Japanese Journal of Infectious Diseases

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Received: January 8, 2021. Accepted: April 5, 2021.
Published online: April 30, 2021.
DOI:10.7883/yoken.JJID.2020.1060

Advance Publication articles have been accepted by JJID but have not been copyedited or formatted for publication.
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Running title: Typing of *Mycobacterium tuberculosis* Strains in Gifu Prefecture

Keywords: *Mycobacterium tuberculosis*; Variable Number Tandem Repeat; Tuberculosis
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**Summary**

To investigate the molecular epidemiological characteristics of *Mycobacterium tuberculosis* strains collected in Gifu Prefecture, Japan, 483 *M. tuberculosis* clinical isolates were used for Japan Anti-Tuberculosis Association (JATA) 18-variable number tandem repeats (VNTR) analysis during 2015–2019. To estimate the lineages of *M. tuberculosis* strains, JATA18-VNTR profiles were applied to a maximum a posteriori method. The results revealed that the ancient Beijing subfamily, accounting for 57.3% (277/483) was the most prevalent *M. tuberculosis* strain. Furthermore, 18 clusters (GC-1–GC-18) were found by minimum spanning tree analysis. The proportion of clustering strains was 9.9% (48/483), and epidemiological links to these clusters were unclear without GC-6 and GC-18. Meanwhile, interestingly, VNTR profiles of GC-7–GC-9 and GC-14 were indistinguishable from the regional epidemic strains of Nagoya City, which has a strong socioeconomic relationship with Gifu Prefecture, but did not match the nationwide epidemic strains. This study suggests that coordinated analyses within prefectures with strong socioeconomic relationships are important.
Tuberculosis (TB) caused by Mycobacterium tuberculosis is a major infectious disease and remains a public health problem in Japan. The incidence of TB in Japan has steadily decreased for decades, with 11.5 cases per 100,000 reported in 2019. The incidence of TB in Gifu prefecture (Gifu), located in the center of Japan (Fig. 1A) and has a strong socioeconomic relationship with Nagoya City (Nagoya) is higher than the average in Japan and the second highest at 14.6/100,000 in 2019 (1).

The surveillance of M. tuberculosis using genotyping methods based on variable number tandem repeats (VNTR) can uncover possible epidemiological links among TB patients (2, 3). The Japan Anti-Tuberculosis Association (JATA)-VNTR typing has become a standard genotyping method for identifying Beijing family strains in Japan (4, 5).

Since 2015, in Gifu, the public health centers and public health institutes have jointly collected M. tuberculosis strains from culture-confirmed TB patients at cooperating hospitals. In this study, we sought to identify the molecular epidemiological characteristics of M. tuberculosis clinical isolates in Gifu by JATA18-VNTR analysis (5). This study was approved by the Research Ethics Committee of the Gifu Prefectural Public Health Center (Approval No. 016).

A total of 487 M. tuberculosis strains were used and collected from culture-confirmed TB patients, including four duplicate samples due to relapse. This sample collection accounts for 58.6% (487/831) of culture-confirmed TB patients in Gifu from 2015 to 2019. In this study, 483 M. tuberculosis strains were analyzed excluding four duplicate samples. The median age of the patient sources of these strains was 81.0 with an interquartile range of 67.0–87.0. Of these strains, 61.7% (298/483) were isolated from males, and 9.7% (47/483) from foreign-born patients,
who were of a younger age than the Japanese patients (Table 1).

JATA18-VNTR loci were amplified by polymerase chain reaction (PCR) using the primers and reaction conditions described previously (4, 6). PCR fragments were analyzed by agarose gel electrophoresis or using the 3500 Genetic Analyzer (Thermo Fisher Scientific, MA, USA). Subsequently, to estimate the lineages of *M. tuberculosis* clinical isolates, JATA18-VNTR profiles were applied to a maximum *a posteriori* method (7). The results showed that 68.9% (333/483) were members of the Beijing family, of which 83.2% (277/333) fall under the ancient Beijing subfamily (Table 1). The ancient Beijing subfamily was found the most prevalent *M. tuberculosis* strain in Gifu, as reported previously for other districts in Japan (6, 8, 9).

Furthermore, to clarify the molecular epidemiological characteristics of the Gifu strains, minimum spanning tree (MST) analysis was carried out using the BioNumerics software (Applied Maths, St-Martens-Latem, Belgium; Fig. 1). In this study, a cluster was defined as two or more strains in which all loci match. MST found 18 clusters (GC-1–GC-18), and the proportion of clustering strains was 9.9% (48/483). Six, nine, and three clusters belonged to the non-Beijing family, ancient Beijing subfamily, and modern Beijing subfamily, respectively (Table 2).

GC-6 was composed of strains from an index case, two the patient’s family, and a contact health personnel. Epidemiological links to other clusters were unclear. Additional epidemiological surveys among patients derived from clustering strains have not been conducted generally in Gifu. However, GC-18 was composed of strains from Japanese who have interacted with foreign technical intern trainees living in the same apartment, as revealed in a subsequent investigation by a public health center. The lack of epidemiological links in most clusters may be due, not only to the difficulty of the epidemiological survey, but also to the possible limitations of the
discriminatory power of the VNTR method (10). Thus, additional analyses such as whole-genome sequencing are necessary to obtain more accurate genotyping results concerning the strains that lack epidemiological links.

The 483 *M. tuberculosis* strains were subsequently compared with the following reference strains: pECT01–pECT09 (4), M-strain previously reported as clustering in Japan (11), NGY-001–NGY-016 clustered in Nagoya, and NGYF-001–NGYF-005 strains isolated from Filipinos (12). The isolated strains from this study did not match pECT01–pECT09 and M-strain, but four NGY strains matched to form four clusters (GC-7–GC-9 and GC-14) (Table 2, Fig. 1A). Our results suggest that the match between the *M. tuberculosis* strains collected in Gifu and those from Nagoya are possibly due to the frequent intermingling of their populations due to their strong socioeconomic ties. Regional epidemic strains are presumed to form in each region, centering in the urban areas such as Nagoya. In Japan, molecular epidemiological information about *M. tuberculosis* is managed in prefectures according to administrative divisions. These results suggest that coordinated genotypic analyses and epidemiological investigations within prefectures with strong socioeconomic relationships are important.

Moreover, the 483 strains were classified according to nationality of the patients and the families of *M. tuberculosis* strains (Fig. 1B–1C). The many strains of the non-Beijing family from the foreign-born patients formed a subtree (Partition-1). Partition-1 consisted of 8 strains isolated from Japanese patients, 19 strains from Filipino and 1 from Cambodian. The median age of the patient sources of the 28 strains was 36.0 (23.5–48.0), while that of the sources of the remaining 122 strains belonging to the non-Beijing family was 82.5 (69.0–87.8). Subsequently, the 28 strains were analyzed by MIRU-VNTRplus (13) using MIRU15-VNTR profiles. The analyses of
additional loci (MIRU16, MIRU40, Mtub30, Mtub39, and ETR-C) were performed with the same method followed for JATA18-VNTR. In consequence, all strains of Partition-1 were estimated to be of East-African Indian lineage, which is reported as the most prevalent in Southeast Asia (14) and the minor in Japan (15). Nevertheless, several strains from Japanese patients were included in Partition-1, and no clusters were found. In this study, epidemiological information such as travel history was not investigated. Thus, we could not establish whether this is the imported regional infection cases that have already been established by the missing link. Henceforth, to monitor the potential for infection in the region, additional epidemiological investigations will be needed on patients from whom strains that accumulated in Partition-1 were isolated.

In conclusion, this study revealed that in Gifu, the ancient Beijing subfamily is the most prevalent *M. tuberculosis* genotype, and the non-Beijing family, mainly isolated from foreign-born patients, belongs to the East-African Indian lineage. Furthermore, the genotype of *M. tuberculosis* strains in Gifu is possibly influenced by those in Nagoya. This result suggests that coordinated analyses within prefectures with strong socioeconomic relationships are necessary.
Acknowledgments

We thank all parties involved in public health centers and hospitals for their cooperation.
Conflict of interest

None to declare
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Figure legends

Fig. 1. Minimum spanning tree based on 18 variable number tandem repeats (VNTR) loci from 483 *Mycobacterium tuberculosis* strains.

(A) The map shows the locations of Gifu Prefecture (Gifu) and Nagoya City (Nagoya) in Japan. Circles correspond to the different types identified with the Japan Anti-Tuberculosis Association (JATA) 18-VNTR loci from 483 *M. tuberculosis* strains and reference strains (pECT01–pECT09, M-strain, NGY-001–NGY-016, and NGYF-001–NGYF-005). A distance of one locus between the two closest VNTR profiles is marked by a thick black line, two loci is a regular line, three loci is a thin line, four loci is a dotted line, and five or larger loci is a thin dotted line.

(B–C) Circles correspond to the different types identified with the 18 loci from 483 *M. tuberculosis* strains.
| Patient nationality | Result of MAP* estimation | Total no. (%) of isolates | No. (%) of patients that were male | median age (Interquartile range) yrs | No. (% of isolates that clusters)** |
|---------------------|---------------------------|--------------------------|-----------------------------------|-------------------------------------|-----------------------------------|
| Japan               |                           |                          |                                   |                                     |                                   |
| Non-Beijing         | 123 (25.5)                | 81 (65.9)                | 83.0 (69.0 - 88.0)                | 19 (15.4)                           |
| Modern Beijing      | 40 (8.3)                  | 25 (62.5)                | 77.0 (56.8 - 86.3)                | 6 (15.0)                            |
| Other country       |                           |                          |                                   |                                     |                                   |
| Non-Beijing         | 27 (5.6)                  | 12 (44.4)                | 27.0 (23.0 - 38.0)                | 0 (0)                               |
| Modern Beijing      | 4 (0.8)                   | 3 (75.0)                 | 25.0 (23.5 - 25.0)                | 1 (25.0)                            |
| Ancient Beijing     | 16 (3.3)                  | 5 (31.3)                 | 28.5 (23.8 - 41.0)                | 2 (12.5)                            |
| Total               |                           |                          |                                   |                                     |                                   |
| Non-Beijing         | 150 (31.1)                | 93 (62.0)                | 77.0 (54.0 - 85.0)                | 19 (12.7)                           |
| Modern Beijing      | 36 (11.6)                 | 30 (83.3)                | 38.5 (40.8 - 83.3)                | 8 (14.3)                            |
| Ancient Beijing     | 277 (57.3)                | 175 (63.2)               | 83.0 (75.0 - 88.0)                | 21 (7.6)                            |
| Total               | 483 (100)                 | 298 (61.7)               | 81.0 (67.0 - 87.0)                | 48 (9.9)                            |

*maximum a posteriori

**The cluster was defined as two or more strains in which all loci of JATA18-VNTR match.

Numbers with different letters are statistically different (a, b: P<0.05, Steel-Dwass test).
Table 2: Clusters and Coincidence detection of strains as previously reported in 18 loci in VNTR typing of Mycobacterium tuberculosis strains collected in Gifu Prefecture, Japan

| Cluster | Number of strains | Reference strains | Result of MAP* estimation | JATA18-VNTR lcs |
|---------|------------------|------------------|--------------------------|-----------------|
| GC-1***| 5                | Non-Beijing       | 2 3 1 3 4 2 5 4 3 12 5 3 5 2 3 5 5 2 |                  |
| GC-2    | 4                | Non-Beijing       | 2 3 1 3 4 2 5 4 3 12 5 3 5 2 3 5 5 2 |                  |
| GC-3    | 2                | Non-Beijing       | 2 3 1 3 4 2 5 4 3 12 5 3 5 2 3 5 5 2 |                  |
| GC-4    | 2                | Non-Beijing       | 2 3 1 3 4 2 5 4 3 12 5 3 5 2 3 5 5 2 |                  |
| GC-5    | 2                | Non-Beijing       | 2 3 1 3 4 2 5 4 3 12 5 3 5 2 3 5 5 2 |                  |
| GC-6    | 4                | Non-Beijing       | 6 8 3 2 8 3 7 4 3 13 7 2 5 9 5 10 10 12 3 |                  |
| GC-7    | 3                | NGY-008          | 3 3 3 4 7 3 7 5 5 7 2 5 10 8 4 9 12 11 |                  |
| GC-8    | 4                | NGY-007          | 3 3 3 4 7 3 7 5 5 7 2 5 10 8 4 12 12 11 |                  |
| GC-9    | 2                | NGY-006          | 3 3 3 4 7 3 7 5 5 7 2 5 10 8 4 10 12 11 |                  |
| GC-10   | 2                | Non-Beijing       | 3 3 3 4 7 3 7 5 5 7 2 5 10 8 4 12 12 10 |                  |
| GC-11   | 2                | Non-Beijing       | 4 3 3 6 3 6 4 5 7 7 3 10 >20 4 17 14 8 |                  |
| GC-12   | 2                | Non-Beijing       | 4 3 3 6 3 6 4 5 7 7 3 10 >20 4 17 14 8 |                  |
| GC-13   | 2                | Non-Beijing       | 3 3 3 6 3 6 4 5 7 7 3 10 >20 4 17 14 8 |                  |
| GC-14   | 2                | NGY-005***        | 4 3 3 6 3 6 4 5 7 7 3 10 >20 4 17 14 8 |                  |
| GC-15   | 2                | Non-Beijing       | 2 1 3 2 6 4 6 4 5 7 8 5 10 9 4 16 11 11 |                  |
| GC-16   | 3                | Modern Beijing    | 4 3 3 3 5 7 3 7 4 5 7 8 3 8 5 4 14 14 10 |                  |
| GC-17   | 2                | Modern Beijing    | 4 3 3 3 5 7 3 7 4 5 7 8 3 8 5 4 14 14 17 |                  |
| GC-18   | 3                | Modern Beijing    | 4 3 3 3 5 7 3 7 4 5 7 8 3 8 5 4 14 14 17 |                  |

* The cluster was defined as two or more strains in which all loci of JATA18-VNTR match, excluding the relapse samples.

** maximum a posteriori

*** Gifu Cluster (GC), **** NGY-010 (QUB3212 >15).
