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
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 vulniers*, *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 [5]. *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. intracellular*-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. intracellular* made it possible to develop effective VNTR methods [11,13].

In this study, PFGE, VNTR, mycobacteria interpersed 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 (rpoF-F: 5′-ACTTGATGGT CAACAGCTCC-3′; rpoB-R: 5′-AGGTCTCCGCCCC 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 SpeI (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,
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 RS6, and the HS6 type was closely related with the RS1 and RS2 types (data not shown).

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

| Set 1          | MIN18 PCR primer pairs (5’–3’, labeling indicated) | Primer concentration |
|---------------|--------------------------------------------------|----------------------|
| MIN19          | GCCGAACCATTTCCTGCAAC(VIC) GGATTCGGCCGCAATTC      | 4µM                  |
| MIN20          | CATGGGCTGCTCCCTACAC(PET) TACGGTGTAAGCATCGAAG     | 5µM                  |
| MIN22          | GCTGAGCTACAGGCGCCTACAC(NED) CGACGCCGATGACGTAAC    | 7.5µM                |

| Set 2          | MIN31 PCR primer pairs (5’–3’, labeling indicated) | Primer concentration |
|---------------|--------------------------------------------------|----------------------|
| MIN32          | TCAGGAATGGGTCCGGTTC(FAM) AGTGTGACGCTGAAAC        | 5µM                  |
| MIN33          | CGACGCCGATCCAGAACACAGVIC GTGCATTCAACACGGAAC(PET) | 7.5µM                |

PCR = polymerase chain reaction.

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

| Antibiotic susceptibility type | Antibacterial drug | AS Type No. (%) |
|-------------------------------|-------------------|-----------------|
| ASCII                         | CLA               |                |
| AST1                          | S                 | R               | R               | R               | S               | R               | R               | R               | 28 (63.6)       |
| AST2                          | S                 | R               | R               | S               | R               | S               | R               | R               | 7 (15.9)        |
| AST3                          | S                 | R               | R               | R               | R               | S               | 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.
Figure 2. Comparison of the dendrograms from the cluster analysis of *Mycobacterium intracellularum* using categorical coefficients.
Figure 3. Dendrogram of the cluster analysis based on DNA restriction banding patterns of the *Mycobacterium intracellulare* clinical isolates using SpeI.
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 \textit{hsp65} and \textit{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 \textit{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 \textit{hsp65} and \textit{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 \textit{rpoB} sequence type of all the INT01 isolates was RS8; however, their \textit{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

| 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 |   |   |   |   |   | 0.0                  |
| MIRU-VNTR | MIN18 |   |   |   | 9 | 31 | 4 |   | 0.464                |
| MIRU-VNTR | MIN20 |   | 11 | 31 | 2 |   |   |   | 0.449                |
| MIRU-VNTR | MIN19 |   | 16 | 14 | 14 |   |   |   | 0.681                |
| MIRU-VNTR | MIN22 |   | 32 | 3 | 9 |   |   |   | 0.434                |
| MIRU-VNTR | MIN31 |   | 35 | 9 |   |   |   |   | 0.333                |
| MIRU-VNTR | MIN33 |   | 26 | 16 | 2 |   |   |   | 0.529                |

**Table 4.** VNTR and MIRU-VNTR allele distribution and allelic diversity of 44 \textit{Mycobacterium intracellulare} clinical isolates.
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. intracellular 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 HSI 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.

Acknowledgments

This research was financially supported through a grant from the Korea Centers for Disease Control and Prevention (2012-N46001-00).

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