Identification of Beijing Lineage *Mycobacterium tuberculosis* with Combined Mycobacterial Interspersed Repetitive Unit Loci 26, 31, and ETR-A

Pei-Ju Chin, Chen-Che Chiu, and Ruwen Jou*

Reference Laboratory of Mycobacteriology, Research and Diagnostic Center, Centers for Disease Control, Department of Health, 161 Kun-Yang St., Nan-Kang, Taipei 115, Taiwan, Republic of China

Received 3 October 2006/Returned for modification 10 November 2006/Accepted 11 January 2007

A rapid method for identification of Beijing lineage *Mycobacterium tuberculosis* is still needed in regions of tuberculosis endemicity, especially if genotyping methods are not readily accessible. After analyzing 1,557 clinical isolates, a PCR method with combined mycobacterial interspersed repetitive unit loci 26, 31, and ETR-A for differentiation of Beijing lineage isolates was established, the sensitivity and specificity of which are 94.7% and 98.5%, respectively.

Beijing lineage *Mycobacterium tuberculosis* isolates were first recognized in 1995 (14). They show closely related IS6110 restriction fragment length polymorphism (RFLP) and identical space oligonucleotide typing (spoligotyping) patterns and accounted for 86% of tuberculosis (TB) isolates from Beijing, China (14), 40.5% of TB isolates from Taiwan (4), and a high proportion of TB isolates from the rest of Asia (3). In contrast, Beijing lineage isolates are relatively rare in other regions of the world, such as Finland (1) and India (1). These isolates are associated with transmission of drug-resistant TB (9) and might potentially become predominant once introduced into a new population (3). Thus, a simple and rapid method for the identification and differentiation of Beijing lineage isolates is needed in the regions of TB endemicity, especially if genotyping methods are not readily available.

Recently, Rao et al. suggested the use of mycobacterial interspersed repetitive unit (MIRU) locus 26 as a signature for Beijing genotype isolates (10). In their study, all Beijing genotype isolates carried seven copies of MIRU locus 26, while none of the non-Beijing genotype isolates did. Nevertheless, only 10 Beijing and 70 non-Beijing genotype isolates were evaluated. Here, we analyzed 1,557 clinical isolates with broad genetic diversity and found that the combination of MIRU loci 26, 31, and ETR-A is more suitable for Beijing lineage isolate identification than MIRU locus 26 alone.

In our study, each isolate was obtained from an individual patient with pulmonary and/or extrapulmonary TB. These isolates were characterized by IS6110 RFLP following standard protocols (12) and spoligotyping with a commercially available kit (Isogen Bioscience BV, Maarssen, The Netherlands) (6). The characteristic Beijing genotype is defined as isolates hybridized only to the last nine spacer oligonucleotides (spacers 35 to 43), whereas the Beijing-like genotype is hybridized to only some of the last nine spacers. The Beijing lineage group includes all isolates with characteristic Beijing genotype and isolates with possible Beijing-like genotypes. All other spoligotypes are defined as non-Beijing genotypes. Of the 1,557 isolates studied, 37.3% (581/1,557), 3.9% (61/1,557), and 58.8% (915/1,557) belonged to the Beijing, Beijing-like, and non-Beijing genotypes, respectively. Furthermore, a total of 479 RFLP profiles were identified among the 642 Beijing lineage isolates, which indicated that they were highly genetically diversified.

In order to test the suggestions of Rao et al., we performed the MIRU-variable-number tandem-repeat (VNTR) assay initially based on a modified high-throughput 15-locus MIRU-VNTR typing system developed by us previously (2). The results demonstrated that only 76.6% (445/581), 54.1% (33/61), and 4.5% (41/915) of Beijing, Beijing-like, and non-Beijing genotype isolates, respectively, had seven copies of MIRU locus 26 (Table 1). Since 23.4% (136/581) of Beijing genotype *M. tuberculosis* isolates did not have seven copies of MIRU locus 26, the application of this locus as the sole specific

<table>
<thead>
<tr>
<th>Copy no.</th>
<th>Beijing (%)</th>
<th>Beijing-like (%)</th>
<th>Non-Beijing (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>1</td>
<td>1 (0.2)</td>
<td>0 (0)</td>
<td>30 (3.3)</td>
</tr>
<tr>
<td>2</td>
<td>8 (1.4)</td>
<td>2 (3.3)</td>
<td>156 (16.6)</td>
</tr>
<tr>
<td>3</td>
<td>4 (0.7)</td>
<td>0 (0)</td>
<td>38 (4.2)</td>
</tr>
<tr>
<td>4</td>
<td>12 (2.1)</td>
<td>19 (31.2)</td>
<td>181 (19.8)</td>
</tr>
<tr>
<td>5</td>
<td>15 (2.6)</td>
<td>1 (1.6)</td>
<td>407 (44.5)</td>
</tr>
<tr>
<td>6</td>
<td>67 (11.5)</td>
<td>3 (4.9)</td>
<td>53 (5.8)</td>
</tr>
<tr>
<td>7</td>
<td>445 (76.6)</td>
<td>33 (54.1)</td>
<td>41 (4.5)</td>
</tr>
<tr>
<td>8</td>
<td>27 (4.7)</td>
<td>2 (3.3)</td>
<td>11 (1.2)</td>
</tr>
<tr>
<td>9</td>
<td>1 (0.2)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>10</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (0.1)</td>
</tr>
<tr>
<td>11</td>
<td>0 (0)</td>
<td>1 (1.6)</td>
<td>1 (0.1)</td>
</tr>
<tr>
<td>12</td>
<td>1 (0.2)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

* Corresponding author. Mailing address: Reference Laboratory of Mycobacteriology, Research and Diagnostic Center, Centers for Disease Control, Department of Health, 161 Kun-Yang St., Nan-Kang, Taipei 115, Taiwan, Republic of China. Phone: (886) 2-26531370. Fax: (886) 2-26531387. E-mail: rwj@cdc.gov.tw.

† Published ahead of print on 24 January 2007.
TABLE 2. Combination of MIRU loci 26, 31, and ETR-A to differentiate 1,557 Mycobacterium tuberculosis isolates

<table>
<thead>
<tr>
<th>Locus 26 copy no.</th>
<th>Locus 31 copy no.</th>
<th>Locus ETR-A copy no.</th>
<th>Beijing lineage</th>
<th>Beijing-like</th>
<th>Non-Beijing</th>
</tr>
</thead>
<tbody>
<tr>
<td>=7</td>
<td>=5</td>
<td>=5</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>=7</td>
<td>=5</td>
<td>≠ 5</td>
<td>328</td>
<td>32</td>
<td>3</td>
</tr>
<tr>
<td>=7</td>
<td>≠ 5</td>
<td>=5</td>
<td>0</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>≠ 7</td>
<td>=5</td>
<td>=5</td>
<td>115</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>≠ 7</td>
<td>=5</td>
<td>≠ 5</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>≠ 7</td>
<td>≠ 5</td>
<td>=5</td>
<td>112</td>
<td>18</td>
<td>3</td>
</tr>
<tr>
<td>≠ 7</td>
<td>≠ 5</td>
<td>≠ 5</td>
<td>0</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>≠ 7</td>
<td>≠ 5</td>
<td>≠ 5</td>
<td>23</td>
<td>10</td>
<td>840</td>
</tr>
</tbody>
</table>

*Classified by spoligotyping.

marker for Beijing genotype isolates needs further assessment and validation. Our previous study revealed that MIRU loci 10, 26, and 31 were highly discriminative; loci 4, 16, 23, 39, 40, ETR-A, and ETR-B were moderately discriminative; and loci 2, 20, 24, 27, and ETR-C were discriminative to a low degree (2). Therefore, we calculated the allelic diversities using the formula \( h = 1 - \frac{1}{n} \sum (n - 1) \), where \( n \) is the number of strains and \( X_i \) is the frequency of the \( i \)th allele at the locus (2), and evaluated the distribution of copy numbers of other MIRU loci among those isolates with discordant genotypes. The meta-analysis of various locus combinations indicated that inclusion of copy number 5 for both MIRU loci 31 and ETR-A could facilitate the differentiation of Beijing lineage isolates. After applying such a locus combination to the 1,557 M. tuberculosis isolates, the sensitivity and specificity reached 94.7% (608/642) and 98.5% (901/915) for Beijing lineage isolates and 98.5% (901/915) and 94.7% (608/642) for non-Beijing genotype isolates (Table 2).

Consequently, a PCR assay using primers designed from MIRU loci 26, 31, and ETR-A is suggested for the initial differentiation between Beijing lineage and non-Beijing genotype M. tuberculosis isolates. The primer sequences of loci 26, 31, and ETR-A were published by Supply et al. (12) as follows: MIRU-26 (forward, TAGGCTACCCGGTCGAATA; reverse, CATAACCGACCCGCAATAG); MIRU-31 (forward, ACTGATGGCTTACACGGCTT; reverse, GTGCCGACGTGGTCTTGAT), and ETR-A (forward, AAATCGGTCCCATCACCTTCTTAT; reverse, CGAAGCCTGGGTCGCCCGATT). The PCR and gel electrophoresis procedures were performed as described previously (11). Briefly, the reactants containing genomic DNA, primer sets, and PCR premixed reagents were subjected to a thermal cycling program starting with a denaturing step of 15 min at 95°C, followed by 40 cycles of 1 min at 94°C and 1 min at 50°C for MIRU locus 26 and 55°C for locus 31 and ETR-A, followed by 1.5 min at 72°C. The reactions were terminated by incubation at 72°C for 10 min. The amplicons were separated by 2% agarose gel electrophoresis.

The study by Rao et al. reveals the feasibility of using the MIRU locus 26 PCR for rapid identification and differentiation of the Beijing genotype of M. tuberculosis. However, the isolates selected by Rao et al. were based on the AmpliBASE-MT spoligotype database: the sizes and the representative features of the samples studied might not be adequate (8). Thus, it is recommended that highly genetically diversified M. tuberculosis isolates be selected for development of diagnostic methods. Thus, appropriate lineage-specific genetic markers for isolates of a certain lineage can be unambiguously suggested.

This work was supported by grant DOH95-DC-2011 from the Taiwan Centers for Disease Control, Department of Health, and joint grant 95-0324-19-F-01-00-00030-35 from the National Science Council and Department of Health, Taiwan, Republic of China.

We would like to thank Chuen-Sheue Chiang for her suggestions in the course of preparing the manuscript. We also would like to thank nine contract mycobacteriology laboratories across Taiwan for providing Mycobacterium tuberculosis complex isolates.

REFERENCES
5. Reference deleted.
7. Reference deleted.