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ORIGINAL ARTICLE

Molecular Typing of *Mycobacterium intracellulare* Using Pulsed-Field Gel Electrophoresis, Variable-Number Tandem-Repeat Analysis, Mycobacteria Interspersed Repetitive-Unit-Variable-Number Tandem Repeat Typing, and Multilocus Sequence Typing: Molecular Characterization and Comparison of Each Typing Methods

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Abstract
Objectives: *Mycobacterium intracellulare* is the major causative agent of non-tuberculous mycobacteria-related pulmonary infections. The strain typing of *M. intracellulare* is important for the treatment and control of its infections. We compared the discrimination capacity and effective value of four different molecular typing methods.
Methods: Antibiotic susceptibility testing, *hsp65* and *rpoB* sequencing, pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), mycobacteria interspersed repetitive-unit-variable-number tandem-repeat analysis (MIRU-VNTR), and VNTR assay targeting 44 *M. intracellulare* isolates obtained from patients with pulmonary infections were performed.
Results: All the antibiotic susceptibility patterns had no association with the molecular and sequence types tested in this study; however, the molecular and

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unit-variable-number tandem-repeat analysis, *Mycobacterium intracellulare*, variable-number tandem-repeat analysis

sequence types were related with each other. PFGE gave best results for discriminatory capacity, followed by VNTR, MLST, and MIRU-VNTR.
Conclusion: The high discriminatory power of PFGE, VNTR, and MLST is enough for differentiating between reinfection and relapse, as well as for other molecular epidemiological usages. The MLST could be regarded as a representative classification method, because it showed the clearest relation with the sequence types.

1. Introduction

The *Mycobacterium avium* complex (MAC) is the major causative agent of nontuberculous mycobacteria (NTM)-related pulmonary infections. The MAC consists of *M. avium, Mycobacterium intracellulare, Mycobacterium chimaera, Mycobacterium colombiense, Mycobacterium arosiense, Mycobacterium vulneris, Mycobacterium marseillense, Mycobacterium timonense,* and *Mycobacterium bouchedurhonense* [1,2]. MAC is usually distributed in environmental systems (such as various soil and water systems) near human settlements [3−5]. Among the various MAC strains, *M. avium* and *M. intracellulare* are the most prevalent forms [6]. *M. intracellulare* is more frequently isolated from patients with MAC-related lung diseases, whereas *M. avium* is generally related with lung diseases in immune-compromised patients [3,4,7]. In Korea, MAC was responsible for 50% of NTM-related lung disease, and it was also reported that *M. intracellulare* is more frequently isolated than *M. avium* [2,4,8,9].

Molecular typing is a useful tool for distinguishing between reinfection and incurable cases in *M. intracellulare*-related lung infections; therefore, the tool is critical and essential not only for treatment but also for the molecular epidemiology [2,10,11].

Pulsed-field gel electrophoresis (PFGE), variable-number tandem-repeat analysis (VNTR), and many other molecular typing methods were used for the differentiation of *M. intracellulare* in various studies [3,8,11,12]. However, because of the unique microbiological nature of *Mycobacterium*, such as slow growth and difficulty involved in performing cell wall lysis owing to its unique cell wall structure, polymerase chain reaction (PCR)-based molecular typing methods are preferred than PFGE, although the latter is considered a "gold standard" molecular typing method in other bacterial species. The recent identification of complete genome sequence of *M. intracellulare* made it possible to develop effective VNTR methods [11,13].

In this study, PFGE, VNTR, mycobacteria interspersed repetitive-unit-VNTR typing (MIRU-VNTR), and multilocus sequence typing (MLST) assays were performed for the *M. intracellulare* strains isolated from patients with pulmonary infections in Korea, and then the discrimination capacity and effective value of these four molecular typing methods were compared.

2. Materials and methods

2.1. Bacterial strains

A total of 44 clinical isolates collected from Korean individuals with pulmonary infections from 2009 to 2011 were collected from the Asan Medical Center, the Seoul National University College of Medicine, Seoul, Korea. The strains were grown in Middlebrook 7H9 broth supplemented with 0.05% Tween 80 and 0.2% glycerol. Mycobacterial DNA was extracted to boil for 20 minutes at 95°C. The clinical strains were identified through *hsp65* and *tuf* sequencing [14−17]. The type strain of *M. intracellulare* used was American Type Culture Collection (ATCC) 13950.

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using Sensititre MIC panel slow growing mycobacteria (Trek Diagnostic Systems, Inc., Cleveland, OH, USA). The strains were cultured in Middlebrook 7H9 broth with 0.05% Tween 80 and 0.2% glycerol containing 3-mm sterilized glass beads. The Sensititre MIC panel slow growing mycobacteria system was prepared according to the manufacturer's instructions. The results were read according to the guidelines of the Clinical Laboratory Standards Institute [18]. The following antibiotics were tested using this method: clarithromycin (CLA), rifabutin, ethambutol, isoniazid, moxifloxacin (MXF), rifampin (RIF), trimethoprim/sulfamethoxazole (SXT), amikacin (AMI), linezolid (LZD), ciprofloxacin (CIP), streptomycin, doxycycline (DOX), and ethionamide.

2.3. *hsp65* and *rpoB* genotyping

Primers previously described were used for the *hsp65* amplification [2]. Primers for *rpoB* genotyping were designed in this study (rpoB-F: 5′-ACTTGATGGT CAACAGCTCC-3′; rpoB-R: 5′-AGGTCTCCGCC GACTACAT-3′). All amplifications were performed using AmpliTaq Gold 360 master mix (Applied Biosystems, Foster City, CA, USA). The amplification conditions were as follows: predenaturation at 94°C for 5 minutes; 30 cycles at 94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute; and a final extension step at 72°C for 10 minutes. The amplified products were purified using QIA quick PCR purification kit (Qiagen, Straße, Hilden, Germany) and sequenced using ABI 3700 DNA analyzer with BigDye Terminator cycle

sequencing kits (Applied Biosystems). The sequences were aligned using CLC Main Workbench 6 (CLC bio, Prismet, Aarhus, Denmark).

2.4. PFGE

Agarose plugs were prepared as previously described [19]. *Salmonella* Breanderup H9812 (ATCC BAA-664) was used as a standard marker (http://www.pulsenetinternational.org). Genomic DNA was digested with *Spe*I (Roche Diagnostics GmbH, Mannheim, Germany) and PFGE was performed using a CHEF Mapper system (Bio-Rad Laboratories, Hercules, CA, USA) [20–22]. This switch time was from 2 seconds to 10 seconds for 13 hours at Block 1 and from 20 seconds to 25 seconds for 6 hours at Block 2 at 14°C. The DNA fingerprinting patterns were analyzed using BioNumerics 7.1 (Applied Maths, Austin, TX, USA). The banding patterns were compared using Dice coefficients with a 1% band position tolerance. The clustering of patterns was performed using unweighted pair group-matching algorithm (UPGMA).

2.5. MLST

The housekeeping genes for MLST assay were selected as in the study by Macheras et al [23]. The fragments from six housekeeping genes, namely, *argH, cya, glpK, gnd, murC,* and *pta,* were amplified using the primer sets designed in this study (Table 1).

The amplification step was performed using AmpliTaq Gold 360 master mix (Applied Biosystems). Conditions for amplification were 5 minutes at 94°C followed by 30 cycles at 94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute with a final extension step at 72°C for 10 minutes. The amplified products were purified using QIA quick PCR purification kit (Qiagen) and sequenced using ABI 3700 DNA analyzer with BigDye Terminator cycle sequencing kits (Applied Biosystems). The sequences were aligned using CLC Main Workbench 6 (CLC bio). The reference sequences of the six housekeeping genes were obtained from *M. intracellulare* ATCC13950 through PCR direct sequencing.

The MLST types were analyzed using BioNumerics 7.1 software (Applied Maths). Pattern clustering was performed using UPGMA.

2.6. VNTR

The primers sets described by Ichikawa et al [3] were used in this study, except VNTR-11, which was not amplified in this study. The amplification was performed using AmpliTaq Gold 360 master mix (Applied Biosystems). The PCR conditions were as follows: 5 minutes at 94°C followed by 30 cycles at 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds with a final extension step at 72°C for 5 minutes.

2.7. MIRU-VNTR

The primer sets proposed by Dauchy et al [10] and Iakhiaeva et al [11] were used, except MIRU 3, which was not amplified in this study. PCR mixtures were prepared using 10 μL of AmpliTaq Gold 360 master mix (Applied Biosystems), 10 ng of template DNA, and 4–7.5 μM of primers (Table 2). The PCR conditions were as follows: 5 minutes at 94°C, followed by 30 cycles at 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 1 minute, with a final extension step at 72°C for 7 minutes.

The size of amplified products was analyzed on ABI 3730XL DNA analyzer (Applied Biosystems). GeneScan 1200 LIZ (Applied Biosystems) was used for fragment analysis.

3. Results

3.1. Antibacterial susceptibilities

The tested isolates were all susceptible to CLA, but 91% of tested isolates were resistant to MXF, RIF, LZD,

Table 1. Primers used for multilocus sequence typing in this study.

Gene	Primer	Sequences (5′–3′)	Amplicon size (bp)	No. of alleles
argH	argH-F	AGTCGTGCAGGGTGACGTAG	495	11
	argH-R	CCACCCTGTTCCGGATGT		
cya	cya-F	CCGTGCGGATCCTTCAGT	660	7
	cya-R	GTGCTGTTGCTGGGCATC		
glpK	glpK-F	ATCGGATCGCCAGTAGGG	670	6
	glpK-R	GACACCTGGGTGTTGTGGAA		
gnd	gnd-F	TGGCGGTCACCACGACAC	555	4
	gnd-R	GATCACGGCGCAGGTCTT		
murC	murC-F	GTTGCTCGGGGTCCAGGT	607	4
	murC-R	GTCGTCGCTGGACCTGTTG		
pta	pta-F	GGTTCGATGCCGAATTGTG	665	8
	pta-R	CACGTCGACATCACCGATCT		

Table 2. Primers used for the mycobacteria interspersed repetitive-unit-variable-number tandem-repeat analysis in this study.

Set	Alias	PCR primer pairs (5′−3′, labeling indicated)	Primer concentration
Set 1	MIN18	GCCGAACCATTTGGCGAAC(VIC)	4μM
		GGATTCGGCCGCGCAATTC	
	MIN19	CATGGTTCGCCCTCTACAC(PET)	5μM
		TAGGGGCAGGTCATCGAAG	
	MIN20	GCTGAGCTACAGCCTCGAC(NED)	7.5μM
		CGACGCCGATGACGTAAAC	
Set 2	MIN22	TCAGGAATGGGTCCGGTTC(FAM)	5μM
		AGCTCGTGACGACGGAAAC	
	MIN31	CGACCGCATCCAGAAACAG(VIC)	5μM
		GCTCTATGACGACCTCAAG	
	MIN33	GTGCAGTTCAACCACGAAC(PET)	7.5μM
		GGCGTTGAACACGTTGGTG	

PCR = polymerase chain reaction.

CIP, and DOX. Only four isolates were susceptible to MXF, RIF, LZD, CIP, and DOX, and these showed the same antibiotic susceptibility pattern, that is, all were susceptible to all of the antibiotics tested except SXT.

A total of 44 isolates were tested in this study, and these were classified into seven antibiotic susceptibility patterns. A total of 28 isolates were included in the major pattern AST1, and these isolates were susceptible to CLA and AMI, but resistant to the remaining five antibiotics. In the second major pattern AST2, seven isolates were included and they were susceptible to CLA, AMI, and SXT, but resistant to the remaining four antibiotics (Table 3). All the antibiotic susceptibility patterns have no relation with the molecular types tested in this study.

3.2. Sequence types *hsp65* and *rpoB*

Based on the sequence of *hsp65*, 44 isolates classified into eight sequence types. The most frequent type was HS1, which included 26 isolates, and the second most frequent was HS6, which included nine isolates. Some minor types (HS7, HS8, and HS9) were closely related with HS1. These three had only 1-bp difference with the HS1 type. The HS2 type also had only 1-bp difference with the HS6 type (Figure 1A).

The 44 isolates were divided into nine sequence types based on their *rpoB* sequence. Among these, the RS8 sequence type was the major one, which included 19 isolates. The RS6 type, which included nine isolates, was the second major type. The RS3 type showed only 2-bp difference with the RS8 type (Figure 1B).

The relationship between *hsp65* sequence type and *rpoB* sequence type was investigated using the composite analysis method of BioNumerics 7.1 software (Applied Maths). The *hsp65* sequence type HS1 was closely related with the *rpoB* sequence types RS8 and RS6, and the HS6 type was closely related with the RS1 and RS2 types (data not shown).

3.3. MLST

MLST identified 25 patterns, of which 16 were unique. The major pattern was ST004, which included six isolates. The ST004 pattern was closely related with the HS1 and RS8 sequence types (Figure 2).

The MLST patterns were divided into two clusters, and computer-calculated genetic similarity between these two clusters was 95.1%. One cluster included HS1 and its related sequence type, and the RS8 and RS67 sequence types, whereas the other cluster included the HS2 and HS6 *hsp65* sequence types and the RS1 *rpoB*

Table 3. Type distribution of antibacterial susceptibility analysis in the clinical isolates of *Mycobacterium intracellulare*.

Antibiotic susceptibility type	Antibacterial drug								AS Type No. (%)
	CLA	MXF	RIF	SXT	AMI	LZD	CIP	DOX	
AST1	S	R	R	R	S	R	R	R	28 (63.6)
AST2	S	R	R	S	S	R	R	R	7 (15.9)
AST3	S	R	R	R	R	R	R	R	4 (9.1)
AST4	S	S	S	R	S	S	S	S	2 (4.5)
AST5	S	R	R	S	R	R	R	R	1 (2.3)
AST6	S	S	S	S	S	S	S	S	1 (2.3)
AST7	S	S	S	R	S	R	S	S	1 (2.3)

AMI = amikacin; AS = antibiotic susceptibility; CIP = ciprofloxacin; CLA = clarithromycin; DOX = doxycycline; LZD = linezolid; MXF = moxifloxacin; R = resistance; RIF = rifampin; S = susceptible; SXT = trimethoprim/sulfamethoxazole.

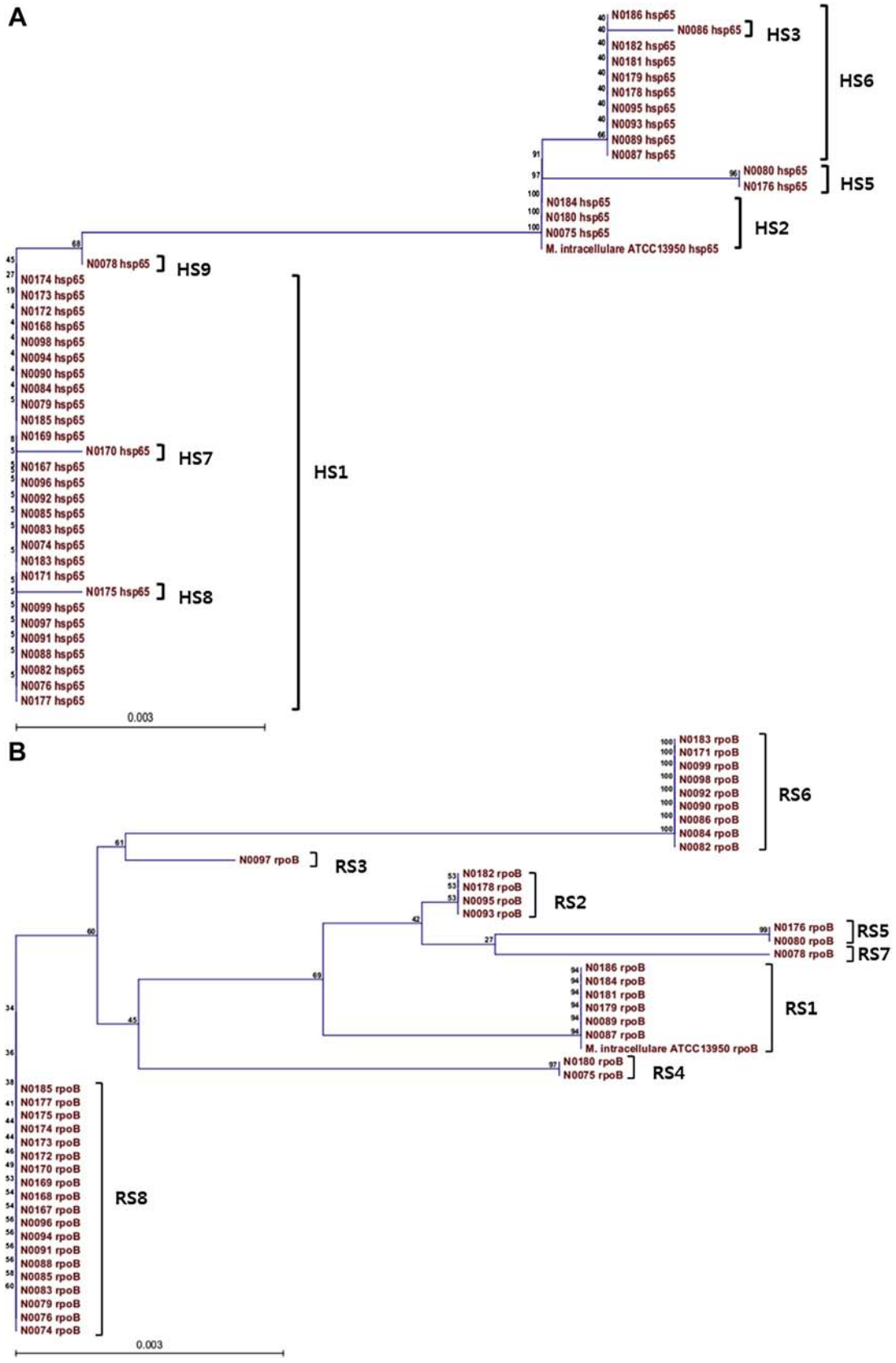
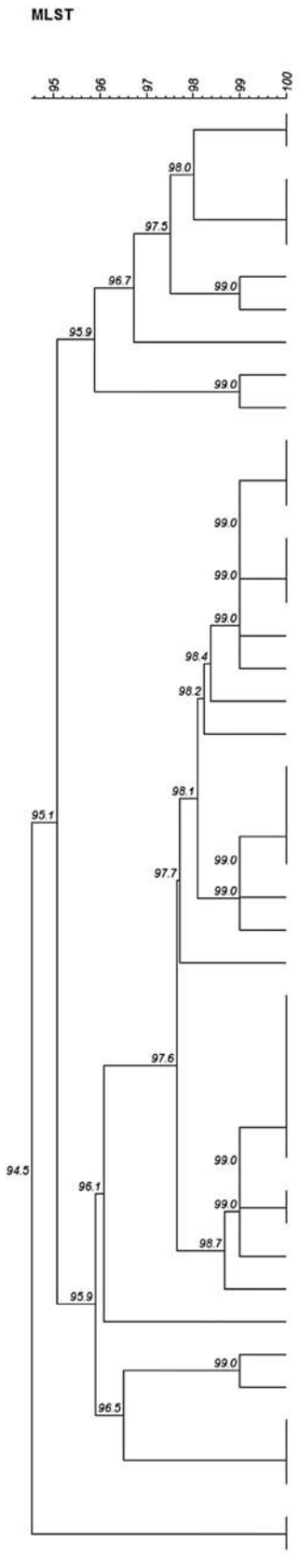


Figure 1. Phylogenetic trees based on the *hsp65* and *rpoB* sequence types in the clinical isolates of *Mycobacterium intracellulare*. Phylogenetic analysis of the (A) *hsp65* sequence type (B) *rpoB* sequence type.



MLST										
argH	cya	glpK	gnd	murC	pta	Lab No	Isolate region	hsp65	rpoB	MLST
10	8	7	8	6	6	N0179	SNUH	HS6	RS1	ST014
10	8	7	8	6	6	N0181	SNUH	HS6	RS1	ST014
12	8	8	8	6	6	N0087	AMC	HS6	RS1	ST013
12	8	8	8	6	6	N0089	AMC	HS6	RS1	ST013
12	8	8	8	6	6	N0186	SNUH	HS6	RS1	ST013
9	8	8	8	5	6	N0075	AMC	HS2	RS4	ST029
9	8	7	8	5	6	N0180	SNUH	HS2	RS4	ST028
15	8	6	8	6	11	N0182	SNUH	HS6	RS2	ST037
10	6	7	8	5	9	ATCC 13950		HS2	RS1	ST002
10	13	7	8	5	9	N0184	SNUH	HS2	RS1	ST030
7	5	6	7	6	6	N0169	SNUH	HS1	RS8	ST007
7	5	6	7	6	6	N0173	SNUH	HS1	RS8	ST007
7	5	6	7	6	6	N0174	SNUH	HS1	RS8	ST007
8	5	6	7	6	6	N0086	AMC	HS3	RS6	ST011
8	5	6	7	6	6	N0171	SNUH	HS1	RS6	ST011
8	5	6	7	6	6	N0172	SNUH	HS1	RS8	ST011
14	5	6	7	6	6	N0167	SNUH	HS1	RS8	ST036
16	5	6	7	6	6	N0183	SNUH	HS1	RS6	ST038
8	5	10	7	6	6	N0098	AMC	HS1	RS6	ST025
7	11	6	7	6	6	N0094	AMC	HS1	RS8	ST024
7	5	6	7	6	8	N0082	AMC	HS1	RS6	ST006
7	5	6	7	6	8	N0090	AMC	HS1	RS6	ST006
7	5	6	7	6	8	N0092	AMC	HS1	RS6	ST006
7	5	6	7	6	8	N0177	SNUH	HS1	RS8	ST006
11	5	6	7	6	8	N0076	AMC	HS1	RS8	ST031
14	5	6	7	6	8	N0185	SNUH	HS1	RS8	ST034
14	12	6	7	6	6	N0170	SNUH	HS7	RS8	ST035
7	7	6	7	6	7	N0079	AMC	HS1	RS8	ST004
7	7	6	7	6	7	N0084	AMC	HS1	RS6	ST004
7	7	6	7	6	7	N0088	AMC	HS1	RS8	ST004
7	7	6	7	6	7	N0091	AMC	HS1	RS8	ST004
7	7	6	7	6	7	N0168	SNUH	HS1	RS8	ST004
7	7	6	7	6	7	N0175	SNUH	HS8	RS8	ST004
7	7	6	7	6	6	N0074	AMC	HS1	RS8	ST008
7	7	6	7	6	6	N0096	AMC	HS1	RS8	ST008
7	7	6	7	6	14	N0078	AMC	HS9	RS7	ST040
7	5	6	7	6	7	N0085	AMC	HS1	RS8	ST023
13	5	9	9	6	6	N0097	AMC	HS1	RS3	ST033
8	5	6	8	7	8	N0080	AMC	HS5	RS5	ST026
8	5	6	7	7	8	N0176	SNUH	HS5	RS5	ST027
12	5	6	8	8	10	N0093	AMC	HS6	RS2	ST012
12	5	6	8	8	10	N0095	AMC	HS6	RS2	ST012
12	5	6	8	8	10	N0178	SNUH	HS6	RS2	ST012
17	5	11	10	9	12	N0083	AMC	HS1	RS8	ST018
17	5	11	10	9	12	N0099	AMC	HS1	RS6	ST018

Figure 2. Comparison of the dendrograms from the cluster analysis of *Mycobacterium intracellulare* using categorical coefficients.

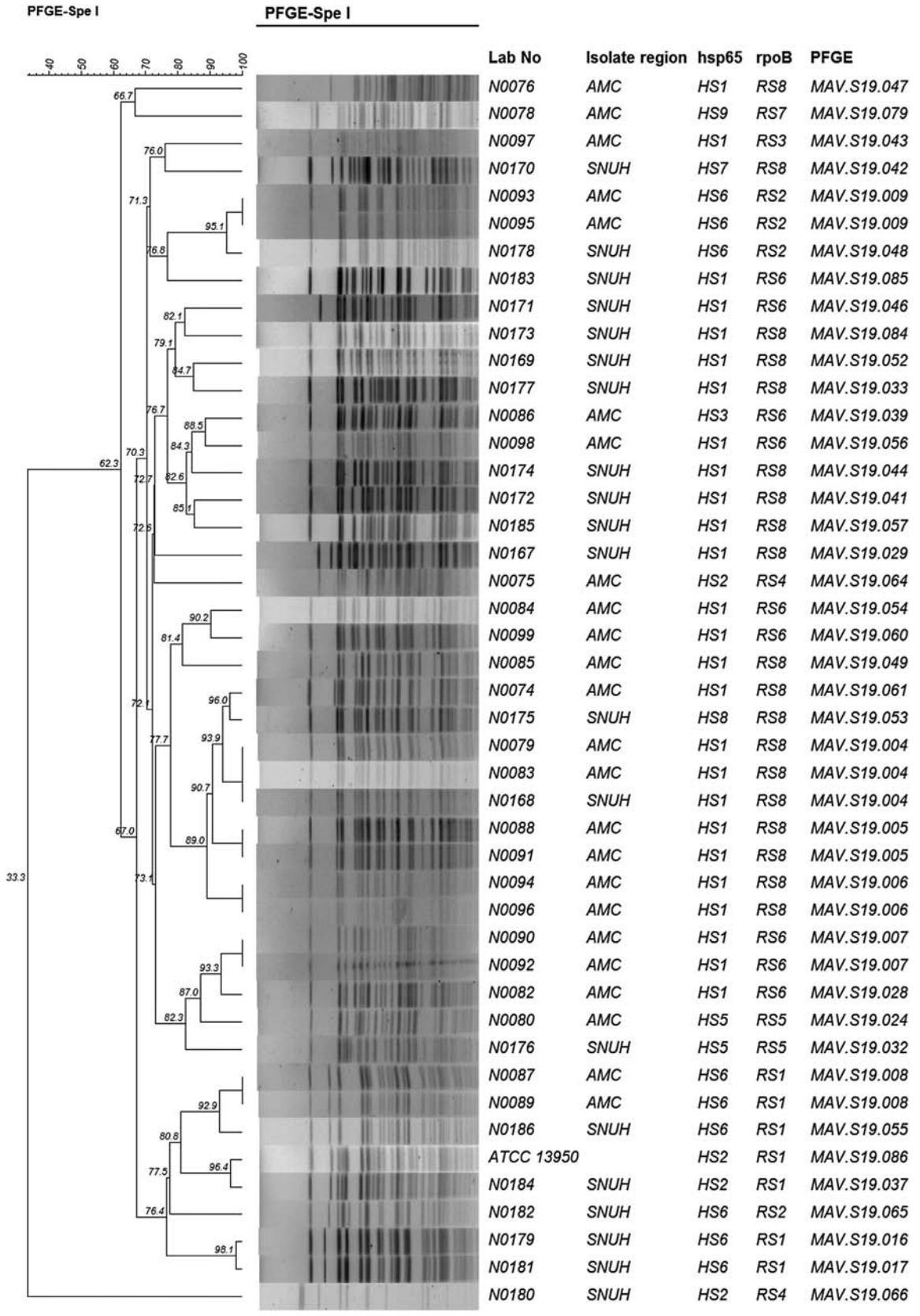


Figure 3. Dendrogram of the cluster analysis based on DNA restriction banding patterns of the *Mycobacterium intracellulare* clinical isolates using *Spe*I.

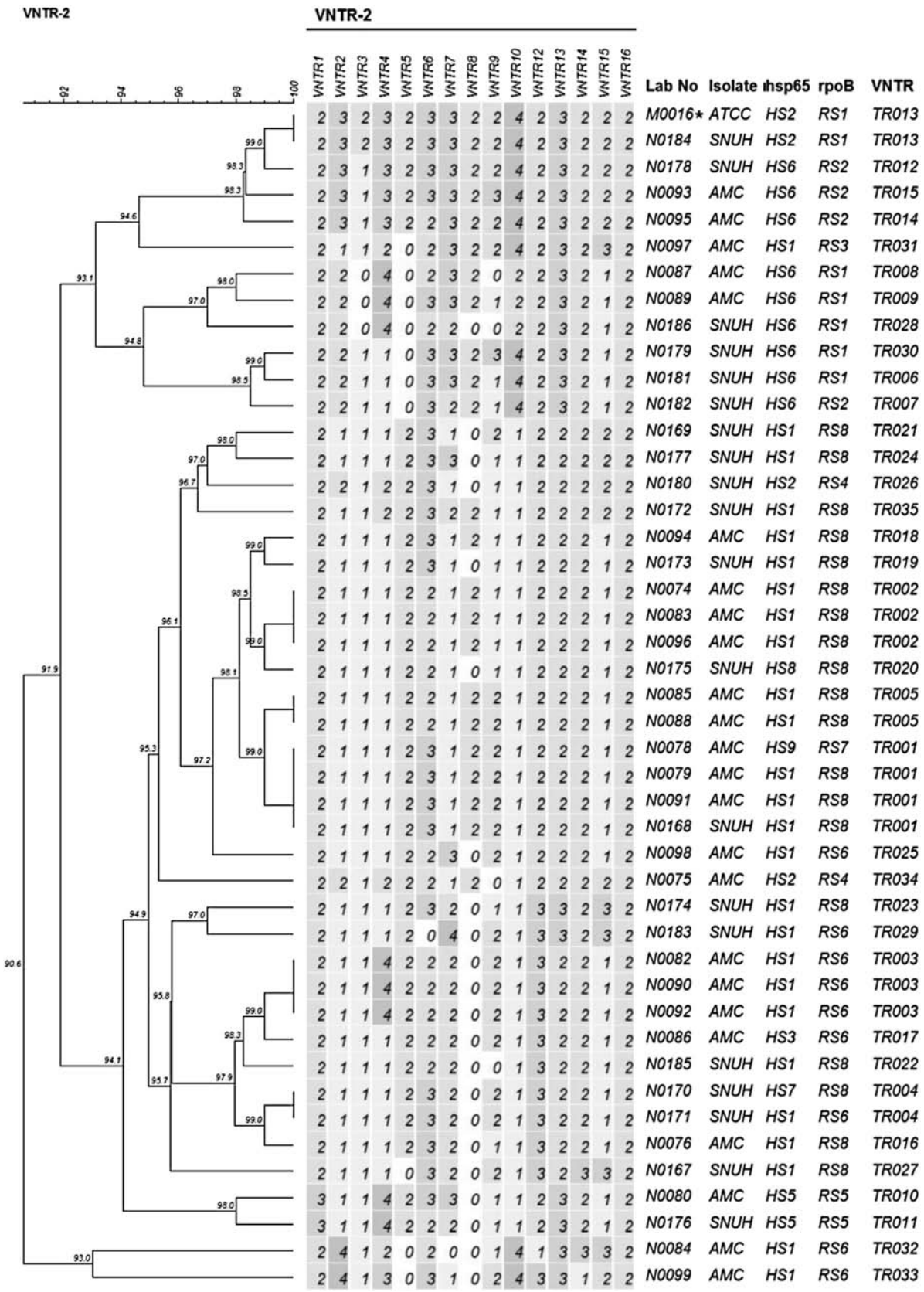


Figure 4. Cluster analysis of *Mycobacterium intracellulare* based on variable-number tandem-repeat analysis profiles. *M0016: Reference strain ATCC 13950.

sequence type. The results indicated that MLST was closely related with the *hsp65* and *rpoB* sequence types.

The diversity index (DI) value for MLST in this study was 0.965, showing that the test has high discriminatory power.

3.4. PFGE

The DI value of PFGE was 0.992, and it had the highest discriminatory power among the four molecular typing methods performed in this study. The 44 *M. intracellulare* isolates were classified into 36 patterns by PFGE. Among these, eight patterns included less than three isolates, and 28 patterns were unique. The major pattern was MAV.S19.004, and it included three isolates, which belong to the HS1 and RS8 sequence types (Figure 3).

The PFGE patterns were divided into two clusters depending on the *hsp65* and *rpoB* sequence types, similar to the results of MLST. The calculated genetic similarity between the two clusters was 67%.

3.5. VNTR

The DI value of VNTR was 0.985, almost as high as the DI value of PFGE. The 44 isolates tested were classified into 35 VNTR patterns, among which 30 patterns were unique (Figure 4). Six VNTR patterns consist of less than four isolates each. The major pattern TR001 included three isolates, which belong to the sequence types HS1 and RS8, and one isolate that belongs to the HS9 and RS7 sequence types.

The allelic diversity of 15 VNTR loci ranged from 0 to 0.707. The VNTR7 locus had the highest DI value (0.707), and the VNTR9 locus was the second with 0.627. However, the VNTR1 locus had a low DI value (0.089) and the VNTR16 locus had no discriminatory power. The copy number of all the tested isolates was only two (Table 4).

3.6. MIRU-VNTR

The DI value of MIRU-VNTR was 0.884, and this was the lowest value among the four molecular typing methods. The MIRU-VNTR method divided the 44 isolates into 16 patterns, among which eight were unique (Figure 5).

The major MIRU-VNTR pattern was INT01, and 12 isolates were included in this pattern. The *rpoB* sequence type of all the INT01 isolates was RS8; however, their hap65 sequence type had two kinds in that 11 isolates were of the HS1 sequence type and one was the HS8 type.

4. Discussion

For treating MAC infections, CLA is commonly used in combination therapy [9,24]. In this study, no resistance to CLA was identified in any of the tested isolates. We classified the tested isolates according to their antibiotic susceptibility patterns. SXT was the major antibiotic drug used for the differentiation of

Table 4. VNTR and MIRU-VNTR allele distribution and allelic diversity of 44 *Mycobacterium intracellulare* clinical isolates.

		No. of alleles in 44 *Mycobacterium intracellulare* clinical isolates							
Methods	Locus	0	1	2	3	4	5	6	Allelic diversity (h)
VNTR	VNTR1	—	—	42	2	—	—	—	0.089
	VNTR2	—	30	8	4	2	—	—	0.503
	VNTR3	3	40	1	—	—	—	—	0.172
	VNTR4	—	26	5	5	8	—	—	0.606
	VNTR5	10	—	34	—	—	—	—	0.359
	VNTR6	1	—	19	24	—	—	—	0.527
	VNTR7	1	16	14	12	1	—	—	0.707
	VNTR8	22	—	22	—	—	—	—	0.512
	VNTR9	4	17	21	2	—	—	—	0.627
	VNTR10	—	31	2	—	10	—	—	0.458
	VNTR12	—	1	31	12	—	—	—	0.439
	VNTR13	—	—	27	17	—	—	—	0.485
	VNTR14	—	1	41	2	—	—	—	0.132
	VNTR15	—	29	10	5	—	—	—	0.513
	VNTR16	—	—	44	—	—	—	—	0
MIRU-VNTR	MIN18	—	—	—	—	9	31	4	0.464
	MIN20	—	—	11	31	2	—	—	0.449
	MIN19	—	—	16	14	14	—	—	0.681
	MIN22	—	—	32	3	9	—	—	0.434
	MIN31	—	—	35	9	—	—	—	0.333
	MIN33	—	—	—	26	16	2	—	0.529

MIRU-VNTR = mycobacteria interspersed repetitive-unit-variable-number tandem-repeat analysis.

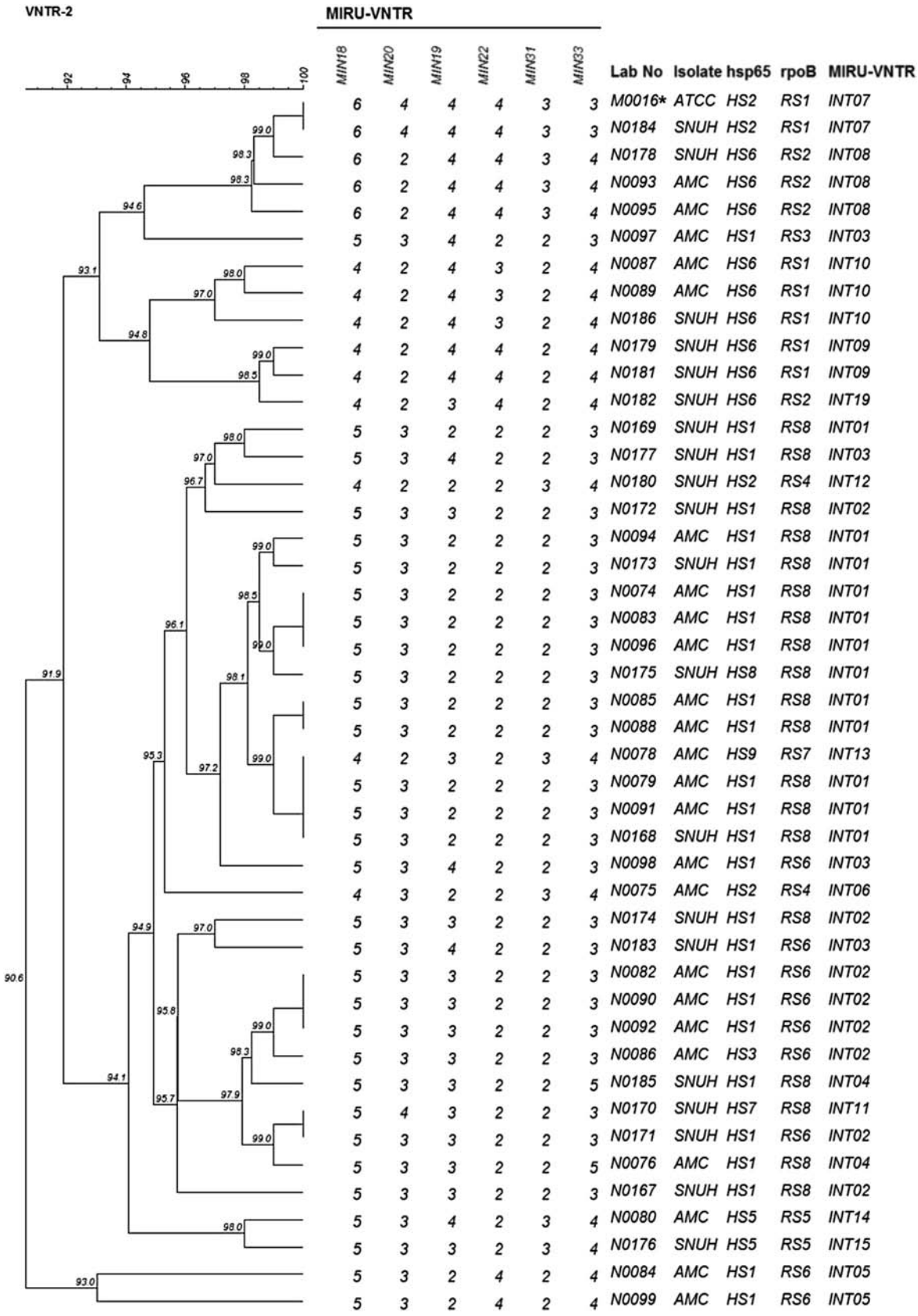


Figure 5. Cluster analysis of *Mycobacterium intracellulare* based on mycobacteria interspersed repetitive-unit-variable-number tandem-repeat analysis profiles. *M0016: Reference strain ATCC 13950.

antibiotic susceptibility patterns in this study; however, all the SXT-susceptible isolates had the same MIC value (2/38 μg/mL), which is close to the break point. It means that almost all the isolates had the same antibiotic susceptibility pattern for SXT, and therefore, there was no necessity to analyze the connection between the antibiotic susceptibility pattern and molecular types.

The *hsp65* sequence type is usually used for the classification of MAC [2], but the *rpoB* sequence type is important for the classification of the whole NTM cluster; therefore, we tried to analyze *rpoB* sequence as well and compared the results with that of *hsp65*. The results showed that there was a clear relation between the major types of both sequence types as described earlier.

In general, the clinical symptom and treatment of MAC infection caused by various species of MAC is no different, and in a previous study, there was almost no relationship between the clinical symptoms and genotype of *M. intracellulare.*

Therefore, further differentiation of *M. intracellulare* is clinically considered unessential [2,4]. However, the molecular typing of *M. intracellulare* is required for determining the reasons for treatment relapse and evaluating disease source to prevent the proliferation of the infection [11].

We investigated the PFGE, VNTR, MIRU-VNTR, and MLST patterns of all the 44 isolates in this study to determine the significance of these four molecular typing methods for discriminating *M. intracellulare* at the subspecies level. Among the four methods, PFGE showed the highest discriminatory power, followed by VNTR. The high discriminatory power of PFGE and VNTR is enough for differentiating between reinfection or relapse, as well as for other molecular epidemiological usages.

The discriminatory power of MIRU-VNTR was low, because MIRU-VNTR in this study used only six loci compared with VNTR that used 15 loci. We used the MIRU-VNTR method developed by Iakhiaeva et al [3]; in their study, multiple isolates obtained from different patients shared the same MIRU-VNTR type. In the study by Ichikawa et al [3], 16 different VNTR loci were used, but again multiple isolates from different patients shared the same MIRU-VNTR type. The finding that different patient isolates with the same VNTR type are mostly clonal by PFGE demonstrates that clonal groups do exist within VNTR *M. intracellulare* and that additional tandem repeats for strain separation may not be useful [11]. However, although we used six loci from the seven loci suggested by Iakhiaeva et al [3], conflicting results were obtained; three isolates, namely, N0086, N0090, and N0092 had the same MIRU-VNTR pattern, and in the VNTR assay, the isolate N0086 had a different VNTR type with N0086 and N0090, but it was almost clonal. In the PFGE result, however, N0086 was not genetically related with N0090 and N0092, and therefore, we concluded that, in contrast to the general expectations, MIRU-VNTR is not enough for the typing of *M. intracellulare*.

In this study, MLST showed a higher discrimination capacity than expected, and also a clear relationship with the *hsp65* and *rpoB* sequence types. Compared with MLST, PFGE and VNTR had higher determinant power, but these two methods had more exceptional case in the relation with the *hsp65* and *rpoB* sequence type. Therefore, if the biological characteristics of each genetic groups identified by MLST can be determined by further research, the MLST method could be regarded as a representative classification method for *M. intracellulare* at the subspecies level.

We also analyzed the isolates included in the major types from each molecular typing and sequence typing methods. *M. intracellulare* isolates N0079 and N0168 belonged to all the major types of commonly tested methods: the HS1 and RS8 types of the *hsp65* and *rpoB* sequence types, the ST004 type of MLST, the TR001 type of VNTR, the INT001 of MIRU-VNTR, and the MAV.S19.004 of PFGE. If the clinical, epidemiological, and microbiological characteristics of these two isolates were reinforced, they could be developed as standard strains for mycobacterial research at least in Korea.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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