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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
¹*Henan Chest Hospital, Zhengzhou, Henan 450003, China*

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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 chosen comprised 1,372 cases of TB, including 916 new cases and 456 relapsed 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 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 3 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 (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-

Table 1. Characteristics of clinical isolates of *M. tuberculosis* with *embB* mutations

Frequency (n)	Base change	Amino acid change
43	ATG→GTG	306 Met→Val
7	ATG→ATA	306 Met→Ile
5	ATG→ATT	306 Met→Ile
3	ATG→CTG	306 Met→Leu
2	ATG→ATC	306 Met→Ile
1	TAT→TGT	319 Tyr→Cys
1	TAT→GAT	319 Tyr→Cys
1	TAT→CAT	333 Tyr→Cys

*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

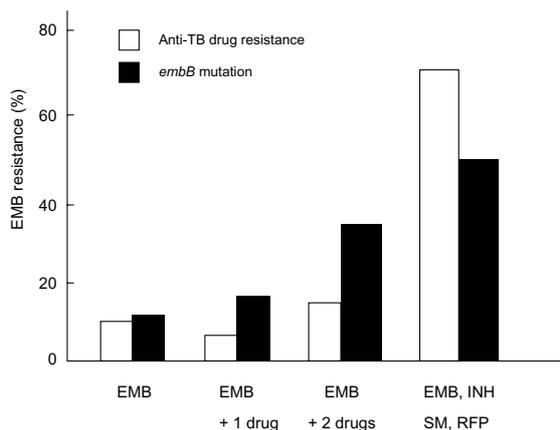


Fig. 1. The relationship between ethambutol to anti-TB drug(s) as evaluated by drug susceptibility testing and *embB* mutation. EMB, ethambutol; INH, isoniazid; SM, streptomycin; RFP, rifampicin.

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 clinical isolates was $>20 \mu\text{g/ml}$.

As shown in Table 2, there have been several previous reports on EMB resistance at the molecular level. Substitutions at codon 306 in the gene *embB* are the most common mutations found in EMB-resistant *M. tuberculosis*, occurring in 48.3-69% of resistant clinical isolates. The results of this study show a relatively low level of EMB resistance (45.2%), although the sample size is the largest of the five reports (133 cases). The *embB* mutation at codon 306 was most common, but three point mutations were found at other codons. Thus, it is possible that an *embB* mutation at another codon also confers EMB resistance.

Sreevatsan et al. (1) reported a point mutation at codon 630 (Thr \rightarrow Ile). We could not detect this mutation in this study, but *embB* point mutations were detected at codons 319 (Tyr \rightarrow Cys) and 333 (Tyr \rightarrow Cys) instead. Further studies are required to determine the importance of such point mutations in EMB resistance.

Mokrousov et al. (4) reported a high frequency of *embB* codon 306 mutations (31.2%) in 154 EMB-susceptible isolates,

Table 2. Frequency of *embB* mutations according to published reports

EMB-resistant isolates	EMB-sensitive isolates	Reference
11/16 (69%)	0/3	Sreevatsan et al., 1997 (1)
52/75 (68%)	0/33	Ramaswamy et al., 2000 (2)
12/21 (57%)	0/5	Lee et al., 2002 (3)
14/29 (48.3%)	48/154 (31.2%)	Mokrousov et al., 2002 (4)
60/133 (45.2%)	3/38 (7.9%)	Current report

whereas other researchers reported no *embB* mutation in EMB-susceptible strains (1-3). The cause of this large discrepancy is unknown, but in this study, the *embB* mutation was detected in three out of 38 EMB-susceptible isolates (7.9%). This mutation was also confirmed by temperature-mediated heteroduplex analysis performed by denaturing high-performance liquid chromatography to identify sequence polymorphisms (10,11). EMB susceptibility testing is currently being performed, because there is a possibility that these three isolates are EMB-resistant. It is interesting that the *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).

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REFERENCES

- Sreevatsan, S. S., Stockbauer, K. E., Pan, X., Kreiswirth, B. N., Moghazeh, S. L., Jacobs, W. R., Telenti, A. and Musser, J. M. (1997): EMB resistance in *Mycobacterium tuberculosis*: critical role of *embB* mutations. *Antimicrob. Agents Chemother.*, 41, 1677-1681.
- Ramaswamy, S. V., Amin, A. G., Goksel, S., Stager, C. E., Dou, S. J., El Sahly, H., Moghazeh, S. L., Kreiswirth, B. N. and Musser, J. M. (2000): Molecular genetic analysis of nucleotide polymorphisms associated with EMB resistance in human isolates of *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.*, 44, 326-336.
- Lee, H. Y., Myoung, H. J., Bang, H. E., Bai, G. H., Kim, S. J., Kim, J. D. and Cho, S. N. (2002): Mutations in the

- embB* locus among Korean clinical isolates of *Mycobacterium tuberculosis* resistant to EMB. Yonsei Med. J., 43, 59-64.
4. Mokrousov, I., Otten, T., Vyshnevskiy, B. and Narvskaya, O. (2002): Detection of *embB* 306 mutations in EMB-susceptible clinical isolates of *Mycobacterium tuberculosis* from Northwestern Russia: implications for genotypic resistance testing. J. Clin. Microbiol., 40, 3810-3813.
 5. Belanger, A. E., Besra, G. S., Ford, M. E., Mikusova, K., Belisle, J. T., Brennan, P. J. and Inamine, J. M. (1996): The *embAB* genes of *Mycobacterium avium* encode an arabinosyl transferase involved in cell wall arabinogalactan biosynthesis that is the target for the antimycobacterial drug EMB. Proc. Natl. Acad. Sci. USA, 93, 11919-11924.
 6. Telenti, A., Philipp, W. J., Sreevatsan, S., Bernasconi, C., Stockbauer, K. E., Wieles, B., Musser, J. M. and Jacobs, W. R. (1997): The *emb* operon, a gene cluster of *Mycobacterium tuberculosis* involved in resistance to EMB. Nature Med., 3, 567-570.
 7. Sugawara, I., Yamada, H., Kazumi, Y., Doi, N., Otomo, K., Aoki, T., Mizuno, S., Udagawa, T., Tagawa, Y. and Iwakura, Y. (1998): Induction of granulomas in IFN- γ gene-disrupted mice by avirulent but not by virulent strains of *Mycobacterium tuberculosis*. J. Med. Microbiol., 47, 871-877.
 8. Victor, T. C., Jordaan, A. M., van Rie, A., van der Spuy, G. D., Richardson, M., van Helden, P. D. and Warren, R. (1999): Detection of mutations in drug resistance genes of *Mycobacterium tuberculosis* by a dot-blot hybridization strategy. Tuber. Lung Dis., 79, 343-348.
 9. Dolin, P. J., Raviglione, M. C. and Kochi, A. (1994): Global tuberculosis incidence and mortality during 1990-2000. Bull. World Health Org., 72, 213-220.
 10. Cooksey, R. C., Morlock, G. P., Holloway, B. P., Limor, J. and Hepburn, M. (2002): Temperature-mediated heteroduplex analysis performed by using denaturing high-performance liquid chromatography to identify sequence polymorphisms in *Mycobacterium tuberculosis* complex organisms. J. Clin. Microbiol., 40, 1610-1616.
 11. Shi, R., Otomo, K., Yamada, H., Tatsumi, T. and Sugawara, I.: Temperature-mediated heteroduplex analysis for the detection of drug-resistant gene mutations in clinical isolates of *Mycobacterium tuberculosis* by denaturing HPLC, SURVEYOR nuclease. Microbes Infect. (in press).