Drug-Susceptible *Mycobacterium tuberculosis* Beijing Genotype Does Not Develop Mutation-Conferred Resistance to Rifampin at an Elevated Rate

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The *Mycobacterium tuberculosis* Beijing genotype has drawn attention because it is often strongly associated with multidrug-resistant tuberculosis (MDR-TB). A possible reason is that the Beijing strains may have an enhanced capacity to develop drug resistance. In this study, we used the Luria-Delbrück fluctuation test to investigate whether strains of Beijing and non-Beijing genotypes exhibit differences in the acquisition of drug resistance. The *M. tuberculosis* reference strain H37Rv and 12 fully drug-susceptible clinical isolates, 6 of which were of the Beijing genotype, were examined. To determine the distribution of rifampin-resistant mutants, 25 independent cultures were made for each strain. The average mutation frequencies for the non-Beijing (H37Rv included) and Beijing genotypes were estimated to be 4.4 × 10⁻⁸ and 3.6 × 10⁻⁸, respectively. The corresponding average mutation rates for the non-Beijing and Beijing strains were 1.3 × 10⁻⁸ and 1.1 × 10⁻⁸ mutations per cell division, respectively. The results suggest that the association of the Beijing genotype with MDR-TB is not due to an altered ability to develop resistance.

Multidrug-resistant tuberculosis (MDR-TB) continues to be a serious problem, particularly in developing countries in Asia (7, 8) but also in the Baltic region (12) and in other parts of the former Soviet Union (22).

In several countries of the Asian continent, the *Mycobacterium tuberculosis* Beijing genotype has been the predominant genotype, with a prevalence of 50 to 80%, since at least the 1950s (1, 20, 24). During the past decade, the Beijing genotype has drawn attention because of its successful spread and outbreaks in different geographical settings worldwide (10, 12, 16, 17). The factors underlying the epidemiology of this genotype are not yet understood.

The strong association of drug resistance with *M. tuberculosis* Beijing strains has raised the question of whether these strains have an enhanced ability to acquire drug resistance (1, 12, 22). There are several reports on the resistance of Beijing strains to antituberculosis agents. In Vietnam (1) and Iran (6), this genotype was associated with resistance to antituberculosis drugs. In Estonia (12), Colombia (14), and Russia (22), there was a clear correlation of the Beijing genotype with MDR-TB. In 1994 in Estonia, almost one-third of all the newly diagnosed pulmonary tuberculosis patients were infected with the Beijing genotype; 70% of them exhibited some resistance, and 35% exhibited resistance to multiple drugs. Of all MDR-TB patients in Estonia, 87.5% were infected with strains of the Beijing genotype family (12). A study in Archangel Oblast, Russia, showed that 44.5% of strains isolated from 1998 to 1999 belonged to the Beijing genotype and that 43% of these strains were MDR. Additionally, almost all of the Beijing strains were part of a cluster, a finding which may reflect a recent transmis-

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**MATERIALS AND METHODS**

*M. tuberculosis* isolates. Eleven out of the 13 *M. tuberculosis* strains analyzed were isolated at the National Estonian Reference Laboratory in Tartu, Estonia. The two additional strains used in the study were susceptible *M. tuberculosis* reference strain H37Rv (ATCC 25618) and *M. tuberculosis* strain Harlingen (11). The identity of the species of each isolate was determined by standard microbiological tests: colony morphology, acid-fast staining, and biochemical tests. Each identification was confirmed by a DNA-RNA hybridization technique (Accu-
The isolates were further analyzed by restriction fragment length polymorphism analysis to visualize their relatedness based on similarities between banding patterns. Extraction of DNA and fingerprinting with B6610 as a probe were performed by standardized methods (23) at SMI. Only strains with different banding patterns were included in the study.

Drug susceptibility testing. Drug susceptibility testing was done at SMI by using a radiometric BACTEC 460 system (Becton Dickinson Diagnostic Systems, Sparks, Md.). Only strains susceptible to the four first-line drugs, rifampin (2 mg/liter), isoniazid (0.2 mg/liter), streptomycin (4 mg/liter), and ethambutol (5 mg/liter), were selected.

Fluctuation test. Each strain was grown at 37°C on Löwenstein-Jensen egg medium for 3 to 4 weeks prior to analysis. Bacterial suspensions were made by using small glass bottles containing 3 ml of phosphate-buffered saline (PBS) and glass beads. These were vortexed for approximately 20 min. The turbidity of the medium for 3 to 4 weeks prior to analysis. Bacterial suspensions were made by vortexing the samples was measured with a spectrophotometer (Ultraspec 2000; Pharmacia) at 600 nm and adjusted to an optimal density (OD) of 0.2.

For each strain, a low-density culture of 125 ml was prepared by using Middlebrook 7H9 broth. To do this, each sample was diluted 10^3 in PBS, except for the final dilution, which was done by using Middlebrook 7H9 broth supplemented with oleic acid-albumin-dextrose-catalase (OADC) enrichment and 0.05% Tween 80 (to reduce clumping). In these low-density (approximately 10^3 cells/ml) cultures, no rifampin-resistant mutants were assumed to be present. Each of the cultures was then divided into 25 individual tubes of 5 ml. A total of 325 cultures were made and incubated at 37°C for 4 weeks.

After incubation, approximately half of the upper phase of each culture was discarded to concentrate the cells to the density needed to select rifampin-resistant mutants. To standardize the inoculum of all vortexed cultures, each culture was adjusted to an OD at 600 nm of approximately 0.8. One milliliter of each well-vortexed culture was transferred to a sterile screw-cap microcentrifuge tube and centrifuged for 1 min. Approximately 700 μl of the supernatant was aspirated by pipetting, leaving a small amount of liquid to allow resuspension of the pellet before inoculation of the plate. The entire volume of the suspended pellet was spread onto one Middlebrook 7H10 agar plate (9 cm) containing OADC and 2 mg of rifampin/liter. Three of the 25 cultures of each strain were diluted by a factor of 10^1, 10^2, and 10^3 in PBS to allow estimation of the total number of cells plated. One hundred microliters of this dilution was plated in duplicate on drug-free Middlebrook 7H10 agar plates (with OADC). All plates were sealed in plastic bags and incubated at 37°C for 4 weeks prior to determination of counts.

To establish reproducibility, the test was performed on two separate occasions for six strains, three of which were of the Beijing genotype.

Estimation of the total number of cells. The total viable cell count for each strain was estimated from three cultures: those with the lowest, the mean, and the highest ODs. The average number of bacteria per milliliter was calculated from dilutions of the three cultures, which were plated in duplicate on Middlebrook 7H10 agar. Colonies were counted after 28 days of incubation.

Estimation of mutation frequency and mutation rate. The mutation frequency for each strain was determined as the ratio of the average number of mutants per milliliter to the total number of cells per milliliter. The mutation rate for each strain was calculated from the “method of means” equation described by Luria and Delbrück (15). The solution is found numerically with MatLab 6.1 software (The Math Works, Inc.) and the function fzero: \[ r = \sum_{i=1}^{N} \frac{N_i}{C} \times \alpha; \] in this equation, \( r \) is the mutation rate, \( r \) is the average number of mutants, \( N_i \) is the total number of cells, and \( C \) is the number of cultures.

RESULTS

The distributions of rifampin-resistant mutants generated in 25 parallel cultures of each of the 13 strains are shown in Table 1. Four out of 325 plates were excluded because of contamination. For 33 of 321 cultures (10.3%), no mutants were obtained. A total of 208 cultures (64.8%) generated 1 to 5 mutants, and 60 (18.7%) generated 6 to 10 mutants. Twenty (6.2%) cultures generated more than 10 mutants. The experimental distributions of the numbers of mutants in a series of randomly selected rifampin-resistant mutants were compared with the experimental distributions of the numbers of mutants in a series of rifampin-resistant mutants obtained from the same laboratory strain. The resulting chi-square values and p values indicated that the experimental distributions did not differ significantly from the theoretical distributions for the number of mutants in rifampin-resistant cultures (Table 2). The results are consistent with the hypothesis that the number of mutants in rifampin-resistant cultures follows a Poisson distribution. The hypothesis can be tested by using a radiometric BACTEC 460 system (Becton Dickinson Diagnostic Systems, Sparks, Md.). Only strains susceptible to the four first-line drugs, rifampin (2 mg/liter), isoniazid (0.2 mg/liter), streptomycin (4 mg/liter), and ethambutol (5 mg/liter), were selected.

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<table>
<thead>
<tr>
<th>Parameter</th>
<th>Non-Beijing</th>
<th>Beijing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Value for the indicated strain of the following genotype:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of cultures</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Vol of cultures (ml)</td>
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<td>1</td>
</tr>
<tr>
<td>Vol of sample (ml)</td>
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<td>1</td>
</tr>
<tr>
<td>No. of bacteria/ml</td>
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<td>$0.5 \times 10^6$</td>
</tr>
<tr>
<td>No. of resistant bacteria</td>
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<td>0</td>
</tr>
<tr>
<td>No. of contaminated plates</td>
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<td>0</td>
</tr>
</tbody>
</table>
increased tendency for acquiring resistance; fluctuation tests with such strains should be carried out to clarify this point.

In future studies, it will be valuable to collect clinical information and determine whether drug-susceptible Beijing strains develop resistance during suboptimal therapy at a higher frequency than do strains with other drug susceptibility genotypes. Krüüner and colleagues (13) recently suggested that treatment failure does not exclusively select for resistant bacteria. For instance, in the Estonian study (13), almost half of the patients instead became reinfected with MDR Beijing strains. One of the strains, which did not develop resistance despite a long period of highly irregular antituberculosis treatment, was Beijing strain E 394/92, included in the present study. In our study, this isolate showed a normal rate of acquisition of resistance. Investigations of possible increased virulence could be more prevalent among patients with acquired immunodeficiency syndrome in Tennessee. J. Med. 307:121–129.

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