiel, A., Dumoutier, N., Dubrou, S. & Vincent, V. (2002). Occurrence of mycobacteria in water treatment lines and in water distribution systems. *Appl Environ Microbiol* 68, 5318–5325.
- Mazurek, G. H., Hartman, S., Zhang, Y., Brown, B. A., Hector, J. S., Murphy, D. & Wallace, R. J., Jr (1993). Large DNA restriction fragment polymorphism in the *Mycobacterium avium*-*M. intracellulare* complex: a potential epidemiologic tool. *J Clin Microbiol* 31, 390–394.
- Mijs, W., de Haas, P., Rossau, R., van der Laan, L., Rigouts, L., Portaels, F. & van Soolingen, D. (2002). Molecular evidence to support a proposal to reserve the designation *Mycobacterium avium* subsp. *avium* for bird-type isolates and '*M. avium* subsp. *hominissuis*' for the human/porcine type of *M. avium*. *Int J Syst Evol Microbiol* 52, 1505–1518.
- Morita, Y., Maruyama, S., Kabeya, H., Nagai, A., Kozawa, K., Kato, M., Nakajima, T., Mikami, T., Katsube, Y. & Kimura, H. (2004). Genetic diversity of the *dnaJ* gene in the *Mycobacterium avium* complex. *J Med Microbiol* 53, 813–817.
- Murcia, M. I., Tortoli, E., Menendez, M. C., Palenque, E. & Garcia, M. J. (2006). *Mycobacterium colombiense* sp. nov., a novel member of the *Mycobacterium avium* complex and description of MAC-X as a new ITS genetic variant. *Int J Syst Evol Microbiol* 56, 2049–2054.
- Ning, Z., Cox, A. J. & Mullikin, J. C. (2001). SSAHA: a fast search method for large DNA databases. *Genome Res* 11, 1725–1729.
- Nishiuchi, Y., Maekura, R., Kitada, S., Tamaru, A., Taguri, T., Kira, Y., Hiraga, T., Hirotani, A., Yoshimura, K. & other authors (2007). The recovery of *Mycobacterium avium*-*intracellulare* complex (MAC) from the residential bathrooms of patients with pulmonary MAC. *Clin Infect Dis* 45, 347–351.
- Pestel-Caron, M., Graff, G., Berthelot, G., Pons, J. L. & Lemeland, J. F. (1999). Molecular analysis of *Mycobacterium avium* isolates by using pulsed-field gel electrophoresis and PCR. *J Clin Microbiol* 37, 2450–2455.
- Reed, C., von Reyn, C. F., Chamblee, S., Ellerbrock, T. V., Johnson, J. W., Marsh, B. J., Johnson, L. S., Trenchel, R. J. & Horsburgh, C. R., Jr (2006). Environmental risk factors for infection with *Mycobacterium avium* complex. *Am J Epidemiol* 164, 32–40.
- Santos, R., Oliveira, F., Fernandes, J., Gonçalves, S., Macieira, F. & Cadete, M. (2005). Detection and identification of mycobacteria in the Lisbon water distribution system. *Water Sci Technol* 52, 177–180.
- Thierry, D., Bauge, S., Poveda, J. D., Vincent, V. & Guesdon, J. L. (1993). Rapid identification of *Mycobacterium avium*-*intracellulare* complex strains: clinical practice evaluation of DT6 and DT1 probes. *J Infect Dis* 168, 1337–1338.
- 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.
- Thorel, M. F., Krichevsky, M. & Levy-Frebault, V. V. (1990). Numerical taxonomy of mycobactin-dependent mycobacteria, emended description of *Mycobacterium avium*, and description of *Mycobacterium avium* subsp. *avium* subsp. nov., *Mycobacterium avium* subsp. *paratuberculosis* subsp. nov., and *Mycobacterium avium* subsp. *silvaticum* subsp. nov. *Int J Syst Bacteriol* 40, 254–260.
- Tortoli, E., Rindi, L., Garcia, M. J., Chiaradonna, P., Dei, R., Garzelli, C., Kroppenstedt, R. M., Lari, N., Mattei, R. & other authors (2004). Proposal to elevate the genetic variant MAC-A, included in the *Mycobacterium avium* complex, to species rank as *Mycobacterium chimaera* sp. nov. *Int J Syst Evol Microbiol* 54, 1277–1285.
- Turenne, C. Y., Semret, M., Cousins, D. V., Collins, D. M. & Behr, M. A. (2006). Sequencing of *hsp65* distinguishes among subsets of the *Mycobacterium avium* complex. *J Clin Microbiol* 44, 433–440.
- Turenne, C. Y., Wallace, R. & Behr, M. A. (2007). *Mycobacterium avium* in the postgenomic era. *Clin Microbiol Rev* 20, 205–229.
- Turenne, C. Y., Collins, M., Alexander, D. C. & Behr, M. A. (2008). *Mycobacterium avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium* are independently evolved pathogenic clones of a much broader group of *M. avium* organisms. *J Bacteriol* 190, 2479–2487.
- Vuorenmaa, K., Ben Salah, I., Barlogis, V., Chambost, H. & Drancourt, M. (2009). *Mycobacterium colombiense* and pseudotuberculous lymphadenopathy. *Emerg Infect Dis* 15, 619–620.

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