Molecular typing of *Mycobacterium intracellulare* using multilocus variable-number of tandem-repeat analysis: identification of loci and analysis of clinical isolates

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In addition to its known status as a disseminated disease in HIV-positive patients, *Mycobacterium avium* complex (MAC) is increasingly recognized as a causative pathogen of respiratory disease in HIV-negative patients. MAC is divided into *Mycobacterium avium*, and the less-epidemiologically studied *Mycobacterium intracellulare*. Genetic typing for *M. intracellulare* using variable number of tandem repeats (VNTR) has not yet been developed. The aim of this study was to identify VNTR loci in the genome of *M. intracellulare* and apply them as an epidemiological tool to clinical isolates. Here, we identified 25 VNTR loci on the *M. intracellulare* genome, of which 16 showed variations among clinical isolates in the number of tandem repeat motifs. Among the 74 *M. intracellulare* isolates, 50 genotypes were distinguished using the 16 VNTR loci, resulting in a Hunter Gaston's discriminatory index of 0.988. Moreover, all 16 VNTR loci were stable in different sets of isolates recovered within time intervals ranging from 2 to 1551 days from 14 separate patients. These results indicate that for use as epidemiological markers of *M. intracellulare*, the loci in this VNTR assay are highly discriminating and stable over time.

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INTRODUCTION

Mycobacterium avium complex (MAC) is an opportunistic pathogen that causes human disease, and has been isolated from a variety of animal hosts and environmental sources such as soil, water and dust (Bauer *et al.*, 1999; Dvorska *et al.*, 2002; De Groote *et al.*, 2006). MAC is divided into

Abbreviations: MAC, *Mycobacterium avium* complex; MLVA, multilocus variable number of tandem repeats analysis; PRA, PCR-restriction enzyme analysis; TR, tandem repeat; VNTR, variable number of tandem repeats.

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Three supplementary figures are available with the online version of this paper.

two closely related organisms, *Mycobacterium avium* and *Mycobacterium intracellulare* (Inderlied *et al.*, 1993), which together account for more than 70 % of non-tuberculous mycobacterial disease in Japan. *M. intracellulare* is more frequently isolated from immunocompetent patients with pulmonary disease than from immunocompromised patients (Kyriakopoulos *et al.*, 1997). Furthermore, immunocompetent patients with MAC disease can be either chronically infected with a given strain and relapse after treatment due to reactivation, or be reinfected by a different strain after cure. Although direct infection from human-to-human contact is considered unlikely (Wolinsky, 1979), and several studies have implicated drinking water or bathrooms as sources of MAC infection (von Reyn *et al.*, 1994; Le Dantec *et al.*, 2002; Nishiuchi

et al., 2007), the source of infection in humans has not yet been clearly identified. The establishment of an accurate and reliable technique for the epidemiological detection and typing of MAC strains is therefore of considerable clinical importance.

PCR-based techniques for the identification and typing of MAC strains have been previously described. Telenti *et al.* (1993) reported PCR amplification of the gene encoding a 65 kDa heat-shock protein (*hsp65*), followed by PCR-restriction enzyme analysis (PRA) to determine mycobacterial species, including *M. avium* and *M. intracellulare*, although only four genotypes of *M. intracellulare* have been distinguished using this technique (Prammananan *et al.*, 2006).

On the other hand, multilocus variable number of tandem repeats (VNTR) analysis (MLVA) has proven to be a rapid and highly discriminating subtyping method for several mycobacterial species (Stragier *et al.*, 2005; Supply *et al.*, 2006). Moreover, in recent studies, Bull *et al.* (2003), Thibault *et al.* (2007) and Inagaki *et al.* (2009) identified loci containing VNTR of specific mycobacterial interspersed repetitive units (MIRUs) in *M. avium*. However, no VNTR loci in *M. intracellulare* have yet been identified.

The aim of this study was to identify VNTR loci based on an intensive screening of tandem repeat (TR) loci in the *M. intracellulare* genome and to examine their epidemiological usefulness when applied to clinical isolates of *M. intracellulare* recovered from different patients.

METHODS

Bacterial strains. Reference strains (*Mycobacterium intracellulare* JATA52-01, derived from *M. intracellulare* ATCC 13950) were purchased from the Japan Anti-Tuberculosis Association. Ninety-three clinical strains, recovered from 74 HIV-negative patients with pulmonary disease, were identified as *M. intracellulare* using the Cobas Amplicor Mycobacterium Test (Roche Diagnostic Systems).

Preparation of genomic DNA. Clinical isolates of *M. intracellulare* were cultured at 37 °C for 1–3 weeks in 7 ml Middlebrook 7H9 liquid medium supplemented with 10 % oleic acid albumin-glucose-catalase enrichment, and 200 µl of this 7 ml was cultured for enrichment in 5 ml Mycobroth liquid medium (Kyokuto) to an OD₅₃₀ of 0.2. DNA was then extracted using InstaGene Matrix (Bio-Rad) according to the manufacturer's instructions.

***hsp65*-PCR-restriction enzyme analysis (*hsp65*-PRA).** A 439 bp fragment of the *hsp65* gene was amplified according to the procedure described by Telenti *et al.* (1993), using primers Tb11 (5'-ACCAA-CGATGGTGTGTCCAT-3') and Tb12 (5'-CTTGTCGAACCG-CATACCCT-3'), and then digested with *Bst*EII or *Hae*III (Takara Bio). After digestion, 12 µl of the restriction digest was loaded onto a 4 % agarose gel (E-gel; Invitrogen) and the gel was electrophoresed by the E-gel power system (Invitrogen). A 50 bp DNA Ladder (Invitrogen) served as an external molecular size marker. Fragments were visualized by ethidium bromide fluorescence, and lengths were calculated by Quantity One (Bio-Rad).

Selection of VNTR loci. The unfinished genome sequence of *M. intracellulare* ATCC 13950 (GenBank accession number

ABIN01000000), which was obtained from the NCBI database (<http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi>) and is presently being sequenced at McGill University, was screened for TRs using the Tandem Repeat Finder (<http://tandem.bu.edu>). Among the TRs found, several different loci were selected using the criteria of: (i) minimum repeat size of 50 bp, allowing differentiation of the polymorphic VNTR loci by size on agarose gels, and (ii) >90 % conservation between the TRs. The VNTR loci used and their characteristics are listed in Table 1. Primers were designed utilizing Primer 3 software (<http://frodo.wi.mit.edu/>), and each primer was designed to anneal to the flanking regions of the TR. To confirm that each VNTR locus was different, VNTR sequences were compared with genome sequence data of *M. intracellulare* ATCC 13950 using BLASTN software.

Final selection of VNTR loci was based on the following criteria of Monteil *et al.* (2007): (i) candidate loci with less than two different alleles were excluded, (ii) when two VNTR loci candidates were isomorphic, one was excluded, and (iii) the full band patterns had to be readable.

VNTR analysis. Mixtures were prepared containing template DNA (1 µl), dNTP mixture (2.5 µl), 10 × PCR buffer (2.5 µl), each of the primer sets (both 12.5 µM), 1.25 U AmpliTaqGold (Applied Biosystems) and DMSO (Wako) to a final concentration of 4 %. Sterilized purified water was added, bringing the total volumes to 25 µl, and the mixtures were then subjected to PCR.

The PCR programme used consisted of an initial denaturing step at 95 °C for 10 min, followed by 38 cycles of denaturing at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 60 s, and a final extension step at 72 °C for 7 min. The amplified DNA products were subjected to electrophoresis on 2 % E-gel (Invitrogen) to determine their size in bp. Template DNA from *M. intracellulare* ATCC 13950 was used as a control in each experiment.

The estimated size of PCR products for the different alleles was deduced from the allele size range, and from the basic unit length. For units that remained incomplete, the calculated values were rounded down to the closest whole number; for example in VNTR-7 loci, (53 bp × 3) + 38 bp units were considered as 3 repeat units (Fig. 1).

The allelic diversity of each VNTR locus was calculated using Selander's formula (Selander *et al.*, 1986) and the discriminatory power of MLVA using Hunter and Gaston's formula (Hunter & Gaston, 1988).

The PCR products obtained from various VNTR loci of *M. intracellulare* and clinical strains were purified using a GenElute PCR DNA Purification kit (Sigma-Aldrich), and direct sequencing analysis was performed with the same primers as used in the PCRs. Alignment of the nucleotide sequences was performed using the CLC Sequence Viewer 4.6.2 (CLC Bio).

Phylogenetic analysis. Phylogenetic analysis was estimated from Manhattan distance matrix data of the allele profiles of VNTR by Fitch–Margoliash criterion without an evolutionary clock using PHYLIP version 3.68c (Nishimori *et al.*, 2002).

RESULTS

Strain identification

All 93 isolates recovered from sputum were identified as *M. intracellulare* using the Cobas Amplicor Mycobacterium Test, and determined as *M. intracellulare hsp65* allelic variant I using *hsp65*-PRA. Although these results were

Table 1. Primer sequences, tandem repeat motifs, conservation, size, and copy number of 16 VNTR loci in *M. intracellulare* ATCC 13950

Locus name	Primer sequence (5'–3')	ORF*	Function†	TR motif	Conservation (%)	Estimated size range (bp)	Repeat×copy no.
VNTR-1	TCGCCGAGGACTTCGTCT	MAV_0742	Putative aminopeptidase 2	CACGCTAGGGTCGATTGCGCGC-TCCTTGCTCGCGCCGCTTCCGG-CGCTCACCGTCGC (57 bp)	100	273	(57 bp × 2) + 12 bp
	GTCACCACGAGGAAGATCG	MAV_0741	Dyp-type peroxidase family protein				
VNTR-2	AGGGTGGTGAACGCGTAG	NF	NA	CGACGGGTCTCGATGGCGCCGA-CCCGCTGCGCCCGGCGCAAGC-GCCGCGCTTGCGAT (57 bp)	99	299	(57 bp × 3) + 5 bp
	CTCTGGCAGCCCGATACC	MAV_3505	Protocatechuate 3,4-dioxygenase, α subunit				
VNTR-3	AGAGGTGCTGCCGATTACAC	MAV_1158	tRNA	TAGTCGGATGACGATGCGGGGC-GCTTGCGGCGCCGAGGAGGAAT-CCGACAATCCGGGCC (58 bp)	100	280	(58 bp × 2) + 25 bp
VNTR-4	TCTTTGTCCGGTTCCTTTTG	MAV_1159	TetR family protein	GGCTAGCGACGATCGCAAGCGC-GGCGTAGCCGGGCGCAGCGGG-TCGTCGCCCCGTT (55 bp)	100	271	(55 bp × 3) + 7 bp
	AGGCTTCAATTCGGGTGAC	MAV_4302	Transposase				
VNTR-5	TTCCGACCACCTACATCGAG	NF	NA	GATGCCCGTGACGATCGCAAGC-GCGGCGGAGCCGGGCGCAGCG-GGTCTCACC (54 bp)	99	223	(54 bp × 2) + 13 bp
	GACGACGACGGTGTGGT	MAV_4692	NA				
VNTR-6	GATCGTCTCGCTGGTGGAC	MAV_4692	NA	AGCTCCCGTGACGATCGCGAGC-GCGGCGTAGCCGGGCGCTGCG-GGTCTCACCATC (56 bp)	98	256	(56 bp × 3) + 7 bp
	GGTCGATCCGGTCAGCTC	MAV_4354	FAD-dependent oxidoreductase				
VNTR-7	CACCTTGATGGGCGATGT	MAV_4353	α -Ketoglutarate-dependent taurine dioxygenase	CGCCGGCGACGATCCAGAGCGC-AGCGATGGGGAGGAGCGGCGC-ACATGACCCC (53 bp)	99	274	(53 bp × 3) + 39 bp
	TTTCATGGTTCGCCCTCTAC	NF	NA				
VNTR-8	GTTCGTCCGAGGTCATGGT	NF	NA	TCGATTGCCCGCCTCCTCACTC-GCGCCGTTCCGGCGCTCACCG-TCGCCGGGCTACGC (57 bp)	97	270	(57 bp × 2) + 3 bp
	TCAAACCTCATTTGCGCTGAG	NF	NA				
VNTR-9	CGACATCTGGTTCTTCGACTC	MAV_5200	O-Methyltransferase, family protein 3	GCCTAGCCCGGCGACGGCGAGC-GCCGCATGGCGCGAGTGAGGA-GCCGGGCAATCGG (56 bp)	98	228	(56 bp × 2) + 5 bp
	GGTCACTGGCTTCTCTCTCTG	NF	NA				
	CACAGCTACGCCGCGAGAC	NF	NA				

Table 1. cont.

Locus name	Primer sequence (5'–3')	ORF*	Function†	TR motif	Conservation (%)	Estimated size range (bp)	Repeat×copy no.
VNTR-10	GGCTGGTTCTTTCTGGTGAC	MAV_0237	Glycosyltransferase, group 2 family protein	CCTAGTGGCGATCGCGAGCGCG-GCGGAGCCGGGCGAAGCGGGT-CGCCACCATCGATA (57 bp)	98	353	(57 bp × 4)
	CGCGTCAAGGAACGTCAT	MAV_0236	O-antigen export system, permease protein				
VNTR-11	TGTTGCGCTGAGGTCATATC	NF	NA	AGCCCGGCGACGGCGAGCGCCG-AAACGGCGCGAGTGAGGAGTC-GGGCAATCCAACCA (57 bp)	96	189	(57 bp × 2) + 6 bp
	ACAGGTTGTCGGTCATTGGT	NF	NA				
VNTR-12	AGACCAACCCAGAAAAGTGC	NF	NA	ATGACTGCGACAGCGACGAAGC-AGAGCGCAGCGATGAAGAGGA-GTGGCGCTCG (53 bp)	97	245	(53 bp × 2) + 6 bp
	GTCGTGATACGCCGAATTG	MAV_4451	30S ribosomal protein S8				
VNTR-13	GTTCAGCGAGCCGGTATCT	MAV_1628	Putative acyl-CoA dehydrogenase	TGAGGAGGAGCGGCGAAGGTGA-CCGCCGGCGACGATGCAGAGC-GAAGCGA (50 bp)	97	292	(50 bp × 3) + 21 bp
	AGCTCTCGCAGCTTGGTTC	MAV_1627	Feruloyl-CoA synthetase				
VNTR-14	ATGCCGTTAGTCTCTCACG	MAV_4662	2,3-Bisphosphoglycerate-dependent phosphoglycerate mutase	GCATCGTCGTCTTCGCGGCTCA-AAGCTCGACTCCTCCTCATCGC-GCTTCGCGCTCT (56 bp)	97	258	(56 bp × 2) + 15 bp
	GCTCGTCGATCCAGAAAGAG	MAV_4664	NA				
VNTR-15	GCAAACGCAGTGGTACTCAG	MAV_2430	Cobalamin biosynthesis protein CobI	CGTAGGGCTCAGTCTGATTGCC-CTACTCCTCCGCACCCCGCCAC-GCGGGCTGCATCGT (58 bp)	93	219	(58 bp × 2) + 13 bp
	GATGATGCCGAGCACCTG	MAV_2429	PPE family protein				
VNTR-16	GGACGCTTTGTATCCGAGTT	MAV_4761	F420-dependent glucose-6-phosphate dehydrogenase	CTCCTGTGCTGATGGTCGCGAC-CCGCCGCGCCCGGCTACGCCG-CGCTTGCGATCG (55 bp)	96	265	(55 bp × 2) + 43 bp
	ACCGTGTCGGTGACTTGAAC	MAV_4762	Metallo-β-lactamase superfamily protein				

*ORF, homologous open reading frame in *M. avium* 104 genome. NF, Not found.

†As predicted from *M. avium* 104. NA, Not applicable.

Fig. 1. Alignment of the *M. intracellulare* ATCC 13950 sequence with sequenced PCR product for clinical strains. The 53 bp repeat units are indicated by arrows. Partial repeats of varying length are present at the end of all the loci; the VNTR-7 locus contains three complete copies of the 53 bp repeat, and also an additional 38 bases corresponding to the beginning of another tandem repeat in *M. intracellulare* ATCC 13950.

consistent with a previous report from Thailand that showed *M. intracellulare* *hsp65* allelic variant I was the most predominant type among pulmonary specimen isolates (Prammananan *et al.*, 2006), there were no PRA profiles that matched the described patterns of non-*M. avium* or non-*M. intracellulare*.

In silico identification and selection of VNTR loci

At the time of this study, only one *M. intracellulare* genome sequence was available online, and using this, 25 loci were initially chosen after comparing the lengths of repeats (repeats of 50–70 bp), sequence identities (>90 % nucleotide sequence identity between repeats) and copy numbers (between two and five copies of the unit repeat). We focused on TRs of the mini-satellite class, defined by a repeat unit size in the range of 10–100 bp, as the corresponding allelic differences of these TRs can be easily resolved by agarose gel electrophoresis.

PCRs, however, gave no amplification or gave amplification of bands associated with TRs in several loci that were excluded from this study (data not shown). Moreover, we excluded the loci where an insertion sequence (*ISMin1*; DDBJ accession no. AB519649) was inserted downstream of the gene (see Supplementary Fig. S1, available with the online version of this paper).

Accordingly, we selected 16 loci based on the three criteria described above; their characteristics, together with the primers used for PCR amplification, are given in Table 1. The gel profile and sequencing data for 16 VNTR loci are indicated in Supplementary Figs S2 and S3. When they

were applied to MLVA in the 93 *M. intracellulare* clinical isolates, and after the exclusion of identical profiles of isolates from the same patients, 74 representative allelic profiles were identified.

Allelic diversity and distribution of VNTR allele numbers

Table 2 shows the allelic diversity of various VNTR loci. The allelic diversity index (*h*) was used to evaluate allelic diversity of the various VNTR loci. Based on this study, nine VNTR loci (VNTR-2, 4, 6, 7, 8, 10, 11, 13 and 15) achieved a high diversity index ($h > 0.5$). Five VNTR loci (VNTR-1, 3, 5, 12 and 14) had a medium diversity index ($h < 0.5$), and VNTR-9 and 2 were the least discriminated loci ($h < 0.1$).

The discriminatory index of MLVA typing was calculated according to Hunter and Gaston's formula (Hunter & Gaston, 1988), in which the closer the discriminatory index is to 1, the higher the discriminatory power of the method. The MLVA method identified 50 distinct genotypes, consisting of 17 clustered and 33 unique, from 74 representative isolates (Fig. 2), giving a Hunter Gaston's Discriminatory Index for MLVA of 0.988.

Stability of the individual VNTR loci

The VNTR profile varied considerably among strains. We therefore explored the frequency of mobility in individual VNTR loci by examining changes in the VNTR pattern during repeated passages *in vitro* and between serial *in vivo* isolates recovered from 14 separate patients.

Table 2. VNTR allelic distribution in *M. intracellulare* clinical isolates

Isolate and locus	No. of isolates with the specified TR allele							Allelic diversity
	0	1	2	3	4	5	6	
VNTR-1	0	5	52	14	0	3	0	0.46
VNTR-2	3	43	18	8	2	0	0	0.58
VNTR-3	7	62	5	0	0	0	0	0.27
VNTR-4	0	32	9	13	20	0	0	0.69
VNTR-5	14	2	55	3	0	0	0	0.40
VNTR-6	0	20	35	16	3	0	0	0.65
VNTR-7	0	20	38	15	1	0	0	0.62
VNTR-8	35	0	37	2	0	0	0	0.52
VNTR-9	2	1	71	0	0	0	0	0.07
VNTR-10	1	44	3	1	20	5	0	0.56
VNTR-11	0	1	34	0	36	2	1	0.54
VNTR-12	0	0	60	14	0	0	0	0.30
VNTR-13	3	1	33	37	0	0	0	0.54
VNTR-14	0	1	65	8	0	0	0	0.21
VNTR-15	0	38	31	5	0	0	0	0.55
VNTR-16	0	4	70	0	0	0	0	0.09

In order to assess the effect of *in vitro* passage, two *M. intracellulare* clinical strains were streaked on 7H11 agar plates and passaged 20 times over 180 days, with a single colony used for inoculation at each passage. After the passages, genomic DNA was extracted and these samples were subjected to VNTR analysis, in which the original cells and the passaged cells showed identical patterns. Furthermore, 14 sets of isolates that were recovered from the same patients at time intervals ranging from 2 to 1551 days were subjected to MLVA and showed identical VNTR profiles, again indicating the stability of the VNTR profiles (Table 3).

DISCUSSION

VNTRs of the mini-satellite class are valuable markers for genotyping several mycobacterial species, including *M. avium*. To our knowledge, this study is the first to perform an exhaustive screening of potential VNTR loci in this genome. This screening was based on *in silico* identification of TRs in the preliminary *M. intracellulare* genome and experimental testing of the polymorphism of the most interesting TR candidates using clinical *M. intracellulare* isolates.

This MLVA exhibited a diversity index value of 0.988, meaning that this method is highly informative, and hence possesses great discriminatory power for identification of genetically similar strains. Although these results were based on a limited number of samples and should therefore be considered preliminary, this MLVA showed a high discriminating power for the genetic diversity found in *M. intracellulare* strains.

With regard to other typing methods, the standard PFGE and restriction fragment length polymorphism methods

are labour-intensive, take approximately 1 week to complete and require a large amount of DNA. In contrast, MLVA typing is a simple and highly reproducible procedure that can be performed with very little DNA and regardless of whether the bacteria are living or dead. It can be performed on mycobacteria killed by heat or alcohol, therefore reducing biohazards. PCR analysis and electrophoresis can be completed in just one day. Results are digital from the outset, simplifying the comparison of large numbers of strains.

In this study, we analysed the genotype stability of MLVA in *M. intracellulare* by using serial isolates from patients infected over long periods (up to 4 years). The 16 VNTR loci were identical within groups of serial isolates in all 14 cases. As these VNTR loci appear to be highly stable over a period as long as 4 years, they all appear suitable for reliable follow-up of patients chronically infected with *M. intracellulare*.

Wallace *et al.* (1998) reported that isolates recovered after apparently successful drug therapy should be genotyped to distinguish between treatment failure and reinfection with another strain. The detection of changes in MLVA should therefore be useful in distinguishing between endogenous reactivation or exogenous reinfection.

Griffith *et al.* (2006) and Prammananan *et al.* (2006) performed PRA of a 441 bp fragment of the *hsp65* gene in *M. intracellulare* isolates from patients with chronic pulmonary disease. Griffith *et al.* (2006) found that all 47 *M. intracellulare* isolates belonged to *M. intracellulare* I, while Prammananan *et al.* (2006) found that 85 % of pulmonary specimen isolates were *M. intracellulare* I. The *hsp65*-PRA type that was present in all strains of *M. intracellulare* I in our study was the same as that in these

Fig. 2. MLVA dendrogram based on the allelic profiles of 74 *M. intracellulare* clinical isolates and *M. intracellulare* ATCC 13950. The Manhattan distance is indicated at the bottom.

studies (Griffith *et al.*, 2006; Prammananan *et al.*, 2006). Our study and these studies (Griffith *et al.*, 2006; Prammananan *et al.*, 2006) indicate that *M. intracellulare* I may possess particular pathogenic characteristics favourable for chronically infecting the pulmonary system. The fact that all clinical strains were identified as *hsp65* allelic variant I suggests, however, that *hsp65*-PRA may not have sufficient discriminatory power for the genetic variants found in *M. intracellulare* clinical strains.

In conclusion, this MLVA method based on VNTR polymorphism provided a rapid and highly discriminating assay for the genetic typing of *M. intracellulare*. In addition, because of the objectivity of the output, the data are amenable to automated computer analysis for easier comparison across laboratories, indicating that MLVA typing may be widely accessible to research and public health laboratories. Elucidation of the transmission routes and pathophysiology of *M. intracellulare* infection may consequently advance through the use of MLVA typing.

Table 3. Stability of VNTR genotypes in serial isolates from 14 chronically infected patients

The following strains were stable over the indicated time frames.

Patient no.	No. of isolates tested	Time span (days)*	Copy no. for VNTR loci:															
			V-1	V-2	V-3	V-4	V-5	V-6	V-7	V-8	V-9	V-10	V-11	V-12	V-13	V-14	V-15	V-16
1	4	1531	2	2	0	4	0	3	1	2	2	2	2	2	3	2	1	2
2	5	973	2	1	1	3	2	2	3	2	2	5	2	2	3	2	2	2
3	2	2	5	0	1	1	2	2	2	0	1	1	1	2	2	3	2	1
4	2	234	2	1	1	1	2	2	2	0	2	1	4	3	2	3	1	2
5	2	210	2	3	1	1	0	2	1	2	2	4	2	2	2	2	1	2
6	2	184	1	2	1	3	2	1	2	0	2	4	4	2	2	2	2	2
7	2	84	2	2	1	1	0	2	1	2	2	4	2	2	3	2	1	2
8	2	39	2	1	1	2	2	1	1	2	2	1	4	2	2	2	2	2
9	2	252	2	1	1	1	0	4	2	0	2	1	2	3	3	3	3	2
10	2	35	2	1	1	1	2	3	2	0	2	1	5	3	3	2	3	2
11	2	321	3	1	1	4	2	2	2	0	2	1	4	2	3	2	1	2
12	2	1134	2	1	1	1	2	1	2	0	2	1	4	3	2	2	3	2
13	2	1551	3	1	1	4	3	2	2	0	2	1	4	2	3	2	1	2
14	2	73	2	1	1	1	2	1	2	2	2	1	4	2	1	2	1	2

*Time between the first and last isolates in each patient.

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REFERENCES

- Bauer, J., Andersen, A. B., Askgaard, D., Giese, S. B. & Larsen, B. (1999). Typing of clinical *Mycobacterium avium* complex strains cultured during a 2-year period in Denmark by using IS1245. *J Clin Microbiol* **37**, 600–605.
- Bull, T. J., Sidi-Boumedine, K., McMinn, E. J., Stevenson, K., Pickup, R. & Hermon-Taylor, J. (2003). Mycobacterial interspersed repetitive units (MIRU) differentiate *Mycobacterium avium* subspecies *paratuberculosis* from other species of the *Mycobacterium avium* complex. *Mol Cell Probes* **17**, 157–164.
- De Groote, M. A., Pace, N. R., Fulton, K. & Falkinham, J. O., III (2006). Relationships between *Mycobacterium* isolates from patients with pulmonary mycobacterial infection and potting soils. *Appl Environ Microbiol* **72**, 7602–7606.
- Dvorska, L., Bartos, M., Ostadal, O., Kaustova, J., Matlova, L. & Pavlik, I. (2002). IS1311 and IS1245 restriction fragment length polymorphism analyses, serotypes, and drug susceptibilities of *Mycobacterium avium* complex isolates obtained from a human immunodeficiency virus-negative patient. *J Clin Microbiol* **40**, 3712–3719.
- Griffith, D. E., Brown-Elliott, B. A., Langsjoen, B., Zhang, Y., Pan, X., Girard, W., Nelson, K., Caccitolo, J., Alvarez, J. & other authors (2006). Clinical and molecular analysis of macrolide resistance in *Mycobacterium avium* complex lung disease. *Am J Respir Crit Care Med* **174**, 928–934.
- 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.
- Inagaki, T., Nishimori, K., Yagi, T., Ichikawa, K., Moriyama, M., Nakagawa, T., Shibayama, T., Uchiya, K., Nikai, T. & Ogawa, K. (2009). Comparison of a variable-number tandem-repeat (VNTR) method for typing *Mycobacterium avium* with mycobacterial interspersed repetitive-unit-VNTR and IS1245 restriction fragment length polymorphism typing. *J Clin Microbiol* **47**, 2156–2164.
- Inderlied, C. B., Kemper, C. A. & Bermudez, L. E. (1993). The *Mycobacterium avium* complex. *Clin Microbiol Rev* **6**, 266–310.
- 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.
- Monteil, M., Durand, B., Bouchouicha, R., Petit, E., Chomel, B., Arvand, M., Boulouis, H. J. & Haddad, N. (2007). Development of discriminatory multiple-locus variable number tandem repeat analysis for *Bartonella henselae*. *Microbiology* **153**, 1141–1148.
- Nishimori, K., Uchida, I., Tanaka, K., Nishimori, T., Imai, K., Kashiwazaki, Y., Murata, N. & Jinma, K. (2002). Molecular epidemiological manual for *Mycobacterium tuberculosis* complex and *Mycobacterium avium* using VNTR (Variable Numbers of Tandem Repeats) typing. *Bull Natl Inst Anim Health* **109**, 25–32 (in Japanese).
- Nishiuchi, Y., Maekura, R., Kitada, S., Tamaru, A., Taguri, T., Kira, Y., Hiraga, T., Hirotsu, 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.

Prammananan, T., Phunpruch, S., Tingtoy, N., Srimuang, S. & Chaiprasert, A. (2006). Distribution of *hsp65* PCR-restriction enzyme analysis patterns among *Mycobacterium avium* complex isolates in Thailand. *J Clin Microbiol* **44**, 3819–3821.

Selander, R. K., Caugant, D. A., Ochman, H., Musser, J. M., Gilmour, M. N. & Whittam, T. S. (1986). Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. *Appl Environ Microbiol* **51**, 873–884.

Stragier, P., Ablordey, A., Meyers, W. M. & Portaels, F. (2005). Genotyping *Mycobacterium ulcerans* and *Mycobacterium marinum* by using mycobacterial interspersed repetitive units. *J Bacteriol* **187**, 1639–1647.

Supply, P., Allix, C., Lesjean, S., Cardoso-Oelemann, M., Rusch-Gerdes, S., Willery, E., Savine, E., de Haas, P., van Deutekom, H. & other authors (2006). Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. *J Clin Microbiol* **44**, 4498–4510.

Telenti, A., Marchesi, F., Balz, M., Bally, F., Böttger, E. C. & Bodmer, T. (1993). Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction analysis. *J Clin Microbiol* **31**, 175–178.

Thibault, V. C., Grayon, M., Boschirol, M. L., Hubbans, C., Overduin, P., Stevenson, K., Gutierrez, M. C., Supply, P. & Biet, F. (2007). New variable number tandem repeat markers for typing *M. avium* subsp. *paratuberculosis* and *M. avium* strains: comparison with IS900 RFLP and IS1245 RFLP typing. *J Clin Microbiol* **45**, 2404–2410.

von Reyn, C. F., Marlow, J. N., Arbeit, R. D., Barber, T. W. & Falkinham, J. O. (1994). Persistent colonisation of potable water as a source of *Mycobacterium avium* infection in AIDS. *Lancet* **343**, 1137–1141.

Wallace, R. J., Jr, Zhang, Y., Brown, B. A., Dawson, D., Murphy, D. T., Wilson, R. & Griffith, D. E. (1998). Polyclonal *Mycobacterium avium* complex infections in patients with nodular bronchiectasis. *Am J Respir Crit Care Med* **158**, 1235–1244.

Wolinsky, E. (1979). Nontuberculous mycobacteria and associated diseases. *Am Rev Respir Dis* **119**, 107–159.

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