Laboratory and Epidemiology Communications

The Molecular Epidemiology of Ethambutol-Resistant
*Mycobacterium tuberculosis* in Henan Province, China

Isamu Sugawara*, Koji Otomo, Hiroyuki Yamada, Guobin Wang¹, Changmei Du¹, Ruiru Shi¹ and Guolong Zhang¹

*Mycobacterial Reference Center, The Research Institute of Tuberculosis, Tokyo 204-0022, Japan and 
¹Henan Chest Hospital, Zhengzhou, Henan 450003, China

Communicated by Masashiko Makino

(Accepted October 17, 2005)

The incidence of tuberculosis (TB) in China is high, according to the Nationwide Random Survey for the Epidemiology of Tuberculosis, 1990, conducted by the Beijing Tuberculosis and Thoracic Tumor Research Institute (Tongzhou, Beijing, China). It is important to obtain fundamental data about drug-resistant TB in China to enable successful treatment of this disease. In 1994, WHO launched the global drug resistance surveillance (DRS) project. Henan province was chosen as the first site in China for collection of data for the DRS in accordance with WHO/IUATLD guidelines. Thirty counties in Henan province were selected as survey sites. The samples of 1,372 cases of TB, including 916 new cases and 456 retreated cases. The enrollment period of the cases comprised 1,372 cases of TB, including 916 new cases and 456 retreated cases. The samples were collected from Henan province as survey sites. The samples chosen comprised 1,372 cases of TB, including 916 new cases and 456 retreated cases. The enrollment period of the TB patients was from April 1 to December 31, 1996. Only genotypic detection of ethambutol (EMB) resistance was performed due to tight restrictions on the research budget; there have been few reports on EMB resistance involving TB patients was from April 1 to December 31, 1996. Only genotypic detection of ethambutol (EMB) resistance was performed due to tight restrictions on the research budget; there have been few reports on EMB resistance involving TB patients was from April 1 to December 31, 1996. Only genotypic detection of ethambutol (EMB) resistance was performed due to tight restrictions on the research budget; there have been few reports on EMB resistance involving large numbers of *Mycobacterium tuberculosis* isolates (1-4). As the *embB* operon, a gene cluster of *M. tuberculosis*, is involved in resistance to EMB (5,6), the study focused on the detection of a point mutation in *embB* codon 306 by DNA sequencing.

The samples of 171 *M. tuberculosis* isolates were recovered from 30 counties in Henan province. The sex ratio (M:F) of the TB patients was 2.2:1, and the mean age was 43.7 years. The mycobacteria were recovered from diseased patients with a variety of distinct clinical manifestations, including pulmonary and extrapulmonary infections. Seventeen reference strains (15 resistant and 2 sensitive) were provided by the Korean Institute of Tuberculosis, Seoul, Korea. The sample included 133 EMB-resistant and 38 EMB-susceptible isolates. The isolates were initially tested for EMB susceptibility in routine diagnostic laboratories by the proportion method with Middlebrook 7H10 medium. The critical concentration of EMB was 2 μg/ml.

Every series of EMB susceptibility tests included the two drug-susceptible reference strains of *M. tuberculosis*. The results of EMB susceptibility tests on the clinical isolates were cross-checked at the Korean Institute of Tuberculosis, with 98% of the results confirmed (laboratory accuracy: 90.8%). Seven hundred and five of the 1,372 isolates were resistant to one of the anti-TB drugs, isoniazid, rifampicin, EMB and streptomycin, by drug susceptibility testing. Ninety-four of the 916 new cases (10.3%) and 93 of the 456 retreated cases were EMB-resistant (20.4%). The 133 EMB-resistant *M. tuberculosis* isolates were investigated by EMB susceptibility testing for *embB* point mutations.

Next, DNA from the *M. tuberculosis* isolates was purified as described previously (7). Primer sets were designed to amplify regions of the *embB* gene; the amplification size was 260 bp (8). Five microliters (300 ng) of genomic DNA was used as the template for amplification in 100 μl of reaction mixture. Reaction mixtures were subjected to PCR in a thermal cycler (Perkin Elmer, Fremont, Calif., USA) as follows: 93°C for 2 min followed by 35 cycles at 93°C for 1 min, annealing at 65°C for 1 min, and an extension step at 72°C for 2 min. Final extension was done at 72°C for 10 min. Efficient amplification was confirmed by gel electrophoresis on 12% polyacrylamide gels. Care was taken to prevent false results due to amplicon contamination. Direct sequencing of amplified PCR products was performed with an ABI PRISM (Model 377; Perkin Elmer). Because the previous reports suggested that mutations at codon 306 of the *embB* gene (the gene encoding arabinosyl transferase) were important in EMB resistance, a 260-bp *embB* region was sequenced in 133 EMB-resistant and 38 EMB-susceptible organisms. A major point mutation was detected in codon 306 with five different kinds of base changes. Five distinct missense mutations were identified in codon 306 (ATG, Met): GTG (Val), ATA (Ile), ATT (Ile), CTG (Leu) and ATC (Ile) (Table 1). Three point mutations were detected in codons 319 and 333 (TAT, Tyr): TGT, GAT and CAT, respectively (Cys). When only epidemiologically distinct organisms were considered, 45.2% of EMB-resistant mycobacteria had an amino acid change in the region of *embB* studied, and most replacements (72%) occurred at position 306. We investigated the relationship between multi-

---

*Corresponding author: Mailing address: Mycobacterial Reference Center, The Research Institute of Tuberculosis, 3-1-24 Matsuyama, Kiyose, Tokyo 204-0022, Japan. Tel: +81-424-93-5075, Fax: +81-424-92-4600, E-mail: sugawara@jata.or.jp

---

<table>
<thead>
<tr>
<th>Frequency (n)</th>
<th>Base change</th>
<th>Amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>43</td>
<td>ATG→GTG</td>
<td>306 Met→Val</td>
</tr>
<tr>
<td>7</td>
<td>ATG→ATA</td>
<td>306 Met→Ile</td>
</tr>
<tr>
<td>5</td>
<td>ATG→ATT</td>
<td>306 Met→Ile</td>
</tr>
<tr>
<td>3</td>
<td>ATG→CTG</td>
<td>306 Met→Leu</td>
</tr>
<tr>
<td>2</td>
<td>ATG→ATC</td>
<td>306 Met→Ile</td>
</tr>
<tr>
<td>1</td>
<td>TAT→TGT</td>
<td>319 Tyr→Cys</td>
</tr>
<tr>
<td>1</td>
<td>TAT→GAT</td>
<td>319 Tyr→Cys</td>
</tr>
<tr>
<td>1</td>
<td>TAT→CAT</td>
<td>333 Tyr→Cys</td>
</tr>
</tbody>
</table>
drug resistance (MDR) and *embB* mutation (Fig. 1) and detected an *embB* mutation in 9.0% of *M. tuberculosis* isolates resistant to EMB alone and in 17% of *M. tuberculosis* isolates resistant to EMB and any one of the other drugs. A higher frequency of *embB* mutations was detected in *M. tuberculosis* isolates resistant to EMB and any two drugs (38%) and in *M. tuberculosis* isolates resistant to EMB, isoniazid, streptomycin and rifampicin (53%).

Molecular epidemiological analysis of EMB resistance was carried out on *M. tuberculosis* isolates from randomly selected TB patients in Henan province, China. Resistance to the anti-TB drugs isoniazid, rifampicin, streptomycin and EMB was detected by drug susceptibility testing in 705 out of 1,372 patients tested (51.4%). MDR occurred in 320 of the 1,372 cases (23.4%). This prevalence is significantly higher than that reported previously in Latvia, Thailand, Mozambique and Uganda by WHO (9). EMB resistance occurred in 187 of the 1,372 cases (13.6%). Ninety-four of 916 new TB cases (10.5%) were EMB-resistant. This tempted us to explore the frequency of EMB resistance at the molecular level, as there have been few reports on the prevalence of EMB resistance in large samples of *M. tuberculosis* isolates. Point mutations of *embB*, the gene encoding arabinosyl transferase, were detected in 60 (45.2%) of the 133 frozen samples of tubercle bacilli. The MIC of these EMB-resistant isolates, *embB* mutation occurs more frequently in strains that are resistant to the four anti-TB drugs than in strains that are resistant to EMB only (Fig. 1). Cross-resistance to the four anti-TB drugs may occur, although the drug targets are clearly different from each other.

Our molecular epidemiologic study revealed a very high incidence of MDR in Henan province, China. There are several possible reasons for the high incidence of MDR. First, the TB control program in Henan province is not operated efficiently due to the poor economic situation in that province. Second, there are no strict laws or regulations guarding against anti-TB drug abuse. Once patients feel better with anti-TB drugs, they stop taking them. Furthermore, anti-TB drugs can be bought at local drug stores without a prescription from medical practitioners. Finally, health education of the public and training of health workers are poor. In a few cases, anti-TB drugs were given to patients with non-TB respiratory diseases. The best treatment for TB patients is adoption of the directly observed therapy short course (DOTS) advocated by WHO (9).

This study was supported in part by the Ministry of Health, Labour and Welfare, Japan. The authors would like to acknowledge the help of Dr. Taiga Tatsumi, Sowa Boeki Co. Ltd., Japan for performing the temperature-mediated heteroduplex analysis by denaturing high-performance liquid chromatography.

### REFERENCES


