Optimal Combination of VNTR Typing for Discrimination of Isolated *Mycobacterium tuberculosis* in Korea

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**Background:** Variable-number tandem repeat (VNTR) typing is a promising method to discriminate the *Mycobacterium tuberculosis* isolates in molecular epidemiology. The purpose of this study is to determine the optimal VNTR combinations for discriminating isolated *M. tuberculosis* strains in Korea.

**Methods:** A total of 317 clinical isolates collected throughout Korea were genotyped by using the IS6110 restriction fragment length polymorphism (RFLP), and then analysed for the number of VNTR copies from 32 VNTR loci.

**Results:** The results of discriminatory power according to diverse combinations were as follows: 25 clusters in 83 strains were yielded from the internationally standardized 15 VNTR loci (Hunter-Gaston discriminatory index [HGDI], 0.9958), 25 clusters in 65 strains by using IS6110 RFLP (HGDI, 0.9977), 14 clusters in 32 strains in 12 hyper-variable VNTR loci (HGDI, 0.9995), 6 clusters in 13 strains in 32 VNTR loci (HDGI, 0.9998), and 7 clusters in 14 strains of both the 12 hyper-variable VNTR and IS6110 RFLP (HDGI, 0.9999).

**Conclusion:** The combination of 12 hyper-variable VNTR typing can be an effective tool for genotyping Korean *M. tuberculosis* isolates where the Beijing strains are predominant.

**Keywords:** *Mycobacterium tuberculosis*; Molecular Epidemiology; Minisatellite Repeats

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**Introduction**

Tuberculosis (TB) is a contagious disease that develops from infection with *Mycobacterium tuberculosis* bacilli in droplets projected by coughing of active TB patients. DNA typing of *M. tuberculosis* is an efficient tool to show the transmission link of TB. In particular, IS6110 restriction fragment length polymorphism (RFLP) has been used for discrimination of Korean *M. tuberculosis* isolates because of high IS6110 copies and diverse patterns. However, IS6110 RFLP has some disadvantages such as a lengthy turnaround time, difficulty in comparison between laboratory DNA typing data, and uncertain discrimination of isolates containing similar fragments sizes for inter- or intra-strains.

Among more than 40 variable-number tandem repeat (VNTR) loci scattered on the *M. tuberculosis* chromosome, 15 and 24 VNTR loci have been proposed as the international standard. However, the discriminatory power is not enough in countries that have a high proportion of Beijing type *M. tuberculosis*. K strains that have 10 IS6110 RFLP copies are most dominant strain and often found in TB outbreak in Korea. K strains occupy about 4–5% out of any group of *M. tuberculosis* isolates isolated in Korea. K strains causes difficulty in discerning the direct transmission link by IS6110 RFLP.
RFLP typing only. Therefore, we attempted to optimize a combination of VNTR loci and IS6110 RFLP for discrimination of Korean M. tuberculosis isolates.

### Materials and Methods

#### 1. Strains

A total of 317 strains were randomly selected among 2,400 strains isolated from smear and culture-positive primary pulmonary TB patients that registered at the Public Health Center (PHC) of Korea between 2006 and 2011. When the strains were clustered in IS6110 RFLP or VNTR typing, we collected

### Table 1. Primer sequences and labeled dyes of 32 VNTR loci for multiplex PCR

| Multiplex | Locus | Alias | Primer sequence | Unit (bp) | Dye |
|-----------|-------|-------|----------------|----------|-----|
| Mix 1     | 2163a | QUB-11a | CTGATGTTGATCGGGAGATGT/ACCTCTGGAATCTGcacGTGCACT | 69 | PET |
| 2996      | MIRU-26 | TAGTTCTACCGTGAAATCTGTCAG/CTAGGGGACCAGGGCGATAG | 51 | VIC |
| 3192      | MIRU-31 | ACTGATTGGCTCTCATAAGGTCTTA/GTGCCGACGGTGCTGTGAG | 53 | NED |
| Mix 2     | 3820  | TGGCGGCTGAATGGAGAG/ACCTCTCACTCTTGGGCGAC | 57 | PET |
| 2163b     | QUB-11b | CGAATGTACGGCGTGAAGA/AGGTGTAGTTGCTGACTCA | 69 | FAM |
| 4156      | ACCGGCAAGGCTATGTACC/TGTCATCTCGACTACTCC | 59 | NED |
| Mix 3     | 3232  | CCCCAGCCCTACGACTGA/CTGGGGCTGTGAGAG | 56 | FAM |
| 3336      | ATCCCCCCGGGCTCCACT/CCCGGCTGACATCC | 59 | PET |
| 4052      | QUB-26 | GTGCCGCGGCTGTCCA/CCCGGCTGACGCTCAG | 111 | NED |
| Mix 4     | 1955  | Mtub21 | AGACGTCAGACCCCCATT/ACCCGGCAAAAGACTCC | 57 | VIC |
| 4120      | GTTCACGGGCTTGGCTTACGG/ATGCCGAGGGGAGGAG | 57 | PET |
| 0424      | Mtub04 | CTTGGCGGCGGACATCA/ACCCGGCAGCCGATCDC | 51 | FAM |
| Mix 5     | 0580  | MIRU-4 | GCCGGAGGCACCGAAG/CGCCAGCAGAAAAGTTCAC | 77 | FAM |
| 2165      | ETR-A  | AAATCGGTGCTCACTCACCTTCT/CGAAGCTGCGGCTGACAT | 75 | NED |
| 1644      | MIRU-16 | TCGGTGACTGGCGGTCAGCCAATGAT/CCCGGCTGACGCTAC | 53 | VIC |
| Mix 6     | 0960  | MIRU-10 | GTCTGTTACCATCGACCTGTCGTC/CCACGGCTGTGAAGTAACCTC | 53 | FAM |
| 3690      | Mtub39 | CGGTGAGGGATGGCAATACC/CTAGCGGAGGCAGGAACTTAG | 58 | VIC |
| 2074      | Mtub24 | TGTGTCACCTCGGAGGAC/TTGCGCAGCCGATCACC | 56 | PET |
| Mix 7     | 2347  | Mtub29 | GCCACGGCGCATTGATAAAAC/AGAACCAGCGTGCTGATGC | 57 | FAM |
| 3007      | MIRU-27 | TCAGAAGCTCTGCGGAGCAG/AAAGAGTGGTCGATGC | 53 | NED |
| 2461      | ETR-B  | CCGAACCCAGAGACAGCATGTGC/CGACGTTGCGGCTGAC | 57 | VIC |
| Mix 8     | 2531  | MIRU-23 | CTTGGCAAGCTGGCGCAAAC/AGCCTCAACGGGTGC | 53 | VIC |
| 4348      | MIRU-39 | CGCATCGAAGACCTGAGCC/CCGAAACCTGCTAGC | 53 | NED |
| 3155      | QUB-15 | GCAGCGGTCAACCCGAGCAG/CGCGCGCGAATCCGGCGATG | 54 | PET |
| Mix 9     | 2687  | MIRU-40 | GGGTTCGCGAGTGAACAGGT/GGATCTGCTCGCCAGATCGA | 54 | NED |
| 0802      | MIRU-40 | GGTTGCTGGTGAACAGGT/GGATCTGCTCGCCAGATCGA | 54 | NED |
| 2372      | ACCTCGGGTCGAGAATC/CCAATGCTGCCAC | 57 | PET |
| Mix 10    | 2401  | Mtub30 | CGTCACCGGCGCGGAGGCA/CTCCCTGCGAAGT | 58 | PET |
| 3171      | Mtub34 | GGTGCGGACACTGCTCCAGATA/AGGTCTCATGAGGAGGGTTCAG | 54 | NED |
| 0577      | ETR-C  | GGTGAGTCGCTGCGAGAATCCGAG/CGGCGCTGACCTCAGCAGGAG | 58 | VIC |
| Single    | 0154  | MIRU-2 | TGGAGCTTGGCAGAATGGCGCAACT/GACTTCGACGCGGCGTCCCAAAT | 53 | FAM |
| Single    | 2059  | MIRU-20 | TCGAGAGATGTGGGCTTACGGAAGT/GAGAGAGGCGGACCAAAGTC | 77 | FAM |

**Note:** VNTR: variable-number tandem repeat; PCR: polymerase chain reaction.
the epidemiological information of the strains from the PHCs and each person who had the clustered strain by documents and telephone calls.

2. IS6110 DNA fingerprinting

For all 317 isolates, DNA isolation and IS6110 RFLP typing were performed as described previously. An RFLP cluster was defined by completely identical patterns after analysis of BioNumerics version 5.1 software (Applied Maths, Kortrijk, Belgium) within two or more isolates. K strains were identified according to the previously reported definition.

3. Variable number of tandem repeats

We selected 32 VNTR loci for this study. Primer sets for polymerase chain reaction (PCR) were prepared for the 32 VNTR loci. PCR was conducted as described previously and VNTR 4052, 3155, and 2074 were designated by the Research Institute of Tuberculosis in Japan (Table 1). Each primer set was labelled with 4 kinds of fluorescent dye for capillary sequencer analysis. The capillary sequencer, a 3500 Genetic analyzer (Applied Biosystems, Foster City, CA, USA), was used for measuring the precise size of PCR products for each VNTR loci. However, in cases of large fragment sizes over 12,000 bp or an ambiguous size in the capillary sequencer, we measured the size by % agarose gel electrophoresis. Clusters were found as the result of comparison of genotyping data by BioNumerics version 5.1 software (Applied Maths). For PCR amplification, we mainly used Ex-Taq polymerase (Takara, Tokyo, Japan), except for Mix4, Mix7, and Mix9, which used KOD FX Taq polymerase (TOYOBIO, Tokyo, Japan). PCR conditions were as follows: pre-denaturation for 5 minutes at 94°C, denaturation for 30 seconds at 94°C, annealing for 30 seconds at 63°C, extension for 1 minute at 72°C, and post-extension for 10 minutes at 72°C, except for Mix9 that had an annealing temperature of 60°C.

4. Comparison of discriminatory power

The allelic diversity (h) of the 32 VNTR loci was calculated by the following formula: \( h = 1 - \sum x_i^2 \), where \( x_i \) is the frequency of the ith allele at the locus. The discriminatory power of each method was calculated by the Hunter-Gaston discriminatory index (HGDI). Ethical clearance was obtained from the Ethics Review Committee of Korean Institute of Tuberculosis.

5. Ethical considerations

Ethical clearance was obtained from the Ethics Review Committee of Korean Institute of Tuberculosis.

Results

1. Analysis of the 32 VNTR loci

As a result of VNTR typing of the 317 M. tuberculosis strains isolated from Korea, VNTR loci showing a high h value of over 0.6 were VNTR 3232, 3820, 4120, 3336, 2163b, 0424, 1955, 4052, 3192, 4156, 2996, and 2163a. Interestingly, VNTR 0802, 3690, 2165, and 0960 revealed a high h value (over 0.6) in non-Beijing M. tuberculosis isolates (Table 2).

Two PCR products amplified from VNTR 3802 and 0580 (MIRU04) were an unusual size, and were regarded as uncountable repeated numbers (Table 2). The imprecise PCR product of the VNTR 3802 loci was located between 8 and 9 copies, and that of VNTR 0580 was located between 3 and 4 copies of the repeated unit. Four PCR products in VNTR 3820, 4052 (QUB26), and 2372 had multiple bands. Non-amplified PCR products were found in VNTR 3232, 4120, and 2163b (QUB11b), 3192 (MIRU31), 2163a (QUB11a), 2165 (ETR-A), and 2074 (Mtub24).

2. Epidemiological linkage of the VNTR clusters

Six clusters in 13 patients were found after VNTR typing of the 32 loci, and only 3 clusters in 6 patients were found when additionally analysed by IS6110 RFLP typing (Table 3). Out of the 3 clusters, we found only one definite epidemiological linkage through personal contact. Patients with the V32C6 cluster were brother and sister. Patient 06-1731 with the cluster was the brother who developed TB in 2006, and was cured completely in 2007. Patient 08-148, who was the sister of patient 06-1731, had developed TB, and was cured in 2008. Even though random selection of PHC strains, we found the strains isolated from brother and sister TB-developed in different year by accident.

Two patients with V32C3 and IS6110 clusters had lived in Jeju province, even though they were not acquainted with each other. The two strains (06-890 and 09-1184) with the C32C4 cluster and identical IS6110 RFLP types were the K strain that is the most frequent endemic strain in Korea (4–5% in any TB population). The other strain (10-335) with the C32C4 cluster and identical IS6110 RFLP types was the K family that exhibited a difference of only one band. Interestingly, another patient (10-179) with the V32C1 cluster also had the K strain. However, we found different copies in VNTR 2163a, 3232, and 3820 compared with that in V32C4 (data not shown).

3. Discriminatory power of VNTR and IS6110 RFLP typing

When the internationally standardized 15 VNTR loci were applied to the 317 strains, we found 25 clusters in 83 strains (HGDI, 0.9958) (Table 4), and at least 7 VNTR loci (3232, 4052, 3192, 2165, 0960, 3820, and 0580) had h values greater than 0.6. As a result, for the Southern Korean TB population, 15 VNTR loci can detect reliable epidemiological linkage.
Table 2. Allelic diversity and $h$ values of 32 *Mycobacterium tuberculosis* VNTR loci determined by 317 Korean isolates

| VNTR locus (alias) | Allele | Multi-locus (n=258) | Total (n=317) | Beijing (n=258) | Non-Beijing (n=59) |
|--------------------|--------|---------------------|---------------|-----------------|-------------------|
| 3232               | 0      | - 2 5 1 13 7 13 8 11 34 28 64 29 51 12 18 2 7 3 1 7 - - - - 1 | 0.894 | 0.866 | 0.866 |
| 3820               | 0      | - 6 1 43 2 21 14 67 16 19 26 7 51 9 8 7 2 7 2 6 1 2 - - | 0.887 | 0.865 | 0.541 |
| 4120               | 0      | - 4 23 32 18 11 31 85 28 48 11 8 7 1 1 3 3 1 - - - - 1 | 0.861 | 0.827 | 0.678 |
| 3336               | 0      | - 1 2 7 19 13 112 11 18 49 18 8 9 43 1 1 - 3 1 1 - - | 0.818 | 0.752 | 0.816 |
| 2163b (QUB1lb)     | 0      | - 3 27 29 42 99 79 29 4 1 - - 1 - - - - - - 1 - - - - | 0.796 | 0.751 | 0.750 |
| 0424 (Mtub04)      | 0      | - 5 102 100 105 5 - - - - - - - - - - - - - - - - | 0.687 | 0.665 | 0.510 |
| 1955 (Mtub21)      | 0      | - 3 9 62 112 126 5 - - - - - - - - - - - - - - - - | 0.678 | 0.588 | 0.450 |
| 4052 (QUB26)       | 0      | - 1 9 1 1 13 18 38 162 66 6 - 1 - - - - - - - - | 0.673 | 0.627 | 0.782 |
| 3192 (MIRU31)      | 0      | - 18 46 70 157 20 1 1 - - - - - - - - - - - - - - | 0.669 | 0.561 | 0.507 |
| 4156 (QUB4156)     | 0      | - 1 8 126 130 52 - - - - - - - - - - - - - - - - | 0.646 | 0.640 | 0.298 |
| 2996 (MIRU26)      | 0      | - 1 2 34 20 40 13 185 16 6 - - - - - - - - - - | 0.623 | 0.482 | 0.622 |
| 2163a (QUB11)      | 0      | - 1 3 - 15 68 7 12 182 9 1 2 - - - - - - - - | 0.601 | 0.483 | 0.606 |
| 3155 (QUB15)       | 0      | - 1 128 6 173 10 - - - - - - - - - - - - - - - - | 0.338 | 0.539 | 0.448 |
| 0802 (MIRU14)      | 0      | - 9 80 202 22 3 1 - - - - - - - - - - - - - - - - | 0.525 | 0.488 | 0.612 |
| 3690 (Mtub39)      | 0      | - 5 26 217 34 29 2 3 1 - - - - - - - - - - - - | 0.504 | 0.426 | 0.742 |
| 2372               | 0      | - 10 49 232 17 7 1 - - - - - - - - - - - - - - | 0.433 | 0.280 | 0.474 |
| 4348 (MIRU39)      | 0      | - 11 70 230 4 - 1 1 - - - - - - - - - - - - - | 0.423 | 0.250 | 0.390 |
| 2165 (ETR-A)       | 0      | - 2 17 43 251 - 1 - - - - - - - - - - - - - - | 0.339 | 0.154 | 0.665 |
| 0960 (MIRU10)      | 0      | - 8 40 255 10 2 2 - - - - - - - - - - - - - - | 0.335 | 0.175 | 0.606 |
| 2461 (Mtub30)      | 0      | - 4 56 - 253 1 1 - - - - - - - - - - - - - - | 0.322 | 0.103 | 0.429 |
| 2074 (Mtub24)      | 0      | - 4 43 259 10 - - - - - - - - - - - - - - | 0.309 | 0.173 | 0.391 |
| 1644 (MIRU16)      | 0      | - 5 10 288 14 - - - - - - - - - - - - - - | 0.171 | 0.139 | 0.299 |
| 3007 (MIRU27)      | 0      | - 2 12 292 11 - - - - - - - - - - - - - - | 0.149 | 0.111 | 0.298 |
| 0580 (MIRU04)      | 0      | - 5 297 5 4 - - - - - - - - - - - - - - | 0.121 | 0.083 | 0.275 |
| 2317 (Mtub29)      | 0      | - 12 4 298 1 2 - - - - - - - - | 0.115 | 0.104 | 0.159 |
| 2531 (MIRU23)      | 0      | - 3 3 - 2 300 8 - 1 - - | 0.104 | 0.061 | 0.270 |
| 2461 (ETR-B)       | 0      | - 16 301 - - - - - - - - - - - - | 0.096 | 0.023 | 0.344 |
| 0577 (ETR-C)       | 0      | - 8 4 302 3 - - - - - - - - | 0.092 | 0.038 | 0.297 |
| 2059 (MIRU20)      | 0      | - 8 305 4 - - - - - - - - | 0.073 | 0.075 | 0.065 |
| 3171 (Mtub34)      | 0      | - 7 307 3 - - - - - - - - | 0.062 | 0.046 | 0.128 |
| 0154 (MIRU02)      | 0      | - 5 310 2 - - - - - - - - | 0.043 | 0.031 | 0.097 |
| 2687 (MIRU24)      | 0      | - 3 17 - - - - - - - - | 0 0 0 |
Table 3. Characteristics of 6 clusters isolated from VNTR typing analysis in 32 VNTR loci

| VNTR cluster | Year | Region | ID      | No. of IS6110 copies | IS6110 RFLP pattern |
|--------------|------|--------|---------|----------------------|---------------------|
| V32C1        | 2006 | Ansan  | 06-681  | 9                    |                     |
|              | 2010 | Jungsun| 10-179  | 10                   |                     |
| V32C2        | 2007 | Pyongtaek | 07-230 | 12                   |                     |
|              | 2007 | Pyongtaek | 07-977 | 13                   |                     |
| V32C3        | 2006 | Jeju   | 06-481  | 17                   |                     |
|              | 2006 | Jeju   | 06-571  | 17                   |                     |
| V32C4        | 2006 | Ansan  | 06-890  | 10                   |                     |
|              | 2009 | Goseung| 09-1184 | 10                   |                     |
|              | 2010 | Pohang | 10-335  | 11                   |                     |
| V32C5        | 2007 | Anyang | 07-2256 | 18                   |                     |
|              | 2007 | Tongyoung | 07-1645 | 19                   |                     |
| V32C6        | 2006 | Busan  | 06-1731 | 16                   |                     |
|              | 2008 | Busan  | 08-148  | 16                   |                     |

VNTR: variable-number tandem repeat; RFLP: restriction fragment length polymorphism.

Discussion

VNTR typing has been proposed as an alternative to IS6110 RFLP typing that bears intrinsic drawbacks for M. tuberculosis DNA typing. However, the disadvantage of VNTR typing is low discriminatory power with few combinations of VNTR loci. Therefore, we tried to find an optimal combination of DNA typing methods to discriminate M. tuberculosis isolated from Korea, which is accompanied by a high proportion of Beijing type 34. Furthermore, compared with the surrounding countries, there is a higher proportion of RD181 among the
ancient Beijing types\(^1\). These peculiar characteristics of Korean \textit{M. tuberculosis} strains lead to an unclear distinction of \textit{M. tuberculosis} isolates with only the 15 international standard VNTR loci.

Recently, the utility of hyper-variable VNTR loci has been used to compensate for the lack of discriminatory power of the 15 VNTR loci\(^{10,11,16}\). Hyper-variable VNTR loci, including 3232, 3820, 4120, 3336, and 2163a, also revealed a high \(h\) value in this study, which was similar to that in other studies\(^{9,11,16}\). In the report Murase et al.\(^9\), more than 4\% of these 5 loci had 15 or more copies, leading to difficulty in interpreting the exact copy number. We also found that 2–4\% had 15 or more copies in VNTR 2163b, 3336, and 4120, and 13–16\% had 15 or more copies in VNTR 3820, and 3232. These hyper-variable VNTR loci are excluded in the international standard 15 VNTR loci\(^7\) and JATA 12\(^9\) because of the absence of PCR products, PCR products that are difficult to interpret with 15 or more copies, and amplification of multiple alleles. Non-amplification of PCR products not only occurred for hyper-variable VNTR loci but also general VNTR loci such as 2165 (ETR-A), and 2074 (Mtub24) in this study (Table 2). The major problem of hyper-variable VNTR loci was a high copy number of the repeats, which required additional analysis such as agarose gel electrophoresis. However, the copies in these hyper-variable VNTR loci are so diverse that they have a higher discriminatory power that is too valuable to be ignored. Iwamoto et al.\(^8\) also recommended that these hyper-variable VNTR loci for second-line typing of clustering following the international standard 15 loci.

MIRU 40, Mtub 21, VNTR 4156, Mtub 04, QUB26, and QUB11b also revealed high \(h\) values in a previous study of South Korean \textit{M. tuberculosis}\(^9\). In this study, we obtained a good result using hyper-variable VNTR loci, and IS6110 RFLP was useful as a secondary tool to discriminate the clusters.

An intriguing characteristic was found after comparing the mode of VNTR loci among Korea, Taiwan, and Japan\(^{4,6}\). Between Taiwan and Korea, there were some differences in the mode of copies of VNTR loci 0154, 3192, 2163b, 4052, 0424, 1955, 2347, and 2401. In particular, 5 copies of VNTR 0154, 0424, and 1955, and 3 copies of VNTR 2347 were modes in Taiwan but rare in Korea. Between Japan and Korea, there were some differences in VNTR 2163b, 1955, 3155, 3232, 3820, and 4120. Notably, 5 copies of VNTR 3155 and 4 copies of VNTR 3336 were more frequent in Japan but rare in Korea. These differences may be the clue for differentiation among the three countries. Compared with Beijing strains, we found that VNTR 2165, 0960, and 2074 loci had excellent higher \(h\) values for the non-Beijing strains, indicating that these VNTR loci may be useful to discriminate \textit{M. tuberculosis} strains in countries with a high proportion of non-Beijing strains. The optimal combination of VNTR loci may be different depending on the proportion of Beijing strains or non-Beijing strains in each country.

We could not find any cluster consisted of only multi-drug resistant (MDR) strains in this study. The strains included in this study were selected randomly among strains collected from PHCs. Therefore, most of them (281 strains) were pan-susceptible, only a few of strains (13 strains) were MDR, and the remains (23 strains) were any drug resistant with non-MDR.

Even though we analysed 32 VNTR loci for discrimination of 317 Korean \textit{M. tuberculosis} isolates, we found 6 clusters. Among them, 3 clusters were not found to be clusters when additionally analysed by the IS6110 RFLP typing method, indicating that IS6110 RFLP typing is very useful for sub-classifying VNTR clusters. In terms of cost efficiency, it is difficult to use 32 VNTR loci for discrimination of \textit{M. tuberculosis}, and the 15 international standard VNTR loci do not have satisfactory discrimination power for Korean strains. Inevitably, we need hyper-variable VNTR loci and the additional IS6110 RFLP typing method for effective discrimination of Korean \textit{M. tuberculosis} strains.

The combination of 12 hyper-variable VNTR typing can be an effective tool for genotyping Korean \textit{M. tuberculosis} isolates in which Beijing strains are predominant.

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