Research article

Transmission of Specific Genotype Streptomycin Resistant Strains of *Mycobacterium tuberculosis* in the Tokyo Metropolitan Area in Japan

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Abstract

**Background:** From 2003 through to 2004, an outbreak of tuberculosis was identified at a university campus in Yokohama City, located in the southern part of the Tokyo Metropolitan Area (TMA). All *Mycobacterium tuberculosis* (*M. tuberculosis*) strains detected with regards to this outbreak turned out to be Streptomycin resistant with matched patterns of 14 IS6110 bands of Restriction Fragment Length Polymorphism (RFLP). The *M. tuberculosis* bacilli, which had the matched IS6110 band patterns with resistance to Streptomycin to those of bacilli isolated in the outbreak, were also concurrently detected through either the population-based or the hospital-based DNA fingerprinting surveillance of *M. tuberculosis* both in Shinjuku City and Kawasaki City respectively.

The aim of the present study is to describe the spread of the specific genotype strains of *M. tuberculosis* in the TMA as observed in the above incident, and to identify the possible transmission routes of the strains among people living in urban settings in Japan.

**Methods:** We applied Variable Numbers of Tandem Repeats (VNTR) analysis to all *M. tuberculosis* isolates which were resistant to Streptomycin with a matched IS6110-RFLP band pattern (M-strains). They were isolated either from cases related to the tuberculosis outbreak that happened at a university, or through DNA fingerprinting surveillance of *M. tuberculosis* both in Shinjuku City...
and in Kawasaki City. For VNTR analysis, 12MIRU loci, 4ETR loci, seven loci by Supply, four loci by Murase (QUB15, Mtub24, VNTR2372, VNTR3336) were selected.

**Results:** Out of a total of 664 isolates collected during the study period, 46 isolates (6.9%) were identified as M-strains. There was a tendency that there was a higher proportion of those patients whose isolates belonged to M4-substrains, with four copies of tandem repeat at the ETR-C locus, to have visited some of the internet-cafés in the TMA than those whose isolates belonged to M5-substrains, with five copies at the ETR-C locus, although statistically not significant (38.1% vs. 10.0%, Exact p = 0.150).

**Conclusion:** Although firm conclusions could not be reached through the present study, it suggested that we have to take into consideration that tuberculosis can be transmitted in congregated facilities like internet cafés where tuberculosis high-risk people and general people share common spaces.

Background

A population-based DNA fingerprinting surveillance of *Mycobacterium tuberculosis* (*M. tuberculosis*) using IS6110 Restriction Fragment Length Polymorphism (IS6110-RFLP) analysis has been conducted since 2002 in Shinjuku City, located in the centre of the Tokyo Metropolitan Area (TMA) with approximately 300,000 residents, in order to evaluate the transmission status of *M. tuberculosis* in an urban setting in Japan [1]. In addition, since 2004 a hospital-based DNA fingerprinting surveillance of *M. tuberculosis* has been conducted at a public hospital in Kawasaki City, located in the south of the TMA with approximately 1.4 million residents [2]. At the same time, from 2003 through to 2004, an outbreak of tuberculosis (TB) was identified at a university campus in Yokohama City, which had the matched IS6110-RFLP band pattern (Figure 1). They were isolated either through the contact investigation of TB with regards to the TB outbreak that happened at a university campus in Yokohama City, from cases with possible links with the other outbreaks as suspected by public health centre (PHC) staff in the TMA, or through the population-based or hospital-based DNA fingerprinting surveillance of *M. tuberculosis* either in Shinjuku City or in Kawasaki City respectively. These two databases, which were independent of each other, were chosen because these were the only ones for us to investigate how these strains spread in the TMA. As the time of this study, there is no database covering the TMA in its entirety. In addition, the database of Kawasaki City, although its coverage among bacillary positive TB patients registered in the City was relatively low, approximately 21% (unpublished data), was chosen because we suspected there existed some link between the M-strains isolated from TB patients at the university campus in Yokohama City and TB bacilli in the Kawasaki City database for the university campus was located very close to the border between the two cities. The study period was set from September 2002 through to January 2008 because the outbreak incident at the university campus in Yokohama City, which prompted us to investigate the transmission situation of the M-strains in the TMA, seemed to have subsided by the end of 2006.

Drug susceptibility tests (DST) were performed at the laboratories where the strains of *M. tuberculosis* bacilli were initially isolated from the patients with TB. The DST results were collected by the PHC in the routine data collection process from doctors treating the patients. The DST was conducted with the proportion method with 1% Ogawa egg slant, with the critical concentration of drug at 0.2 mcg/ml for Isoniazid, 40 mcg/ml for Rifampicin, 10 mcg/ml for Streptomycin, and 2.5 mcg/ml for Ethambu-
tol. The DST results were verified at the Research Institute of Tuberculosis (RIT), Tokyo, unless the laboratories where the DST was performed had been included in the network of quality assurance by RIT.

Once isolated at laboratories, cultures of *M. tuberculosis* from each patient were sent to RIT, for DNA fingerprinting analysis. The standardised method of DNA fingerprinting using IS6110 by the restriction fragment length polymorphism analysis was applied [4]. The 27-locus VNTR analysis was performed using primers designed specifically to each of the loci selected. In the current VNTR analysis, 12 loci by Mazars et al. i.e., MIRU 2, 4, 10, 16, 20, 23, 24, 26, 27, 31, 39, 40 [5]; four loci by Frothingham et al. i.e., ETR A, B, C, F [6]; supplemented with seven loci recommended by Supply et al. i.e., QUB11b, QUB26, Mtub04, Mtub21, Mtub30, Mtub39, VNTR4156 [7]; and the other four loci recommended by Murase et al. i.e., QUB15, Mtub24, VNTR2372, VNTR3336 [8] were selected. The last four loci were added in order to increase the discriminatory power in the context of high prevalence of Beijing genotype *M. tuberculosis* strains in Japan [9,10]. The VNTR profile of each of the analyzed *M. tuberculosis* was described as a set of figures of tandem repeat copy number according to this loci set order as mentioned above. The VNTR analysis was made following the previously described methods using Ex Taq with GC PCR buffer I (Takara Bio) [8,11]. In brief, the PCR mixture was prepared in a 20 ml volume with 16GC PCR buffer I, 0.5 U Ex Taq, 200 mM each of four dNTPs, 0.5 mM each of the primer set and 10 ng template DNA. PCR was carried out for all loci under the following conditions: initial denaturation at 94 uC for 5 minutes, and then 35 cycles of 94 uC for 30 seconds, 63 uC for 30 seconds and 72 uC for 3 minutes, followed by a final extension at 72 uC for 7 minutes [8].

The M-strains are defined in this paper as *M. tuberculosis* strains which have a matched IS6110-RFLP band pattern including those with one band difference, if any (Figure 1); are resistant to Streptomycin; have any of the following VNTR profiles, either M4-substrain with four tandem repeat copies at the ETR C locus, i.e., 223315173533-4243-8844433-4337, or M5-substrain with five tandem repeat copies at the same locus including one copy number difference at the VNTR4156 locus, i.e., either 223315173533-4253-8844433-4337 or 223315173533-4253-8844434-4337 (Table 1)

The epidemiological linkage, among those from whom M-strain was isolated, was investigated through routine or outbreak-related contact investigations by the PHC staff or by the infection control team at the above-mentioned university. The investigation was made mainly through interviews with the patients by the staff mentioned above. Most of the interviews had been done before the results of the molecular analyses became available.

An internet-café described in this paper is in general a type of cafeteria, being popular among youngsters for passing time and having a rest, which is commonly located in buildings close to a railway or an underground station in urban settings and is usually opened for 24 hours a day. At the place, they can spend some time by using a computer with Internet access. It is also commonly used as a temporary residence for homeless people to stay during night as its price is relatively inexpensive at night [12].

According to the Ethical Guidelines for Epidemiological Research by the government of Japan, informed consent does not necessarily need to be obtained from research subjects to conduct an observational study using the existing epidemiological data if the study does not use any human biological specimens [13]. Therefore informed consent was not obtained for this study because the present study was an observational study using anonymous and unlinked data of TB patients, whose data sources were existing three databases that had already been collected and analyzed. The informed consent, however, had been routinely verbally obtained regarding the DNA fingerprinting surveillance of *M. tuberculosis* from each of the bacillary positive tuberculosis patients registered in the Shinjuku PHC by the PHC staff, and the documented consent forms were also routinely obtained from each of the TB patients by the hospital staff in Kawasaki City. The study protocol was approved by the Institutional Review Board Committee of the Research Institute of Tuberculosis with the reference number 21-1.

Chi-square test was applied for the comparison between two sample proportions and Student’s t test was applied for the comparison between two sample means. The p-value of 0.05 was set as a statistically significant level.
Table 1: Patient and VNTR Profiles whose M. tuberculosis Indicated M-strains

| No. | M-strain Type (*1) | Year Patient Registered | TB diagnosis (*2) | sex (*3) | Age (years old) | Internet-café use (*4) | Homeless (*5) | Drug Resistance (*6) | VNTR profiles (*7) |
|-----|---------------------|-------------------------|-------------------|---------|----------------|------------------------|---------------|----------------------|------------------|
| 1   | M5                  | 2004                    | univ              | M       | 22             | No                     | No            | SM                   | 223315173533-42  |
|     |                     |                         |                   |         |                |                        |               | 53-884443             | 4-4337           |
| 2   | M5                  | 2004                    | univ              | M       | 22             | No                     | No            | SM                   | 223315173533-42  |
|     |                     |                         |                   |         |                |                        |               | 53-884443             | 4-4337           |
| 3   | M5                  | 2004                    | univ              | M       | 22             | No                     | No            | SM                   | 223315173533-42  |
|     |                     |                         |                   |         |                |                        |               | 53-884443             | 4-4337           |
| 4   | M5                  | 2004                    | univ              | M       | 22             | Yes                    | No            | SM                   | 223315173533-42  |
|     |                     |                         |                   |         |                |                        |               | 53-884443             | 4-4337           |
| 5   | M5                  | 2004                    | univ              | F       | 21             | No                     | No            | SM                   | 223315173533-42  |
|     |                     |                         |                   |         |                |                        |               | 53-884443             | 4-4337           |
| 6   | M5                  | 2004                    | univ              | M       | 22             | No                     | No            | SM                   | 223315173533-42  |
|     |                     |                         |                   |         |                |                        |               | 53-884443             | 4-4337           |
| 7   | M5                  | 2004                    | univ              | M       | 22             | No                     | No            | SM                   | 223315173533-42  |
|     |                     |                         |                   |         |                |                        |               | 53-884443             | 4-4337           |
| 8   | M5                  | 2004                    | univ              | M       | 22             | No                     | No            | SM                   | 223315173533-42  |
|     |                     |                         |                   |         |                |                        |               | 53-884443             | 4-4337           |
| 9   | M5                  | 2004                    | univ              | M       | 22             | No                     | No            | SM & INH             | 223315173533-42  |
|     |                     |                         |                   |         |                |                        |               | 53-884443             | 4-4337           |
| 10  | M5                  | 2004                    | univ              | M       | 22             | No                     | No            | SM & INH             | 223315173533-42  |
|     |                     |                         |                   |         |                |                        |               | 53-884443             | 4-4337           |
| 11  | M5                  | 2004                    | univ              | M       | 22             | No                     | No            | SM                   | 223315173533-42  |
|     |                     |                         |                   |         |                |                        |               | 53-882443             | 4-4337           |
| 12  | M5                  | 2005                    | univ              | F       | unknown        | No                     | No            | SM                   | 223315173533-42  |
|     |                     |                         |                   |         |                |                        |               | 53-884443             | 4-4337           |
| 13  | M5                  | 2005                    | univ              | M       | 22             | No                     | No            | SM                   | 223315173533-42  |
|     |                     |                         |                   |         |                |                        |               | 53-884443             | 4-4337           |
| 14  | M5                  | 2005                    | univ              | M       | 24             | No                     | No            | SM                   | 223315173533-42  |
|     |                     |                         |                   |         |                |                        |               | 53-884443             | 4-4337           |
| 15  | M5                  | 2005                    | univ              | M       | 21             | No                     | No            | SM                   | 223315173533-42  |
|     |                     |                         |                   |         |                |                        |               | 53-884443             | 4-4337           |
| 16  | M5                  | 2005                    | univ              | M       | 22             | No                     | No            | SM                   | 223315173533-42  |
|     |                     |                         |                   |         |                |                        |               | 53-884443             | 4-4337           |
| 17  | M5                  | 2005                    | univ              | M       | 23             | No                     | No            | SM                   | 223315173533-42  |
|     |                     |                         |                   |         |                |                        |               | 53-884443             | 4-4337           |
| 18  | M5                  | 2005                    | univ              | M       | 22             | No                     | No            | SM                   | 223315173533-42  |
|     |                     |                         |                   |         |                |                        |               | 53-884443             | 4-4337           |
| 19  | M5                  | 2006                    | s                 | M       | 58             | No                     | Yes           | SM                   | 223315173533-42  |
|     |                     |                         |                   |         |                |                        |               | 53-884443             | 4-4337           |
| 20  | M5                  | 2007                    | s                 | M       | 39             | Yes                    | Yes           | SM                   | 223315173533-42  |
|     |                     |                         |                   |         |                |                        |               | 53-884443             | 4-4337           |
Table 1: Patient and VNTR Profiles whose *M. tuberculosis* Indicated M-strains (Continued)

| No. | Year | Gender | Age | Status | SM | VNTR Profile |
|-----|------|--------|-----|--------|----|--------------|
| 21  | 2003 | s      | 59  | No     | No | SM           |
| 22  | 2004 | univ   | 22  | Yes    | No | SM           |
| 23  | 2004 | univ   | 24  | No     | No | SM           |
| 24  | 2004 | Ob     | 24  | No     | No | SM           |
| 25  | 2004 | Ob     | 35  | No     | No | SM           |
| 26  | 2004 | ka     | 49  | No     | Yes| SM           |
| 27  | 2004 | ka     | 31  | No     | No | SM           |
| 28  | 2004 | ka     | 41  | No     | No | SM           |
| 29  | 2004 | s      | 65  | Yes    | Yes| SM           |
| 30  | 2005 | Ob(ka) | 47  | Yes    | Yes| SM           |
| 31  | 2005 | Ob(s)  | 28  | Yes    | No | SM           |
| 32  | 2005 | Ob     | 31  | Yes    | Yes| SM           |
| 33  | 2005 | Ob     | 25  | Yes    | Yes| SM           |
| 34  | 2005 | ka     | 30  | No     | No | SM           |
| 35  | 2005 | ka     | 56  | No     | No | SM           |
| 36  | 2005 | ka     | 45  | Yes    | Yes| SM           |
| 37  | 2006 | ka     | 67  | No     | No | SM           |
| 38  | 2006 | ka     | 58  | No     | No | SM           |
| 39  | 2006 | s      | 33  | No     | No | SM           |
| 40  | 2007 | ka     | 46  | No     | No | SM           |
| 41  | 2007 | ka     | 54  | No     | No | SM           |
Table 1: Patient and VNTR Profiles whose M. tuberculosis Indicated M-strains (Continued)

| ID | M  | Year | KA | Gender | Age | History | Resistance | VNTR Profile |
|----|----|------|----|--------|-----|---------|------------|--------------|
| 42 | M4 | 2007 | ka | M      | 44  | No      | No         | 223315173533-4243-884443 3-4337 |
| 43 | M4 | 2007 | ka | F      | 39  | No      | No         | 223315173533-4243-884443 3-4337 |
| 44 | M4 | 2007 | ka | M      | 55  | No      | No         | 223315173533-4243-884443 3-4337 |
| 45 | M4 | 2007 | s  | M      | 50  | Yes     | Yes        | 223315173533-4243-884443 3-4337 |
| 46 | M4 | 2007 | s  | M      | 45  | No      | Yes        | 223315173533-4243-884443 3-4337 |

*1: "M4" indicates the M4-substrain with four tandem repeat copies at the ETR C locus. "M5" indicates the M5-substrain with five tandem repeat copies at the ETR C locus.

*2: "univ" indicates a TB patient detected through the contact investigation related to the tuberculosis outbreak at the university campus mentioned in the text.

*3: "Ob" indicates a TB patient detected through contact investigations related to tuberculosis outbreak incidents other than the one at the university campus mentioned above.

*4: "s" indicates a TB patient detected through the population-based DNA fingerprinting surveillance of M. tuberculosis in Shinjuku City.

*5: "ka" indicates a TB patient detected through the hospital-based DNA fingerprinting surveillance of M. tuberculosis in Kawasaki City.

"M" indicates male and "F" indicates female

"SM" stands for Streptomycin.

"INH" stands for Isoniazid.

"Yes" indicates a TB patient who claimed to have a past history of the use of some internet cafés in the TMA within the past two years.

"No" indicates a TB patient who did not claim to have a past history of the use of some internet cafés in the TMA within the past two years.

Results

Out of a total of 664 isolates collected during the study period; 464 isolates from the TB patients registered in Shinjuku City, 176 isolates from those in Kawasaki City, 20 isolates from those related to the outbreak at a university campus in Yokohama City, and the other four isolates from those related to the outbreak at a university campus in Shinjuku City, in Kawasaki City. It was therefore suspected that all of these 46 isolates were further divided into two groups based on the VNTR analysis on the ETR-C locus. 26 M-strains had four copies of tandem repeat at the ETR-C locus (No.21 through 46), designated as M4-substrains, i.e., 223315173533-4243-884443-4337. Among these M4-substrains, only one isolate showed six copies of tandem repeat at the QUB11b locus (No.42). The other 20 M-strains, which have five copies of tandem repeat at the ETR-C locus, designated as M5-substrains, i.e., either 223315173533-4253-884443-4337 or 223315173533-4253-884443-4337, eight isolates showed three copies and 12 isolates showed four copies of tandem repeat at the VNTR4156 locus. 18 isolates (No.1 through No.18) were obtained from the patients related to the outbreak-related contact investigations at the university campus in Yokohama City. It was therefore suspected that all of these
of bacillary positive tuberculosis patients city-wide, suggested that its prevalence might not be high enough to be considered as an endemic strain [1]. It showed that only eight out of 464 isolates (1.7%) belonged to M-strains in Shinjuku City. In addition, the data from a nationwide drug resistance survey of M. tuberculosis in Japan in 2002 suggested that thus far, M-strains have not spread widely and nationwide [15]. It showed that four among 325 randomly selected samples of M. tuberculosis from the survey indicated the matched IS6110-RFLP band pattern as that of M-strain, however none of them was identified as M-strain as described above (unpublished data).

The annual notification rate of bacillary TB patients in Japan has been declining from 14.6 in 1980 to 12.0 per 100,000 population in 2006. In accordance with the decline of the number of TB patients, the prevalence of TB infection was estimated to decline among all age groups especially among young age groups [16]. Meanwhile, TB patients have been geographically concentrated mainly in urban settings in Japan where there are a number of people such as homeless people who have higher chances to be infected with and develop TB. For instance, the annual notification rates of bacillary TB patients per 100,000 population in mega-cities like Tokyo Metropolitan (15.2), Osaka City (35.4), Nagoya City (20.0), and Kawasaki City (16.1) were far above the national average (12.0) in 2006 [17]. Considering the very low level of TB infection...
among young age groups, it is expected that a TB outbreak could be highly possible among young age groups once a contagious TB patient has a contact with them. For instance, Nakamichi et al. reported outbreak incidents of TB including homeless people and the workers at several public saunas in Shinjuku City where homeless patients often stayed with other homeless workers [18]. They pointed out the possibility of transmission of TB in such places where quite a few homeless people stay for a long time as temporary residence.

We reported relatively high genotype clustering rate among homeless people (unadjusted Odds Ratio: 3.21, 95% Confidence Intervals 1.98–5.21) in Shinjuku City and many genotype clusters were a mixture of homeless and non-homeless patients, which may indicate transmission between these two population segments [1]. In line with this, the present study suggested that specific Streptomycin resistant M4-substrains have been transmitting among both homeless people and contacts with them in internet-cafés. Internet-cafés in addition to public saunas are currently used as temporary residence for a considerable number of homeless people in urban areas in Japan. Furthermore, they are the sites where many youngsters stay concurrently. This implies that not only close contact among family members or friends but also casual contact like those who share a common floor or space in an internet-café could be a focus of transmission of TB. Bifani et al. reported a spread of multiple drug resistant M. tuberculosis widely across several states around New York City, and warned significant risk for TB control in the future [19]. The present study similarly showed a spread of Streptomycin resistant specific genotype tuberculosis strains in urban settings in Japan.

The epidemiological investigation in the context of the present study has been limited to those whose strains sharing the closely related genotype caused the outbreak in a university campus, therefore this limits the ability to draw conclusions clearly as to the specific risk factors for acquisition of this strain. Nevertheless, the results of current molecular as well as epidemiological analysis successfully described the tip of the iceberg regarding the spread of specific Streptomycin resistant M. tuberculosis strains in the urban context in the TMA. The M-strains, which are resistant to Streptomycin, caused an outbreak of TB at a university involving numerous young age group people and also spread significantly in the urban area. All of the 46 TB patients infected with M-strains were not chronic or treatment failure cases but initial cases, which implies that the M-strains successfully transmit among people and induce development of active TB.

Conclusion
Although firm conclusions could not be reached through the present study, it suggested that we have to take into consideration that TB could transmit in congregated facilities like internet-cafés where TB high-risk people and general people share common spaces for relatively long time. The results of this study also imply that we further need to monitor the spread of this strain across the TMA and clarify the transmission routes precisely so that we will be able to control the transmission effectively in an urban context in Japan.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
All authors contributed to conduct the present study significantly. AO, MM, NH, GO, MN, HM, and MO collected data and analysed. YM and SM performed DNA fingerprinting analysis. AO wrote the manuscript and YM, NH, GO, KI, MO, NY, SK, TM, and NI edited it. All the 14 authors approved the contents of the manuscript.

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