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Short Communication

Molecular Genotyping of *Mycobacterium tuberculosis* in Mie Prefecture, Japan, Using Variable Numbers of Tandem Repeats Analysis

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SUMMARY: The variable numbers of tandem repeats (VNTR) analysis is a method frequently employed as a molecular epidemiological tool for *Mycobacterium tuberculosis* genetic fingerprinting. In this study, we characterized the population of *M. tuberculosis* circulating in Mie Prefecture, Japan, and assessed the utility of the proposed JATA12- and 15-VNTR analyses of 158 *M. tuberculosis* clinical isolates using 25 VNTR loci. The results revealed that the ancient Beijing sublineage is the most prevalent *M. tuberculosis* strain in Mie Prefecture, accounting for 85.0% of 113 Beijing lineage isolates. Our results also showed that JATA-VNTR using well-selected loci is as reliable as standardized 15-locus MIRU-VNTR. Furthermore, JATA15-VNTR analysis reliably improved the discriminatory power compared with basic JATA12-VNTR analysis. In summary, our data suggest that JATA-VNTR is a useful tool for discrimination of *M. tuberculosis* in areas where ancient Beijing strains are frequently isolated.

Tuberculosis (TB) is a major infectious disease and remains a major public health problem in Japan. In recent years, molecular typing methods have become useful tools for the control of TB and help to indicate possible epidemiological links between TB patients.

IS*6110* restriction fragment length polymorphism (RFLP) typing has been used as the gold standard genotyping method of *Mycobacterium tuberculosis*. However, this method has disadvantages in that the culture of TB bacilli is time-consuming, RFLP typing is technically demanding, and comparison of data from different laboratories is difficult. On the other hand, the variable numbers of tandem repeats (VNTR) typing is considerably faster, requires only small amounts of DNA, and can be easily digitized to share data among other laboratories. This technique not only facilitates the correct identification of isolates in putative outbreaks but also aids in the estimation of unidentified transmissions in surveillance studies.

In Europe, standardized mycobacterial interspersed repetitive unit (MIRU)-VNTR typing has been reported (1,2). The newly proposed MIRU-VNTR systems using 15 and 24 loci were demonstrated to have higher discriminatory powers with a worldwide collection of *M. tuberculosis* strains (2). However, a recent study showed that this typing method could not sufficiently differentiate *M. tuberculosis* strains, including many Beijing genotype strains (3,4).

Meanwhile, the Japan Anti-Tuberculosis Association (JATA)-VNTR has become a standard genotyping method for *M. tuberculosis*, and it has great potential for widespread use in Japan. Although JATA-VNTR is thought to be a specialized combination of VNTR for discrimination of Japanese *M. tuberculosis* strains, few studies have assessed the utility of JATA-VNTR analyses (5–7).

In this study, we investigated the current population structure of *M. tuberculosis* strains circulating in Mie Prefecture, and assessed the utility of the proposed JATA12- and 15-VNTR in typing these clinical isolates using 25 VNTR loci.

A total of 158 *M. tuberculosis* strains were used in this study, of which 4 were isolates from 1 group of epidemiologically linked patients. The remaining 154 strains without epidemiological links were randomly collected at the Mie Prefecture Health and Environment Research Institute from culture-confirmed pulmonary TB patients in the National Hospital Organization Mie Chuo Medical Center from 2007 to 2010. This sample collection accounts for 28.1% (158/562) of sputum smear-positive patients in Mie Prefecture from 2007 to 2010.

Twenty-five VNTR loci (JATA and additional 10 loci; MIRU 4, 40, 16, 23, 27, and 39; ETR-C and F; Mtub30 and 39) in the VNTR typing method were used in this study (Table 1). Each locus was amplified by PCR with the primers described previously (2,7,8). The PCR fragments were analyzed by gel electrophoresis using 1.7–2.0% NuSieve 3:1 agarose (Lonza, Rockland, Maine, USA). To evaluate the discriminatory power of the typing methods and the allelic diversity of each VNTR locus, the Hunter-Gaston Discrimination Index (HGDI) was used as described previously (9). To identify a strain belonging to the Beijing lineage, the DNA

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Table 1. The allelic profiles and diversity of each of the 25 VNTR loci in *M. tuberculosis* isolates from Mie Prefecture (2007–2010)

Alias	Synonym	Locus	Copy number of tandem repeat unit(s)															Multiple	Allelic diversity		
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	over		All	Beijing	non-Beijing
Mtub04	J 01	424		8	40	24	83	2	1											0.63	0.49	0.60
ETR-C		577				14	139	2												0.19	0.14	0.31
MIRU 4	ETRD	580		5	139	6	3	2	1											0.21	0.09	0.47
MIRU 40		802		21	19	110	3	1	1											0.47	0.15	0.72
MIRU 10	J 02	960		26	12	92	13	8			4				1				1	0.62	0.50	0.77
MIRU 16		1644	1	5	14	122	12													0.38	0.33	0.49
Mtub21	J 03	1955	2	19	21	86	17	1		1	2	1		1						0.63	0.39	0.73
QUB 18	J 13	1982	1		6	2		30	3	7	29	7	59	7	4			1	1	0.78	0.69	0.62
Mtub24	J 04	2074		8	40	90	16	1											1	0.59	0.57	0.63
QUB 11b	J 05	2163			20	33	14	10	43	25	6	1		1					2	0.83	0.78	0.82
QUB 11a	J 14	2163			19	1	6	11	1	11	63	23	2	2	1			12		0.74	0.58	0.80
ETR-A	J 15	2165			10	32	107	6			1									0.48	0.20	0.55
VNTR 2372	J 06	2372		13	26	85	28	4	1										1	0.65	0.54	0.66
Mtub30		2401		3	67	1	87													0.52	0.43	0.40
MIRU 23		2531		5			2	120	26		1	1							1	0.38	0.17	0.52
MIRU 26	J 07	2996		6	5		11	27	23	77	5	3							1	0.71	0.53	0.70
MIRU 27	QUB 5	3007		2	7	147	1													0.12	0.14	0.09
QUB 15	J 08	3155			18	5	120	13				1				1				0.41	0.41	0.38
MIRU 31	J 09	3192			9	39	32	71	5	2										0.70	0.57	0.54
ETR-F		3239	3	2	18	130	1	1												0.28	0.20	0.48
QUB 3336	J 10	3336				1	4	1	9	87	6	2	20	5	14	4	1		1	0.66	0.42	0.83
Mtub39		3690		8	12	101	16	15	4	1	1									0.57	0.34	0.82
QUB 26	J 11	4052			16	7	10	8	5	31	70	9	1					1		0.75	0.65	0.85
QUB 4156	J 12	4156		2	13	51	41	48												0.72	0.67	0.58
MIRU 39		4348		21	23	108	3												2	0.49	0.20	0.66

Table 2. Comparison of the discriminatory power of VNTR analyses

Typing method	Total no. of type patterns	No. of unique types	No. of clusters	No. of clustered isolates (%)[1)]	Maximum no. of isolates in a cluster	HGDI[2)]
15MIRU-VNTR	135	122	13	36 (22.8)	6	0.996
JATA12-VNTR	138	126	12	32 (20.3)	5	0.997
JATA15-VNTR	148	141	7	17 (10.8)	5	0.999
25-loci VNTR	149	143	6	15 (9.5)	5	0.999
JATA15 + 3 loci[3)]	149	143	6	15 (9.5)	5	0.999

[1)]: The clustering rate (%) was defined as *Nc/N*, where *N* represents the total number of strains in the study and *Nc* represents the number of strains in clusters of 2 or more strains.
[2)]: The Hunter-Gaston Discrimination Index (HGDI) was calculated as described previously (9).
[3)]: Additional 3 loci represent MIRU39, Mtub30, and Mtub39.

was subjected to PCR amplification using Ex Taq HS version, as previously reported (10). Furthermore, to classify Beijing lineage into the ancient and modern Beijing sublineages, IS*6110* insertion in the NTF region was also analyzed (11).

In this study on 158 *M. tuberculosis* isolates from Mie Prefecture, 71.5% (113/158) were found to be members of Beijing lineage. Furthermore, it was confirmed that the ancient Beijing sublineage is the most prevalent *M. tuberculosis* in Mie Prefecture, accounting for 85.0% of 113 Beijing lineage isolates. Of the 17 putative modern Beijing isolates, none was found to harbor 2 IS*6110* within the NTF region, indicating the absence of W strains from this sample collection. A recent report suggested that the modern Beijing sublineage, which has high transmissibility, is currently increasing in Japan (12). Furthermore, the modern sublineage is considered to be more virulent and to have higher fitness in human hosts than the ancient sublineage (6,11–13). Therefore, it is essential to continuously monitor the population shift for a long period in Mie Prefecture.

We first analyzed the genotypes of the 158 *M. tuberculosis* clinical isolates from Mie Prefecture using the proposed JATA-VNTR analysis. As a result, the use of JATA12-VNTR generated 12 VNTR clusters, with a clustering rate of 20.3% (Table 2). In contrast, use of the JATA15-VNTR dramatically increased the discriminatory power, with a clustering rate of 10.8%. When comparing the HGDI of each JATA-VNTR analysis, JATA15-VNTR achieved a good discriminatory power (HGDI, 0.999) with respect to that of JATA12-VNTR analysis (HGDI, 0.997) (Table 2).

With 25-loci VNTR typing of all isolates, 6 VNTR clusters were found. The use of an additional 10 loci

(with the exception of JATA loci) did not significantly affect discriminatory power, although the clustering rate decreased slightly to 9.5% (Table 2).

In addition, the use of 15MIRU-VNTR generated 13 VNTR clusters, with a clustering rate of 22.8%, indicating that the discriminatory power of 15MIRU-VNTR (135 types; HGDI, 0.996) was inferior to that of other VNTR combinations (Table 2).

The discriminatory power of the VNTR typing system can be improved by supplementation of an extra number of VNTR loci, however, it takes time and effort for analysis. Consequently, probed for other combinations of loci to find those with a discriminatory power close to that of 25-loci VNTR typing on the basis of a reduced number of VNTR loci. Compared with the allelic diversity of an additional 10 loci, 3 additional loci (MIRU 39, Mtub30, and Mtub39) had a relatively higher allelic diversity compared with other additional loci (0.49, 0.52, and 0.57, respectively) (Table 1). By use of these 3 additional loci with JATA15-VNTR, the clustering rate decreased to 9.5% (which is identical to that of 25-loci VNTR typing) (Table 2). Date from these results indicate that the discriminatory power of JATA15-VNTR plus the 3 additional loci combination was equivalent to that of 25-loci VNTR typing (Table 2). However, additional work using many samples is needed to properly confirm the utility of this 18-locus VNTR combination.

Following 25-loci VNTR analysis, the largest cluster contained 5 strains, while other clusters contained 2 strains. In the largest cluster, 4 strains were isolated from epidemiologically linked patients. However, the remaining strain was isolated from a patient whose epidemiological backgroud was unknown. Three clusters had VNTR profiles as 413264745785–74.10–233443, 333473755725–10.84–243443, and 434363745883–884–233443 (listed in the order JATA12-15-MIRU 04, 16, 40, ETR-C, Mtub30, 39 locus), respectively. These cluster profiles were consistent with those of putative expanding cluster types (pECTs) previously reported in Osaka City (7). Because these strains of pECTs are likely highly transmissible, it will be necessary to monitor their emergence from a public health perspective.

The allelic diversity of the VNTR loci varied significantly at each locus (Table 1). Among the 25 loci investigated in this study, QUB 18 (0.78), QUB 11b (0.83), QUB 11a (0.74), MIRU 26 (0.71), MRU 31 (0.70), QUB 26 (0.75), and QUB 4156 (0.72) had high allelic diversity (HGDI, $\geqq$0.70). These high discriminatory loci are included in the JATA-VNTR combination, indicating that the proposed JATA-VNTR contains well-selected loci and can be useful in discriminating clinical isolates in areas where the Beijing lineage is dominant.

The optimal VNTR combination to use for strain typing of *M. tuberculosis* will depend on the number of isolates to be typed, the resources available for typing, and the degree of discriminatory power required. Moreover, a recent study showed that the allelic diversity of each VNTR locus differs according to the strain lineage circulating in each region (14). Therefore, accumulation of local findings of VNTR analyses and selection of optimal VNTR loci are needed to establish this technology as a more powerful tool for the molecular epidemiology of *M. tuberculosis*. Furthermore, use of conventional epidemiological data in combination with these VNTR techniques suitable for the local region could help open a new era against TB in Japan. To the best of our knowledge, this study is the first report of the molecular genotyping of *M. tuberculosis* in this region of Japan, and this information is expected to improve our understanding of TB transmission in this region.

In conclusion, our study revealed that the ancient Beijing sublineage is the most prevalent *M. tuberculosis* genotype in Mie Prefecture, accounting for 85.0% of Beijing lineage isolates. Our results also showed that JATA-VNTR using well-selected loci is a reliable method compared with 15MIRU-VNTR, and that JATA15-VNTR analysis reliably improved the discrimination of *M. tuberculosis* isolates compared with basic JATA12-VNTR analysis. In summary, our data suggest that JATA-VNTR is a useful tool for discrimination of *M. tuberculosis* in areas where ancient Beijing strains are dominant.

Conflict of interest None to declare.

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