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Mycobacterial Interspersed Repetitive Units-Variable Number of Tandem Repeats를 이용한 국내 결핵균의 균주 감별

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Strain Typing of *Mycobacterium tuberculosis* Isolates from Korea by Mycobacterial Interspersed Repetitive Units-Variable Number of Tandem Repeats

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Background : *Mycobacterium tuberculosis* is one of the most clinically significant infectious agents. Especially during mass outbreaks, accurate identification and monitoring are required. The proportion of Beijing family members is very high among infecting strains, and spoligotyping is not suitable for strain typing. Therefore, we studied the homogeneity of isolates using the mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR) method and identified its utility for carrying out molecular epidemiologic analysis.

Methods : Eighty-one clinical *M. tuberculosis* isolates that had previously been analyzed by spoligotyping were used in this study. We used the 12 standard MIRU loci and further four exact tandem repeat (ETR) loci (ETR-A, -B, -C, and -F). Four strains each of randomly selected Beijing and Beijing-like families were subjected to IS6110- restriction fragment length polymorphism analysis.

Results : All 81 samples showed amplification products of all VNTR loci, and all of them showed differences in at least one locus. The calculation of the Hunter-Gaston diversity index (HGDI) for MIRU-VNTR gave the value of 0.965. Discriminatory index in the six loci (MIRU-10, -16, -26, -31, -39, and ETR-F) were found to be highly discriminated (HGDI >0.6). Beijing and Beijing-like family isolates were discriminated into different MIRU-VNTR types.

Conclusions : MIRU-VNTR analysis by using well-selected loci can be useful in discriminating the clinical *M. tuberculosis* isolates in areas where the Beijing family is predominant. *(Korean J Lab Med 2009;29:314-9)*

Key Words : *Mycobacterium tuberculosis, Molecular strain typing, MIRU-VNTR*

INTRODUCTION

Mycobacterium tuberculosis is one of the most clinically significant infectious agents, killing about 3 million persons each year and infecting at least 10 million more. It has been estimated that as much as one third of the world's population is infected [1, 2]. There is no exception in South

Korea. Mass outbreaks of tuberculosis have been sporadic but frequent in high school students, military personnel, and North Korean refugees [3].

During mass outbreaks, accurate identification and monitoring of large numbers of *M. tuberculosis* strains are required to analyze transmission of the infection. Because of these demands, various genotyping methods have been applied, and efforts continue to find powerful new approaches. Established methods include IS6110-restriction fragment length polymorphism (RFLP), random amplification of polymorphic DNA (RAPD), interspersed repetitive sequence-polymerase chain reaction (IRS-PCR), and direct repeat (DR) spoligotyping [4]. At present, IS6110-RFLP is the gold standard [5], but this method is labor intensive and requires culturing of the slowly growing mycobacteria, which is inconvenient and delays the results [6]. Moreover, strains with low IS6110 copy numbers cannot be analyzed adequately, and such strains are spreading worldwide. Also, the Beijing family carries 20 to 25 copies of IS6110 and exhibits highly similar RFLP patterns and thus likewise cannot be analyzed accurately [5-7]. The spoligotyping method is simple, rapid, robust, and economical [4, 5]. However, its differentiating power is less than that of IS6110 typing when high copy-number strains are being analyzed [5, 8].

A new PCR-based method relies on mycobacterial interspersed repetitive units-variable number tandem repeats (MIRU-VNTR). Each isolate is typed according to the number of copies of repeated units at 12 independent loci scattered throughout the genome. This method has been evaluated in several studies in different settings [6, 9, 10]. The high resolution, fast turnaround time, ability to compare results easily between laboratories, and the possibility of high-throughput analysis make MIRU-VNTR an attractive method for fingerprinting large numbers of *M. tuberculosis* isolates. However, the 12-locus MIRU-VNTR provides limited information about Beijing family isolates [7, 11]. Therefore, the newly proposed variable-loci MIRU-VNTR typing method was evaluated for its ability to differentiate *M. tuberculosis* Beijing family strains [12-14]. In our previous study, the proportion of Beijing and Beijing-like families was about 80% [15], so a sophisticated molecular epidemiologic analytic method other than IS6110-RFLP or spoligotyping is required. We investigated the utility of MIRU-VNTR as a molecular strain typing tool for differentiating *M. tuberculosis* isolated in South Korea.

MATERIALS AND METHODS

1. Sample collection and bacterial strains

A total of 81 isolates were randomly selected from among 187 isolates used in the previous study [15], which were clinical isolates originally cultured at Pusan National University Hospital or the Korean Institute of Tuberculosis. Therefore, the same spoligotyping data were used. All strains were grown on Löwenstein-Jensen medium suspended in 400 μL of TE buffer (10 mM Tris hydrochloride, 1 mM EDTA, pH 8.0). The DNA extraction process for MIRU-VNTR analysis was as follows. After sterilization for 20 min at 80℃, lysozyme was added to a final concentration of 1 mg/mL, and the mixture was incubated for 24 hr at 37℃. Next, 6 μL of proteinase K (at a 10 mg/mL concentration) and 70 μL of 10% sodium dodecyl sulfate (SDS) were added, and incubation was continued for 10 min at 65℃. An 80 μL volume of N-cetyl-N,N,N-trimethyl ammonium bromide was added. The cups were vortexed briefly and incubated for 10 min at 65℃. An equal volume of chloroform-isoamyl alcohol (24:1 vol/vol) was added, and the mixture was vortexed for 10 sec. After centrifugation for 5 min, 0.6 volume of isopropanol was added to the supernatant liquid to precipitate the DNA. After 20 min at −20℃ and centrifugation for 15 min, the pellet was washed once with 70% ethanol, and the air-dried pellet was dissolved in 20 μL of 0.1× Tris-EDTA (TE) buffer [16].

2. MIRU-VNTR

The isolates were genotyped by PCR amplification of the original 12 MIRU-VNTR loci (MIRU-02, -04, -10, -16, -20, -23, -24, -26, -27, -31, -39, -40) designated by Supply et al. [14, 17] and 4 exact tandem repeat (ETR) loci (ETR-A, -B, -C, -F) containing VNTRs of interspersed sequences. The

primers and DNA sequences for their amplification are des-cribed in Table 1. The amplification profile consisted of 30 cycles of 30 sec at 95℃, 30 sec at 61℃, and 1 min at 72℃. The amplified PCR products were analyzed on a 2% agarose gel, and the number of tandem repeats was calculated. For discrimination analysis, the Hunter-Gaston diversity index (HGDI) was calculated as described elsewhere [18] and used for comparison of the discriminatory power of each VNTR

locus.

3. IS6110-RFLP

Among 81 *M. tuberculosis* isolates, randomly selected four Beijing family and four Beijing-like family members were subjected to IS6110-RFLP analysis performed by an internationally standardized method [19]. Briefly, mycobac-

Table 1. Primer sequences for amplifying MIRU-VNTR loci

Primer	Sequence (5′-3′)	Primer	Sequence (5′-3′)
MIRU-2F	TGGACTTGCAGCAATGGACCAACT	MIRU-2R	TACTCGGACGCCGGCTCAAAAT
MIRU-4F	GCGCGAGAGCCCGAACTGC	MIRU-4R	GCGCAGCAGAAACGTCAGC
MIRU-10F	GTTCTTGACCAACTGCAGTCGTCC	MIRU-10R	GCCACCTTGGTGATCAGCTACCT
MIRU-16F	TCGGTGATCGGGTCCAGTCCAAGTA	MIRU-16R	CCCGTCGTGCAGCCCTGGTAC
MIRU-20F	TCGGAGAGATGCCCTTCGAGTTAG	MIRU-20R	GGAGACCGCGACCAGGTACTTGTA
MIRU-23F	CTGTCGATGGCCGCAACAAAACG	MIRU-23R	AGCTCAACGGGTTCGCCCTTTTGTC
MIRU-24F	CGACCAAGATGTGCAGGAATACAT	MIRU-24R	GGGCGAGTTGAGCTCACAGAA
MIRU-26F	TAGGTCTACCGTCGAAATCTGTGAC	MIRU-26R	CATAGGCGACCAGGCGAATAG
MIRU-27F	TCGAAAGCCTCTGCGTGCCAGTAA	MIRU-27R	GCGATGTGAGCGTGCCACTCAA
MIRU-31F	ACTGATTGGCTTCATACGGCTTTA	MIRU-31R	GTGCCGACGTGGTCTTGAT
MIRU-39F	CGCATCGACAAACTGGAGCCAAAC	MIRU-39R	CGGAAACGTCTACGCCCCACACAT
MIRU-40F	GGGTTGCTGGATGACAACGTGT	MIRU-40R	GGGTGATCTCGGCGAAATCAGATA
ETR-AF	AAATCGGTCCCATCACCTTCTTAT	ETR-AR	CGAAGCCTGGGGGTGCCCGCGATTT
ETR-BF	GGCATGCCGGTGATCGAGTGG	ETR-BR	GGCATGCCGGTGATCGAGTGG
ETR-CF	GTGAGTCGCTGCAGAACCTGCAG	ETR-CR	GGCGTCTTGACCTCCACGAGTG
ETR-FF	CTCGGTGATGGTCCGGCCGGTCAC	ETR-FR	GGAAGTGCTCGACAACGCCATGCC

Abbreviation: MIRU-VNTR, mycobacterial interspersed repetitive units-variable number of tandem repeats.

Table 2. Distribution of 81 *M. tuberculosis* isolates containing variable numbers of each VNTR locus and allelic diversity

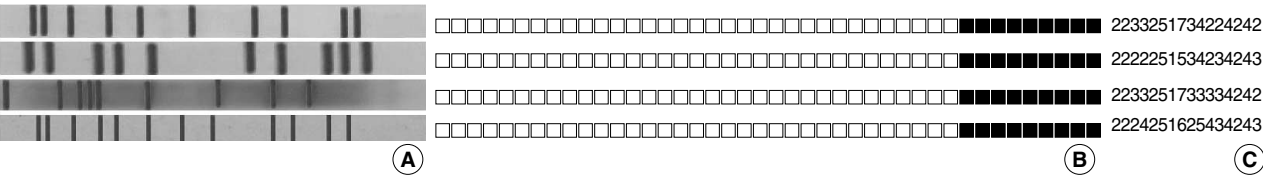| VNTR Loci	N of VNTR repeats										HGDI*
	0	1	2	3	4	5	6	7	8	9	
MIRU-2		18	61	2							0.388
MIRU-4	1	3	77								0.096
MIRU-10		4	35	34	8						**0.633**
MIRU-16		2	32	35	12						**0.643**
MIRU-20		4	77								0.095
MIRU-23					6	69	4	1	1		0.269
MIRU-24		78	3								0.072
MIRU-26			1	12	12	9	12	28	5	2	**0.808**
MIRU-27		1	23	50	7						0.537
MIRU-31		1	8	17	24	20	11				**0.789**
MIRU-39		10	34	30	7						**0.672**
MIRU-40		1	25	47	7	1					0.567
ETR-A		2		5	73	1					0.185
ETR-B		3	72	6							0.206
ETR-C			1	5	74	1					0.163
ETR-F	1	10	33	26	10	1					**0.709**

*Boldface type indicates loci showing high discriminatory power as defined by Sola et al. [20].
Abbreviations: MIRU-VNTR, mycobacterial interspersed repetitive units-variable number of tandem repeats; HGDI, Hunter-Gaston diversity index; ETR, exact tandem repeat.

terial DNA 4.5 μg was digested with *Pvu*II (New England Biolab, Ipswich, MA, USA) 10 U for at least 2 hr. The digested DNA was separated on a 0.8% agarose gel, the gel was reacted with 0.25 M HCl for 10 min and 0.4 M NaOH for 20 min, and the degraded DNA was transferred to an N⁺ Hybond membrane (Amersham Biosciences, Piscataway, NJ, USA) using the capillary method. Hybridizing DNA was detected with an enhanced chemiluminescent labeling kit (ECL, Amersham Biosciences, Piscataway, NJ, USA). The oligonucleotides INS-1 (5′-CGTGAGGGCATCGAGGTGGC) and INS-2 (5′-GCGTAGGCGTCGGTGACAAA) were used to amplify a 245-bp fragment. After hybridization with probes for a day, the membrane was exposed to X-ray film for 30 min followed by development.
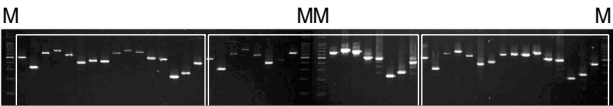
Fig. 1. MIRU-VNTR electrophoresis patterns of three *M. tuberculosis* isolates. Lane M, 100-bp molecular marker. Other lanes in each isolate represent the 16 VNTR loci. Order of the 16 VNTR loci is the same as in Table 1 and 2.
Abbreviation: See Table 1.

RESULTS

All 81 samples showed amplification products of all VNTR loci, and all of them showed differences in at least one locus (Table 2). Fig. 1 shows three isolates' electrophoretic patterns. The results of allelic diversity and HGDI are summarized in Table 2. The calculation of the HGDI for MIRU-VNTR gave the value of 0.965. The discriminatory index in the seven loci (MIRU-4, -20, -23, -24; ETR-A, -B, and -C) showed poor discriminatory power (HGDI<0.3). Three loci (MIRU-2, -27, and -40) were dispersed to discriminate the isolates moderately (0.3≤HGDI≤0.6). Lastly, six loci (MIRU-10, -16, -26, -31, -39, and ETR-F) were highly discriminated (HGDI>0.6). Beijing family isolates having exactly the same spoligotyping pattern were discriminated into different MIRU-VNTR types, as were Beijing-like family isolates (Fig. 2, 3)

DISCUSSION

Identification of homogeneity between strains of *M. tuberculosis* can help not only in certifying the route of infection and the pattern of spread in a nationwide outbreak, but

Fig. 2. Comparison of molecular strain typing patterns of four *M. tuberculosis* Beijing family isolates showing the diversity of MIRU-VNTR. (A) IS6110 RFLP analysis pattern, (B) Spoligotyping pattern, and (C) MIRU-VNTR pattern. Order of the 16 VNTR loci is the same as in Table 1 and 2. All isolates belonging to the Beijing family defined by Kremer et al. [21] hybridized to the last nine probes in spoligotyping, but differed in at least three loci in MIRU-VNTR analysis.
Abbreviations: RFLP, restriction fragment length polymorphism; MIRU-VNTR, mycobacterial interspersed repetitive units-variable number of tandem repeats.
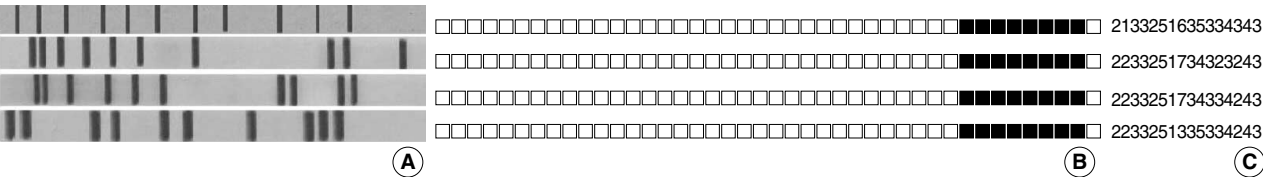
Fig. 3. Comparison of molecular strain typing patterns of four *M. tuberculosis* Beijing-like family isolates showing diversity of MIRU-VNTR. (A) IS6110 RFLP analysis pattern, (B) Spoligotyping pattern, and (C) MIRU-VNTR pattern. Order of the 16 VNTR loci is the same as in Table 1 and 2. All isolates belonging to the Beijing-like family defined by Kremer et al. [21] hybridized exclusively with most of the last nine probes in spoligotyping, but differed from each other in at least two loci in MIRU-VNTR analysis.
Abbreviations: See Fig. 2.

also in recognizing whether a particular infection represents reactivation or reinfection. There have been many studies for identification of strain homogeneity, and many methods that can detect homogeneity rapidly and accurately have been introduced. After the development of the RFLP method for IS6110 in 1991 [22], it became the gold standard for *M. tuberculosis* typing [5]. However, this method is labor intensive [10] and requires culturing for 3 to 4 weeks because it uses colonies, not DNA amplification. Another epidemiologic analysis, the spoligotyping method, is simple, rapid, robust, and economical when only one or two colonies are available or for use in smears because it is based on PCR. However, the differentiating power is less than that of IS6110 typing when high copy-number strains are being analyzed [5, 8], and it cannot differentiate strains within the Beijing family [23].

The MIRU-VNTR is a relatively new method that has been standardized [9, 14, 24]. This method is considerably faster than IS6110-RFLP typing and is applicable to crude DNA extracts from early mycobacterial cultures. It thus is an invaluable tool for genotyping and provides data in a simple format based on the number of repetitive sequences in polymorphic microsatellite and minisatellite regions [4, 6], making the results easy to compare and exchange [9, 25]. A total of 41 loci have been identified in the genome of *M. tuberculosis*, of which 12 (MIRU-2, -4, -10, -16, -20, -23, -24, -26, -27, -31, -39, and -40) are hypervariable repetitive units [9, 17]. We used four additional loci (ETR-A, -B, -C, and -F) [10]. In contrast to previous studies [13, 14, 24], we found the diversities of MIRU-4, -23, -24, ETR-A, -B, and -C to be insufficient to classify the organisms in our collection. For example, the same 2251424 MIRU-VNTR profile of MIRU-4, 20, 23, and 24, and ETR-A, B, and C loci were observed in more than half of the tested isolates. However, our study showed that the VNTR method can be effective in differentiating strains because each of four randomly selected isolates of the Beijing and Beijing-like families had different MIRU-VNTR types. These results show that the VNTR method makes it possible to distinguish domestic strains of similar spoligotypes that are hard to differentiate with IS6110-RFLP. We did not apply this method

for tracing of TB outbreak, but other studies showed its technological reliability [6, 26]. In conclusion, MIRU-VNTR analysis using well-selected loci can be useful in discriminating clinical *M. tuberculosis* isolates in areas where the Beijing family is predominant.

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