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Variable number tandem repeat analysis of *Mycobacterium bovis* isolates from Gyeonggi-do, Korea

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Bovine tuberculosis (TB) is a major zoonosis that's caused by *Mycobacterium bovis* (*M. bovis*). Being able to detect *M. bovis* is important to control bovine TB. We applied a molecular technique, the variable number tandem repeat (VNTR) typing method, to identify and distinguish the *M. bovis* isolates from Gyeonggi-do, Korea. From 2003 to 2004, 59 *M. bovis* clinical strains were isolated from dairy cattle in Gyeonggi-do, Korea, and these cattle had tuberculosis-like lesions. Twenty-four published MIRU-VNTR markers were applied to the *M. bovis* isolates and ten of them showed allelic diversity. The most discriminatory locus for the *M. bovis* isolates in Korea was QUB 3336 (h = 0.64). QUB 26 and MIRU 31 also showed high discriminative power (h = 0.35). The allelic diversity by the combination of all VNTR loci was 0.86. Six loci (MIRU 31, ETR-A and QUB-18, -26, -3232, -3336) displayed valuable allelic diversity. Twelve genotypes were identified from the 59 *M. bovis* isolates that originated from 20 cattle farms that were dispersed throughout the region of Gyenggi-do. Two genotypes [designation index (d.i.) = e, g] showed the highest prevalence (20% of the total farms). For the multiple outbreaks on three farms, two successive outbreaks were caused by the same genotype at two farms. Interestingly, the third outbreak at one farm was caused by both a new genotype and a previous genotype. In conclusion, this study suggests that MIRU-VNTR typing is useful to identify and distinguish the *M. bovis* isolates from Gyeonggi-do, Korea.

Keywords: bovine tuberculosis, Korea, *Mycobacterium bovis*, VNTR typing

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Introduction

Mycobacterium bovis (*M. bovis*) is the cause of bovine tuberculosis (TB), which is a major zoonosis. *M. bovis* has a broad range of hosts that includes humans [12]. Bovine TB affects more than 500 dairy cattle each year in Korea and it is responsible for major agricultural economic losses. Bovine TB especially affects people in the developing countries. In some of these countries, *M. bovis* is responsible for 5-10% of all human TB and 30% of all the child TB patients [26].

Diagnosis is important to control bovine TB and to help block transmission not only to animals, but also to humans. To control bovine TB, it is necessary to know how many and which *M. bovis* strains are dispersed in the field. Classical bacteriological methods are important for isolating pathogenic bacteria from the samples, but these methods are unable to distinguish strains among the same species. The advent of molecular techniques has greatly contributed to the identification and typing of *M. bovis* [13]. The molecular techniques also enable identification of *M. bovis* in a short time because *M. bovis* requires 3-4 weeks to grow [8]. Moreover, the molecular typing method can distinguish *M. bovis* from other *M. tuberculosis* complex and it can discriminate between clinical *M. bovis* isolates. This epidemiological information is useful for tracing the outbreaks and transmission among domestic or wild animals [24,25]. Molecular typing methods such as restriction fragment length polymorphism (RFLP), spoligotyping and variable number tandem repeat (VNTR) analysis have been used. One RFLP method is IS*6110* RFLP, which analyzes the polymorphism of the insertion sequence *6110* (IS*6110*), which is the typical repetitive sequence of the *Mycobacterium tuberculosis* complex. IS*6110* RFLP has shown high discriminatory power and

sensitivity and it has been widely used for molecular typing of the *M. tuberculosis* complex [2,14,23]. However, the IS*6110* RFLP method is not applicable to the typing of *M. bovis* because *M. bovis* has only a single or a few IS*6110* copies [6].

VNTR typing is a PCR-based typing method that analyzes the variations in the number of tandem repeated sequences that are distributed across several loci of the genome [10,22]. In eukaryotes, tandem repeated sequences, such as the microsattellites of 1-15 bp and minisatellites of 10-100 bp, have been found and used clinically [20]. In mycobacteria, repeated sequences similar to the minisatellites in eukaryotes have been identified from the genome sequences of *M. tuberculosis* H37Rv and *M. bovis* AF2122/976 [4]. Based on the minisatellites of mycobacteria, the VNTR methods have been applied to typing *M. bovis*. One VNTR method is the mycobacterial interspersed repetitive units (MIRUs). MIRUs are scattered across 41 locations in the *M. tuberculosis* H37Rv genome and these are composed of 51-77 bp repetitive sequences [20]. Twelve of the 41 loci have shown polymorphism and these 12 loci have been used for typing the *M. tuberculosis* complex [11,20]. Additionally, Queen's University Belfast (QUB) VNTRs have been applied to *M. bovis* strains [15,19]. The VNTR typing methods have shown good stability, reproducibility and high discriminatory power for the *M. tuberculosis* complex [10,12].

In this study, we applied the VNTR typing methods on *M. bovis* strains that were isolated in Gyeonggi-do, Korea and we examined the prevalence and distribution of the genotypes of the *M. bovis* isolates.

Materials and Methods

Study collection and bacteriology

Fifty-nine *M. bovis* isolates originating from dairy cattle that had tuberculosis-like lesions, and the cattle were from Gyeonggi-do livestock, were included in this study; the isolates were taken by the Veterinary Service from 2003 to 2004. Samples from the hilar lymph nodes of cattle suspected to be infected with bovine TB were collected, homogenized with sterile saline solution and decontaminated with N-acetyl-L-cysteine-4% NaOH for 15 min at room temperature. After centrifugation at 1,000 × *g* for 20 min, the isolates were cultured on Lowenstein-Jensen media (Difco, USA) for 3 to 4 weeks at 37°C. Bacteriological identification of *M. bovis* was based on acid-fast staining, the nitrate reductase test and the tiophene-2-carboxylic acid hydrazine assay (Becton Dickinson, USA). *M. tuberculosis* H37Rv (ATCC 27294) was used as a reference strain because its genomic sequence information is available.

DNA preparation

The genomic DNA was extracted from the *M. bovis* isolates as described [15]. In brief, the *M. bovis* isolates from the slopes of the Lowenstein-Jensen media were grown for 3 to 4 weeks at 37°C in Middlebrook 7H9 liquid medium (Difco, USA) that was supplemented with oleic acid-albumin-dextrose-catalase and Tween 80. The cultures of *M. bovis* were collected by centrifugation at 10,000 × *g* for 10 min and they were resuspended with 250 μl of sterile distilled water. The suspended cultures were boiled for 5 min in a water bath, and the supernatant was collected after removing the cellular debris by centrifugation. The DNA concentration was measured with a spectrophotometer at wavelength of 260 nm (Pharmacia Biotech., USA). The DNA was kept at −20°C until it was used for PCR reaction.

VNTR-PCR analysis

Smart *Taq* Pre-Mix (Solgent, Korea) was used for the PCR amplification. Twelve MIRU, 3 ETR (A to C), 7 QUB (11a, 11b, 18, 26, 1895, 3232, 3336), 2 VNTR (0424, 1955) primer pairs were used (Table 1). The PCR reaction was performed with a 20 μl PCR mixture that contained 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.5 μM of each primer, 1.5 units of *Taq* DNA polymerase (Perkin-Elmer Biosystems, USA) and 20 ng genomic DNA as a template. PCR amplification was carried out in a Geneamp PCR system 2700 (Applied Biosystems, USA). The initial denaturation at 95°C for 10 min was followed by 30 cycles of 30 sec at 94°C, 60 sec at 58°C and 90 sec at 72°C. The reaction was terminated by a 7 min step at 72°C. Genomic DNA of *M. tuberculosis* H37Rv and sterile distilled water were used as the positive and negative controls, respectively, in each set of reactions. The PCR products were analyzed by performing electrophoresis with using 1.5% agarose gels in 1 × Tris-boric acid-EDTA buffer (pH 7.2). The TriDye 100-bp DNA ladder (New England Biolabs, USA) was used for estimating the size of the PCR products.

Allelic diversity

The discriminatory power of the individual and combined VNTR markers was assessed by calculating the allelic diversity (h) with using the following equation: $h = 1 - \sum x_i^2[n/(n-1)]$, where n is the number of isolates and xi is the frequency of the ith allele at the locus [17].

Table 1. Primer sequences and the size of the repeat units of the VNTR loci in this study

Locus	Alias	PCR primer sequence (5'-3')†	Repeat unit size (bp)
MIRU 2	VNTR 154	TGGACTTGCAGCAATGGACCAACT	53
		TACTCGGACGCCGGCTCAAAAT	
MIRU 4*	VNTR 580	CAGGTCACAACGAGAGGAAGAGC	77
	ETR-D	GCGGATCGGCCAGCGACTCCTC	
MIRU 10	VNTR 960	GTTCTTGACCAACTGCAGTCGTCC	53
		GCCACCTTGGTGATCAGCTACCT	
MIRU 16	VNTR 1644	TCGGTGATCGGGTCCAGTCCAAGTA	53
		CCCGTCGTGCAGCCCTGGTAC	
MIRU 20	VNTR 2059	GCCCTTCGAGTTAGTATCGTCGGTT	77
		CAATCACCGTTACATCGACGTCATC	
MIRU 23	VNTR 2531	CAGCGAAACGAACTGTGCTATCAC	53
		CGTGTCCGAGCAGAAAAGGGTAT	
MIRU 24	VNTR 2687	CGACCAAGATGTGCAGGAATACAT	54
		GGGCGAGTTGAGCTCACAGAA	
MIRU 26	VNTR 2996	TAGGTCTACCGTCGAAATCTGTGAC	51
		CATAGGCGACCAGGCGAATAG	
MIRU 27	VNTR 3007	TCGAAAGCCTCTGCGTGCCAGTAA	53
		GCGATGTGAGCGTGCCACTCAA	
MIRU 31*	VNTR 3192	ACTGATTGGCTTCATACGGCTTTA	53
	ETR-E	GTGCCGACGTGGTCTTGAT	
MIRU 39	VNTR 4348	CGCATCGACAAACTGGAGCCAAAC	53
		CGGAAACGTCTACGCCCCACACAT	
MIRU 40	VNTR 802	AAGCGCAAGAGCACCAAG	54
		GTGGGCTTGTACTTGCGAAT	
ETR-A	VNTR 2165	ATTTCGATCGGGATGTTGAT	75
		TCGGTCCCATCACCTTCTTA	
ETR-B	VNTR 2461	GCGAACACCAGGACAGCATCATG	57
		GGCATGCCGGTGATCGAGTGG	
ETR-C	VNTR 0577	GACTTCAATGCGTTGTTGGA	58
		GTCTTGACCTCCACGAGTGC	
QUB 11a	VNTR 2163a	CCCATCCCGCTTAGCACATTCGTA	69
		TTCAGGGGGGATCCGGGA	
QUB 11b	VNTR 2163b	CGTAAGGGGGATGCGGGAAATAGG	69
		CGAAGTGAATGGTGGCAT	
QUB 18	VNTR 1982	CCGGAATCTGCAATGGCGGCAAATTAAAAG	78
		TGATCTGACTCTGCCGCCGCTGCAAATA	
QUB 26	VNTR 4052	AACGCTCAGCTGTCGGAT	111
		GGCCAGGTCCTTCCCGAT	
QUB 1895	VNTR 1895	GTGAGCAGGCCCAGCAGACT	57
		CCACGAAATGTTCAAACACCTCAAT	
QUB 3232	VNTR 3232	TGCCGCCATGTTTCATCAGGATTAA	56 (57)
		GCAGACGTCGTGCTCATCGATACA	
QUB 3336	VNTR 3336	ATCCCCGCGGTACCCATC	59
		TTCTACGACTTCGCAACCAAGTATC	
VNTR 0424		CTTGGCCGGCATCAAGCGCATTATT	51
		GGCAGCAGAGCCCGGGATTCTTC	
VNTR 1955		AGATCCCAGTTGTCGTCGTC	57
		CAACATCGCCTGGTTCTGTA	

*MIRU 4 and 31 are also known as ETR-D and E, respectively. †Forward and reverse primers, respectively.

Table 2. VNTR analysis of the *M. bovis* isolates

Isolates	MIRU												ETR			QUB							VNTR		VNTR profile*	Farm
	2	4	10	16	20	23	24	26	27	31	39	40	A	B	C	11a	11b	18	26	1895	3232	3336	0424	1955		
1	2	3	2	2	2	4	2	5	3	1	2	2	7	5	4	10	4	3	4	4	6	3	2	3	5 1 7 5 3 4 4 6 3 2	A
2	2	3	2	2	2	4	2	5	3	1	2	2	7	5	4	10	4	3	4	4	6	3	2	3	5 1 7 5 3 4 4 6 3 2	A
3	2	3	2	2	2	4	2	5	3	2	2	2	7	5	4	10	4	3	3	4	9	10	2	3	5 2 7 5 3 3 4 9 10 2	B
4	2	3	2	2	2	4	2	5	3	1	2	2	7	5	4	10	4	3	4	4	10	4	2	3	5 1 7 5 3 4 4 10 4 2	F
5	2	3	2	2	2	4	2	5	3	1	2	2	7	5	4	10	4	3	4	4	10	4	2	3	5 1 7 5 3 4 4 10 4 2	C
6	2	3	2	2	2	4	2	5	3	1	2	2	7	5	4	10	4	3	4	4	10	4	2	3	5 1 7 5 3 4 4 10 4 2	C
7	2	3	2	2	2	4	2	5	3	1	2	2	7	5	4	10	4	3	4	4	10	3	2	3	5 1 7 5 3 4 4 10 3 2	C
8	2	3	2	2	2	4	2	5	3	1	2	2	7	5	4	10	4	3	4	4	10	4	2	3	5 1 7 5 3 4 4 10 4 2	F
9	2	3	2	2	2	4	2	5	3	1	2	2	7	5	4	10	4	3	4	4	10	4	2	3	5 1 7 5 3 4 4 10 4 2	F
10	2	3	2	2	2	4	2	5	3	1	2	2	7	5	4	10	4	3	4	4	10	4	2	3	5 1 7 5 3 4 4 10 4 2	F
11	2	3	2	2	2	4	2	5	3	1	2	2	7	5	4	10	4	3	4	4	10	4	2	3	5 1 7 5 3 4 4 10 4 2	F
12	2	3	2	2	2	4	2	5	3	2	2	2	7	5	4	10	4	3	3	4	9	10	2	3	5 2 7 5 3 3 4 9 10 2	D
13	2	3	2	2	2	4	2	5	3	2	2	2	7	5	4	10	4	3	3	4	9	10	2	3	5 2 7 5 3 3 4 9 10 2	D
14	2	3	2	2	2	4	2	5	3	1	2	2	7	5	4	10	4	3	4	4	10	3	2	3	5 1 7 5 3 4 4 10 3 2	E
15	2	3	2	2	2	4	2	5	3	1	2	2	7	5	4	10	4	3	4	4	10	3	2	3	5 1 7 5 3 4 4 10 3 2	E
16	2	3	2	2	2	4	2	5	3	1	2	2	7	5	4	10	4	3	4	4	10	3	2	3	5 1 7 5 3 4 4 10 3 2	E
17	2	3	2	2	2	4	2	5	3	1	2	2	7	5	4	10	4	3	4	4	10	3	2	3	5 1 7 5 3 4 4 10 3 2	E
18	2	3	2	2	2	4	2	5	3	1	2	2	7	5	4	10	4	3	4	4	10	3	2	3	5 1 7 5 3 4 4 10 3 2	E
19	2	3	2	2	2	4	2	5	3	1	2	2	7	5	4	10	4	3	4	4	10	3	2	3	5 1 7 5 3 4 4 10 3 2	E
20	2	3	2	2	2	4	2	5	3	1	2	2	7	5	4	10	4	1	4	4	10	4	2	3	5 1 7 5 1 4 4 10 4 2	F
21	2	3	2	2	2	4	2	5	3	1	2	2	7	5	4	10	4	1	4	4	10	4	2	3	5 1 7 5 1 4 4 10 4 2	F
22	2	3	2	2	2	4	2	5	3	1	2	2	7	5	4	10	4	3	4	4	10	4	2	3	5 1 7 5 3 4 4 10 4 2	F
23	2	3	2	2	2	4	2	5	3	1	2	2	7	5	4	10	4	1	4	4	10	4	2	3	5 1 7 5 1 4 4 10 4 2	F
24	2	3	2	2	2	4	2	5	3	1	2	2	7	5	4	10	4	1	4	4	10	4	2	3	5 1 7 5 1 4 4 10 4 2	F
25	2	3	2	2	2	4	2	5	3	1	2	2	7	5	4	10	4	1	4	4	10	4	2	3	5 1 7 5 1 4 4 10 4 2	F
26	2	3	2	2	2	4	2	5	3	1	2	2	7	5	4	10	4	1	4	4	10	4	2	3	5 1 7 5 1 4 4 10 4 2	F
27	2	3	2	2	2	4	2	5	3	1	2	2	7	5	4	10	4	1	4	4	10	4	2	3	5 1 7 5 1 4 4 10 4 2	F
28	2	3	2	2	2	4	2	5	3	1	2	2	7	5	4	10	4	1	4	4	10	4	2	3	5 1 7 5 1 4 4 10 4 2	F
29	2	3	2	2	2	4	2	5	3	1	2	2	7	5	4	10	4	1	4	4	10	4	2	3	5 1 7 5 1 4 4 10 4 2	G
30	2	3	2	2	2	4	2	5	3	2	2	2	7	5	4	10	4	3	3	4	10	10	2	3	5 2 7 5 3 3 4 10 10 2	H
31	2	3	2	2	2	4	2	5	3	2	2	2	7	5	4	10	4	3	3	4	10	10	2	3	5 2 7 5 3 3 4 10 10 2	H
32	2	3	2	2	2	4	2	5	3	2	2	2	7	5	4	10	4	3	3	4	10	10	2	3	5 2 7 5 3 3 4 10 10 2	H
33	2	3	2	2	2	4	2	5	3	1	2	2	4	5	4	10	4	3	4	4	10	3	2	3	5 1 4 5 3 4 4 10 3 2	I
34	2	3	2	2	2	4	2	5	3	1	2	2	4	5	4	10	4	3	4	4	10	3	2	3	5 1 4 5 3 4 4 10 3 2	I
35	2	3	2	2	2	4	2	5	3	1	2	2	4	5	4	10	4	3	4	4	10	3	2	3	5 1 4 5 3 4 4 10 3 2	I
36	2	3	2	2	2	4	2	5	3	1	2	2	4	5	4	10	4	3	4	4	10	3	2	3	5 1 4 5 3 4 4 10 3 2	I
37	2	3	2	2	2	4	2	5	3	1	2	2	4	5	4	10	4	3	4	4	10	3	2	3	5 1 4 5 3 4 4 10 3 2	I
38	2	3	2	2	2	4	2	5	3	2	2	2	7	5	4	10	4	3	3	4	10	10	2	3	5 2 7 5 3 3 4 10 10 2	H
39	2	3	2	2	2	4	2	5	3	1	2	2	7	4	4	10	4	3	4	4	10	3	2	3	5 1 7 4 3 4 4 10 3 2	J
40	2	3	2	2	2	4	2	5	3	1	2	2	6	5	4	10	4	3	4	4	10	3	3	3	5 1 6 5 3 4 4 10 3 3	K
41	2	3	2	2	2	4	2	5	3	1	2	2	6	5	4	10	4	3	4	4	10	3	2	3	5 1 6 5 3 4 4 10 3 2	L
42	2	3	2	2	2	4	2	5	3	1	2	2	6	5	4	10	4	3	4	4	10	3	2	3	5 1 6 5 3 4 4 10 3 2	L
43	2	3	2	2	2	4	2	5	3	1	2	2	6	5	4	10	4	3	4	4	10	3	2	3	5 1 6 5 3 4 4 10 3 2	L
44	2	3	2	2	2	4	2	4	3	1	2	2	7	5	4	10	4	3	4	4	7	3	2	3	4 1 7 5 3 4 4 7 3 2	M
45	2	3	2	2	2	4	2	5	3	1	2	2	7	5	4	10	4	3	4	3	10	3	2	3	5 1 7 5 3 4 3 10 3 2	N
46	2	3	2	2	2	4	2	5	3	1	2	2	7	5	4	10	4	3	4	3	10	3	2	3	5 1 7 5 3 4 3 10 3 2	D

Table 2. Continued

Isolates	MIRU												ETR			QUB							VNTR		VNTR profile*	Farm
	2	4	10	16	20	23	24	26	27	31	39	40	A	B	C	11a	11b	18	26	1895	3232	3336	0424	1955		
47	2	3	2	2	2	4	2	5	3	2	2	2	7	5	4	10	4	3	3	4	10	10	2	3	5 2 7 5 3 3 4 10 10 2	O
48	2	3	2	2	2	4	2	5	3	2	2	2	7	5	4	10	4	3	3	4	10	10	2	3	5 2 7 5 3 3 4 10 10 2	O
49	2	3	2	2	2	4	2	5	3	2	2	2	7	5	4	10	ND†	3	3	4	10	10	2	3	5 2 7 5 3 3 4 10 10 2	O
50	2	3	2	2	2	4	2	5	3	2	2	2	7	5	4	10	4	3	3	4	10	10	2	3	5 2 7 5 3 3 4 10 10 2	O
51	2	3	2	2	2	4	2	5	3	1	2	2	7	5	4	10	4	3	4	4	10	3	2	3	5 1 7 5 3 4 4 10 3 2	P
52	2	3	2	2	2	4	2	5	3	1	2	2	7	5	4	10	4	3	4	4	10	3	2	3	5 1 7 5 3 4 4 10 3 2	P
53	2	3	2	2	2	4	2	5	3	1	2	2	7	5	4	10	4	3	4	4	10	3	2	3	5 1 7 5 3 4 4 10 3 2	P
54	2	3	2	2	2	4	2	5	3	2	2	2	7	5	4	10	4	3	3	4	10	10	2	3	5 2 7 5 3 3 4 10 10 2	Q
55	2	3	2	2	2	4	2	5	3	2	2	2	7	5	4	10	4	3	3	4	10	10	2	3	5 2 7 5 3 3 4 10 10 2	Q
56	2	3	2	2	2	4	2	5	3	1	2	2	7	5	4	10	4	3	4	4	10	4	2	3	5 1 7 5 3 4 4 10 4 2	R
57	2	3	2	2	2	4	2	5	3	1	2	2	7	5	4	10	4	3	4	4	10	3	2	3	5 1 7 5 3 4 4 10 3 2	S
58	2	3	2	2	2	4	2	5	3	1	2	2	7	5	4	10	4	3	4	4	10	3	2	3	5 1 7 5 3 4 4 10 3 2	S
59	2	3	2	2	2	4	2	5	3	2	2	2	7	5	4	10	4	3	3	4	10	10	2	3	5 2 7 5 3 3 4 10 10 2	T

*The VNTR profiles were determined by the MIRU-VNTR loci that showed polymorphism. †Not determined.

Table 3. Allelic distribution at each VNTR locus

No. of copies	VNTR locus number																							
	MIRU												ETR			QUB							VNTR	
	2	4	10	16	20	23	24	26	27	31	39	40	A	B	C	11a	11b*	18	26	1895	3232	3336	0424	1955
0																								
1										45								9						
2	59		59	59	59		59			14	59	59											58	
3		59							59									50	14	2		27	1	59
4						59		1					5	1	59		58		45	57		18		
5								58						58										
6													4								2			
7													50								1			
8																								
9																					3			
10																59					53	14		
11																								
12																								
Allelic diversity (h)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.35	0.00	0.00	0.26	0.02	0.00	0.00	0.00	0.25	0.35	0.05	0.18	0.64	0.02	0.00

*The locus QUB 11b did not amplify in one sample.

Results

Analysis of the MIRU-VNTR loci in the *M. bovis* isolates

All 59 *M. bovis* strains were isolated from 20 dairy cattle farms in Gyeonggi-do, Korea. MIRU-VNTR analysis was performed on all the 59 *M. bovis* isolates by using 24 published markers (Table 1), which included 12 MIRU, 3 ETR, 7 QUB and 2 VNTR loci. Ten of the 24 VNTR markers showed genetic polymorphism (Table 2). The loci that showed polymorphism were two MIRU (26, 31), two ETR (A, B), five QUB (18, 26, 1895, 3232, 3336), and one

VNTR (0424). Twelve different VNTR profiles were obtained by these 10 loci.

The allelic diversity (*h*) differed for the individual loci, ranging from 0.00 to 0.64 (Table 3). The QUB 3336 locus showed the highest discriminatory power (*h* = 0.64, Fig. 1). QUB 26 and MIRU 31 also showed high allelic diversity (*h* = 0.35), and four other loci (MIRU 26, ETR B, QUB 1895, VNTR 0424) showed low discriminative power (*h* = 0.02-0.05). In 12 MIRUs, two loci displayed allelic diversity and the other ten loci showed no allelic diversity. In the QUBs, 5 of 7 loci showed allelic diversity. The ETRs and QUBs had more polymorphic regions than did the MIRUs in the *M. bovis* isolates from Gyeonggi-do, Korea.

The discriminatory power of combining the VNTR loci was evaluated and compared (Table 4). When all the VNTRs were combined together, there were 12 different alleles and the allelic diversity (*h*) was 0.86. The combination of the three most polymorphic VNTR markers (MIRU 31, QUB 26, QUB 3336) showed three different alleles and high allelic diversity (*h* = 0.64). When the discriminative loci (ETR A, QUB 18 and QUB 3232) were added to this combination (MIRU 31, QUB 26, QUB 3336), nine different alleles were identified and the allelic diversity was enhanced (*h* = 0.84). With using all the QUBs and all the ETRs, eight and five different alleles were identified and the allelic diversities (*h*) were 0.77 and 0.57, respectively. With using all 12 MIRUs, three different alleles were identified and the allelic diversity (*h*) was 0.38.

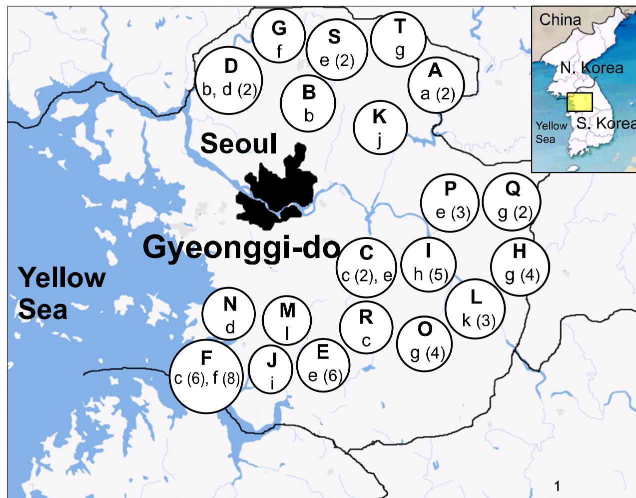

Fig. 1. The geographical origin of the *M. bovis* strains isolated from cattle are aligned with the corresponding VNTR genotype. A capital letter indicates the cattle farm. A lower-case letter indicates the genotypes of the *M. bovis* strains at each cattle farm and the number in parenthesis indicates the number of outbreaks of the genotype of the *M. bovis* isolates.

Table 4. The allelic diversities determined by the combinations of different VNTR loci

Combination of VNTR loci	No. of alleles	Largest group (%)	Allelic diversity (h)
MIRUs	3	75	0.38
ETRs	5	59	0.57
QUBs	8	37	0.77
MIRU 31, QUB 26, 3336	3	46	0.64
MIRU 31, ETR-A, QUB 18, 26, 3232, 3336	9	25	0.84
All VNTRs	12	20	0.86

Table 5. The VNTR profiles of the *M. bovis* isolates according to the cattle farm

Farm	VNTR allele profile[†]	Designation index	No. of *M. bovis* isolates
A	5 1 7 5 3 4 4 6 3 2	a	2
B	5 2 7 5 3 3 4 9 10 2	b	1
C	5 1 7 5 3 4 4 10 4 2	c	2
C	5 1 7 5 3 4 4 10 3 2	e	1
D (1)*	5 2 7 5 3 3 4 9 10 2	b	1
D (2)*	5 1 7 5 3 4 3 10 3 2	d	2
E	5 1 7 5 3 4 4 10 3 2	e	6
F (1)*	5 1 7 5 3 4 4 10 4 2	c	1
F (2)*	5 1 7 5 3 4 4 10 4 2	c	4
F (3)*	5 1 7 5 1 4 4 10 4 2	f	8
F (3)*	5 1 7 5 3 4 4 10 4 2	c	1
G	5 1 7 5 1 4 4 10 4 2	f	1
H (1)*	5 2 7 5 3 3 4 10 10 2	g	3
H (2)*	5 2 7 5 3 3 4 10 10 2	g	1
I	5 1 4 5 3 4 4 10 3 2	h	5
J	5 1 7 4 3 4 4 10 3 2	I	1
K	5 1 6 5 3 4 4 10 3 3	j	1
L	5 1 6 5 3 4 4 10 3 2	k	3
M	4 1 7 5 3 4 4 7 3 2	l	1
N	5 1 7 5 3 4 3 10 3 2	d	1
O	5 2 7 5 3 3 4 10 10 2	g	4
P	5 1 7 5 3 4 4 10 3 2	e	3
Q	5 2 7 5 3 3 4 10 10 2	g	2
R	5 1 7 5 3 4 4 10 4 2	c	1
S	5 1 7 5 3 4 4 10 3 2	e	2
T	5 2 7 5 3 3 4 10 10 2	g	1

*Indicates the cattle farms that had several outbreaks. The number in parentheses represents the order of the outbreaks. [†]The VNTR profiles are based on MIRU 26, 31, ETR-A, B, QUB 18, 26, 1895, 3232, 3336 and VNTR 0424.

Fig. 2. PCR products of the various *M. bovis* isolates with using primers that amplify QUB3336. Lane M: 100 bp DNA ladder, lane 1-12 and lanes 15-26: *M. bovis* isolates, lanes 13 and 27: *M. tuberculosis* H37Rv, lanes 14 and 28: negative controls.

Table 6. Genotype prevalence of the *M. bovis* isolates

Designation index	VNTR allele profile	No. of farm (%)	No. of *M. bovis* isolates (%)
A	5 1 7 5 3 4 4 6 3 2	1 (5)	2 (3.39)
B	5 2 7 5 3 3 4 9 10 2	2 (10)	2 (3.39)
C	5 1 7 5 3 4 4 10 4 2	3 (15)	9 (15.25)
D	5 1 7 5 3 4 3 10 3 2	2 (10)	3 (5.08)
E	5 1 7 5 3 4 4 10 3 2	4 (20)	12 (20.34)
F	5 1 7 5 1 4 4 10 4 2	2 (10)	9 (15.25)
G	5 2 7 5 3 3 4 10 10 2	4 (20)	11 (18.64)
H	5 1 4 5 3 4 4 10 3 2	1 (5)	5 (8.47)
I	5 1 7 4 3 4 4 10 3 2	1 (5)	1 (1.69)
J	5 1 6 5 3 4 4 10 3 3	1 (5)	1 (1.69)
K	5 1 6 5 3 4 4 10 3 2	1 (5)	3 (5.08)
L	4 1 7 5 3 4 4 7 3 2	1 (5)	1 (1.69)
Total	12	20 (100)	59 (100)

VNTR profiles of the *M. bovis* isolates in the region of Gyeonggi-do

The VNTR profiles of the 59 *M. bovis* isolates were examined. Twelve genotypes were identified from the *M. bovis* isolates originating from the 20 dairy cattle farms in Gyeonggi-do.

Most of the farms had one genotype that was identical to the *M. bovis* isolates in the region of Gyeonggi-do (Fig. 1). Interestingly, there were three farms that had two *M. bovis* genotypes (Table 5). Two genotypes (designation index (d.i.) = c, e) coexisted at farm C. There were multiple TB outbreaks at farms D, F and H. The genotype (d.i. = g) of the *M. bovis* isolates was identical at the first and second outbreaks on farm H. Interestingly, the genotype of *M. bovis* was different between the first (d.i. = b) and second outbreaks (d.i. = d) on farm D. The identical genotype (d.i. = c) of the *M. bovis* isolates was identified at the first and second outbreaks, but a new genotype (d.i. = f) of *M. bovis* appeared at the third outbreak as the main genotype with a previous genotype (d.i. = c) being noted at farm F.

The prevalence of the genotypes of the *M. bovis* isolates was examined. Two genotypes of the *M. bovis* isolates (d.i. = e, g) were prevalent in Gyeonggi-do. These two genotypes of the *M. bovis* isolates were identified at four cattle farms (20% of the total cattle farms) and they accounted for 20.34% and 18.64% of the total *M. bovis* isolates, respectively. Two genotypes (d.i. = c, f) of the *M. bovis* isolates accounted for about 15% of the total *M. bovis* isolates individually and these were found at 2-3 cattle farms. Most of the remaining genotypes were identified from one *M. bovis* isolate at one cattle farm.

Discussion

Advances in molecular typing techniques have contributed to the epidemiological study of infectious diseases such as bovine TB. Molecular typing techniques have enabled us to identify and distinguish *M. bovis* isolates, and the data from molecular typing allow us to discern the genotypes of *M. bovis* that are prevalent in a specific area and how multiple outbreaks occur. Moreover, this epidemiological information could trace the origin of outbreaks and advise us on how to block the transmission of bovine TB. The MIRU-VNTR typing is a powerful tool for identifying and genotyping the *M. tuberculosis* complex. Various combinations of tandem repeats such as MIRUs, ETRs and VNTRs have been proven to be useful for identifying the *M. tuberculosis* complex, including *M. bovis* [7,16,21]. Spoligotyping is the method to detect the presence or absence of a 43 spacer DNA sequence between direct repeats (DR) in the DR region of *M. tuberculosis* complex strains, and IS*6110* typing is a RFLP method that uses the element IS*6110* as a probe. Spoligotyping and IS*6110* RFLP are also useful tools, but spoligotyping and IS*6110* RFLP have been reported to be less discriminative that the other typing methods for *M. bovis* because most of the *M. bovis* isolates have one or few copies of DR or IS*6110*, respectively [15,18]. The MIRU-VNTR method has proven to be more reproducible, stable and sensitive for the *M. tuberculosis* complex than IS*6110* RFLP [9].

In this study, we analyzed 59 *M. bovis* isolates from

Gyenggi-do, Korea by using 24 published novel VNTR markers. Among the four sets of the MIRU-VNTR loci, the ETRs and QUBs have shown high polymorphism, but the MIRUs have shown very low polymorphism. This result is consistent with previous studies that the QUBs and ETRs showed highly discriminative power both for *M. bovis* and *M. tuberculosis* [5,19], yet the MIRUs have a high discriminative power for *M. tuberculosis*, but not for the *M. bovis* isolates [1,3,7,11,21]. Three loci (QUB 26, QUB 3336, MIRU 31) showed high discriminative power (h = 0.35, 0.64, 0.35, respectively), and ETR A, QUB 18 and QUB 3232 showed moderate allelic diversity (0.26, 0.25, 0.18, respectively). This result is consistent with other reports that five loci (QUB 3232, ETR (A, B), MIRU (24, 26)) were highly discriminative for the *M. bovis* isolates from Chad [7], and six loci, QUB (11a, 26, 3232), ETR (A, B), MIRU 26, were highly polymorphic for the *M. bovis* isolates from Northern Ireland [16]. The *M. bovis* isolates from Belgium were highly discriminated by seven loci, QUB (11a, 11b, 3232, 3336), ETR (A, B), MIRU 26 [1]. However, QUB 11a and MIRU 26 showed low discriminative power for the *M. bovis* isolates from Korea in our study. Also, MIRU 31 (corresponding to ETR E) showed higher polymorphism (h = 0.35) for the *M. bovis* isolates from Korea than that reported in previous study [7].

There were 12 genotypes of *M. bovis*, and these genotypes were dispersed throughout the region of Gyeonggi-do, Korea. There were two prevalent genotypes (d.i. = e, g) of *M. bovis* in the area of Gyeonggi-do, and these two main genotypes were identified in about 20% of the 59 total *M. bovis* isolates and at 20% of the total 20 total cattle farms. This data could not be compared because there have been no previous studies on the genotypes of *M. bovis* isolates in Korea.

There were three cattle farms on which multiple outbreaks took place. One outbreak took place because of a relapse of a previous *M. bovis* strain, and another outbreak took place successively because of two different genotypes of the *M. bovis* strains. Interestingly, the pattern of the outbreaks at one cattle farm indicated a combination of the relapse of a previous *M. bovis* strain and the introduction of a new genotype of the *M. bovis* strain. Two *M. bovis* genotypes coexisted at the third outbreak on this farm, and this might reflect the current state of the Korean livestock industry.

This study suggests the VNTR markers that have high discriminative power could be used to investigate the *M. bovis* isolates from all over Korea, and the information on the genotypes of the *M. bovis* isolates might provide important information on how to control bovine TB in Korea.

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