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

Jim Werngren* and Sven E. Hoffner

Department of Bacteriology, Swedish Institute for Infectious Disease Control, Solna, Sweden

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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-

sion (22). The largest outbreak of MDR-TB in North America was described by Bifani et al. in 1996 (2); it was caused by strain W, which is a member of the Beijing genotype family.

M. tuberculosis lacks plasmids and cannot transfer DNA between strains. The adaptation of a strain to antibiotics is thus caused by spontaneous chromosomal mutations, which can result in the selection of resistant strains during suboptimal chemotherapy. The Luria-Delbrück fluctuation test (15) has widely been used as a reference method to calculate and compare spontaneous mutation rates and is commonly applied in investigations of somatic cell genetics and cancer biology (3, 21, 25, 26). To our knowledge, fluctuation analysis has been used only once to determine the acquisition of resistance to different antibiotics by *M. tuberculosis* reference strain H37Rv (5).

In the present work, we have examined the hypothesis that an elevated spontaneous mutation rate for an isolate is associated with an increased acquisition of drug resistance, which may result in a more rapid selection of resistant bacteria and thus in an increase in the risk of development of MDR-TB. Our aim was to use fluctuation analysis to determine whether drug-susceptible Beijing isolates in vitro generate a larger number of rifampin-resistant mutants and are therefore more prone to becoming drug resistant than non-Beijing isolates. Additionally, we evaluated the fluctuation test, since its reproducibility for use with different strains of the tubercle bacillus has not been demonstrated.

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-

* Corresponding author. Mailing address: Department of Bacteriology, Swedish Institute for Infectious Disease Control (SMI), S-171 82 Solna, Sweden. Phone: 46-8-457 2443. Fax: 46-8-30 17 97. E-mail: jim.werngren@smi.ki.se.

Probe; GenProbe Inc., San Diego, Calif.) at the Swedish Institute for Infectious Disease Control (SMI).

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 IS6110 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 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 10^5 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 μ l 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 10^4 , 10^5 , and 10^6 in PBS to allow estimation of the total number of cells plated. One hundred microliters of each 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: $r = a \times N_i \ln(N_i \times C \times a)$; in this equation, a 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 parallel cultures are shown in Fig. 1. No differences in growth rates were observed for the different strains, as reflected by the time of appearance of colonies on solid medium. Colonies on

nonselective agar were visible on the plates after approximately 2 weeks. For the resistant mutants, colonies were often visible only after 3 weeks. Although all cultures were adjusted to an OD at 600 nm of 0.8 prior to plating, slight variations in viable counts were observed. All strains showed similar mutation frequencies and mutation rates. The average mutation frequencies for non-Beijing and Beijing strains were 4.4×10^{-8} and 3.6×10^{-8} , respectively. The average mutation rates for non-Beijing and Beijing strains were 1.3×10^{-8} and 1.1×10^{-8} mutations per cell division, respectively. Overall, the rates of mutation to resistance to rifampin were between 10^{-9} and 10^{-8} mutations per cell division. None of the isolates was found to be a mutator strain (i.e., a strain with a significantly enhanced mutation rate). The mutation frequencies for the six retested strains agreed well with the primary results (Fig. 2). Five of the six strains showed a reproduced mutation frequency with a small difference of one mutant per 10^8 CFU. The mutation frequency for the other strain differed more, but by less than 10 cells, with about seven mutants per 10^8 CFU. The average mutation frequencies for the six isolates tested on occasions A and B were 3.1×10^{-8} and 4.6×10^{-8} , respectively.

DISCUSSION

Our results showed that rifampin-resistant mutants appeared rarely for both Beijing and non-Beijing strains. Using rifampin as a marker to determine spontaneous mutation frequencies and rates, we found that Beijing strains exhibited an acquisition of resistance comparable to that of other *M. tuberculosis* strains not belonging to the Beijing genotype. The mutation frequencies for the 12 tested clinical isolates were in agreement with that described earlier for *M. tuberculosis* H37Rv (5). Remarkably, the rates of mutation to rifampin resistance were higher (10^{-9} to 10^{-8} mutations per cell division) for all strains than was previously reported for H37Rv (5). This difference may have been due to the small size of each sample (0.1 ml) used in the earlier study, which probably contained too few mutants for exact calculations.

However, the reproduced results indicate that this test is robust for investigation of the distributions of in vitro-selected mutants of *M. tuberculosis*, at least when it is performed under strictly standardized conditions. To our knowledge, there are no studies investigating *M. tuberculosis* mutator strains and their mutation rates. Generally, mutator cells have mutation rates that are >10-fold higher than those of wild-type cells (9, 18, 25), a difference that would have been discovered in our test. Our finding that drug-susceptible Beijing strains are no more prone to generating resistant cells than are non-Beijing strains suggests that an increased spontaneous mutation rate is not responsible for the association of the Beijing genotype with MDR-TB. Qian and colleagues (19) recently demonstrated that strains of the Beijing genotype acquire a point mutation (Ser531Leu) in the *rpoB* gene, conferring high-level rifampin resistance, at a frequency similar to that for their non-Beijing counterparts. In the present study, we included a number of fully drug-susceptible Beijing strains isolated from patients undergoing antituberculosis therapy. However, we cannot exclude the possibility that MDR Beijing isolates exhibit an in-

TABLE 1. Distributions of rifampin-resistant mutants from 13 *M. tuberculosis* strains included in the fluctuation test

Parameter	Value for the indicated strain of the following genotype:															
	Non-Beijing								Beijing							
	H37Rv	E 865/94	E 729/94	E 740/94	E 1221/94	E 1449/94	Harlingen	E 26/95	E 80/95	E 55/94	E 26/94	E 3942/94	E 47/94			
No. of cultures	25	25	25	25	25	25	25	25	25	25	25	25	25			
Vol of cultures (ml)	5	5	5	5	5	5	5	5	5	5	5	5	5			
Vol of sample (ml)	1	1	1	1	1	1	1	1	1	1	1	1	1			
No. of bacteria/ml	2.3×10^8	0.5×10^8	1.3×10^8	1.0×10^8	1.6×10^8	1.0×10^8	1.0×10^8	0.8×10^8	1.2×10^8	0.6×10^8	0.8×10^8	0.9×10^8	0.9×10^8			
No. of resistant bacteria																
0	0	1	2	2	3	1	0	4	2	8	2	2	6			
1	0	3	3	1	7	1	2	6	6	8	7	2	7			
2	0	5	6	3	5	2	2	6	1	1	8	5	1			
3	1	2	3	10	3	4	1	3	7	4	2	4	1			
4	2	4	3	5	0	5	4	1	2	0	4	1	3			
5	5	2	1	0	3	3	9	0	3	1	2	3	0			
6	3	3	1	1	1	2	5	2	3	2	0	1	3			
7	2	0	3	0	0	0	0	0	1	0	0	1	1			
8	3	1	0	0	1	2	0	0	0	0	0	0	0			
9	3	0	0	2	0	1	1	1	0	0	0	2	0			
10	1	1	1	0	0	0	0	1	0	0	0	3	1			
11	2	1	0	0	0	1	0	0	0	0	0	0	1			
12	1	0	1	1	1	0	0	0	0	0	0	0	1			
13	1	0	0	0	0	0	0	0	0	0	0	0	1			
14	0	0	0	0	0	1	1	0	0	0	0	0	0			
15	0	0	0	0	0	1	0	0	0	0	0	0	1			
16	0	0	0	0	1	0	0	0	0	0	0	1	0			
17	1	0	0	0	0	0	0	0	0	0	0	0	0			
18	0	0	1	0	0	0	0	0	0	0	0	0	0			
19	0	0	0	0	0	0	0	0	0	0	0	0	0			
20	0	0	0	0	0	0	0	0	0	0	0	0	0			
21	0	0	0	0	0	0	0	1	0	0	0	0	0			
No. of contaminated plates	0	2	0	0	0	1	0	0	0	1	0	0	0			
Average no. of mutants/ml	7.7	4.0	4.3	3.7	3.4	5.4	4.9	3.3	3.0	1.6	2.2	4.8	3.8			
Mutation frequency	3.3×10^{-8}	8.0×10^{-8}	3.3×10^{-8}	3.7×10^{-8}	2.1×10^{-8}	5.4×10^{-8}	4.9×10^{-8}	4.1×10^{-8}	2.5×10^{-8}	2.7×10^{-8}	2.8×10^{-8}	5.3×10^{-8}	4.2×10^{-8}			
Mutation rate	8.6×10^{-9}	2.4×10^{-8}	9.6×10^{-9}	1.1×10^{-8}	6.5×10^{-9}	1.5×10^{-8}	1.4×10^{-8}	1.3×10^{-8}	7.9×10^{-9}	1.0×10^{-8}	9.4×10^{-9}	1.5×10^{-8}	1.2×10^{-8}			

no. of cultures

FIG. 1. Overall distribution of mutants obtained in the fluctuation test.

creased 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 3942/94, included in the present study. In our study, this isolate showed a normal rate of acquisition of resistance. Investigations of possible increased virulence, fitness, or transmissibility despite drug resistance will be needed to further the understanding of the correlation of drug resistance and Beijing strains. A few studies in which the virulence of the Beijing genotype is discussed have been reported (4, 10, 27). In conclusion, the data presented in this study reject the tenet that Beijing genotype strains would be more prone to gaining drug resistance. Hypothetically, MDR Beijing genotype strains with increased virulence could be more prevalent among cases of tuberculosis and thus be associated with MDR-TB. To clarify whether the Beijing genotype spreads more effectively due to increased virulence, further investigations are required.

FIG. 2. Fluctuation test carried out on two different occasions (A (♦) and B (■)) and showing the reproducibility of the mutation frequencies for six strains (mean number of mutants per 10^8 cells).

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