

Drug Susceptibility of *Mycobacterium tuberculosis* Beijing Genotype and Association with MDR TB

Jurriaan E.M. de Steenwinkel,
Marian T. ten Kate, Gerjo J. de Knecht,
Kristin Kremer, Rob E. Aarnoutse,
Martin J. Boeree, Henri A. Verbrugh,
Dick van Soolingen,
and Irma A.J.M. Bakker-Woudenberg

To determine differences in the ability of *Mycobacterium tuberculosis* strains to withstand antituberculosis drug treatment, we compared the activity of antituberculosis drugs against susceptible Beijing and East-African/Indian genotype *M. tuberculosis* strains. Beijing genotype strains showed high rates of mutation within a wide range of drug concentrations, possibly explaining this genotype's association with multidrug-resistant tuberculosis.

The emergence of *Mycobacterium tuberculosis* resistance to antituberculosis (anti-TB) drugs is a major public health challenge that is threatening World Health Organization targets set for the elimination of TB (1). Approximately 500,000 cases of multidrug-resistant TB (MDR TB) are diagnosed annually, but the true magnitude of the MDR TB problem is not known because adequate laboratory tools are lacking. Multiple factors contribute to low cure rates, treatment failures, and relapses: poor-quality guidance regarding treatment, HIV co-infection, transmission of resistant forms of TB, underdeveloped laboratory services, and unavailability of alternative drug treatments. However, the evolution of *M. tuberculosis* is an additional factor that presumably fuels

the worldwide problem of emerging resistance. The Beijing genotype is significantly associated with drug resistance (2,3), especially in geographic areas where prevalence of resistance to anti-TB drugs is high, and it is associated with recent TB transmission (2–6). There are also indications that the population structure of *M. tuberculosis* in areas with a high prevalence of anti-TB drug resistance is changing rapidly toward an increase in Beijing genotype strains (2,6–8).

The World Health Organization target rates for detecting and curing TB in Vietnam have been met; however, the rate of TB infection is not decreasing as expected (4,5). Earlier in this country, the Beijing genotype was strongly correlated with MDR TB and treatment failures (9). Extensive molecular epidemiologic studies showed that the Beijing and East-African/Indian (EAI) genotypes are predominating in Vietnam; each lineage causes ≈40% of the TB cases. According to the single-nucleotide polymorphism typing described by Hershberg et al. (10), the Beijing genotype is a representative of the modern lineage, and the EAI genotype is believed to represent an evolutionary lineage more closely related to the common ancestor of the *M. tuberculosis* complex.

We compared the in vitro activity of anti-TB drugs against susceptible Beijing and EAI *M. tuberculosis* isolates from Vietnam and determined the in vitro mutation frequency of these strains during drug exposure. We also determined time-kill kinetics of anti-TB drugs and assessed the emergence of resistant mutants and the concentration range within which resistant mutants and no susceptible mycobacteria were selected. The concentration at which resistant mutants did not emerge (the mutant prevention concentration) was also ascertained. By using this approach, we established an in vitro model for determining differences in the ability of *M. tuberculosis* strains to resist anti-TB drug treatment.

The Study

Results of a liquid culturing system (BD BACTEC MGIT 960 System; BD Diagnostics, Sparks, MD, US) (for details, see the online Technical Appendix, wwwnc.cdc.gov/EID/pdfs/11-0912-Techapp.pdf) showed that all 5 Beijing and 5 EAI genotype strains were susceptible to isoniazid (INH), rifampin (RIF), moxifloxacin (MXF), and amikacin (AMK). MICs were determined by using the agar proportion method (11), which showed that ranges were small for the Beijing and EAI genotype strains: INH, 0.062–0.125 mg/L; RIF, 0.125–1 mg/L; MXF, 0.125–0.5 mg/L; and AMK, 0.5–2 mg/L. Duplicate values showed only minor differences.

We determined the mutation frequencies of the Beijing and EAI genotype strains by using previously defined critical drug concentrations of 1 mg/L for INH, RIF, and

Author affiliations: Erasmus University Medical Center, Rotterdam, the Netherlands (J.E.M. de Steenwinkel, M.T. ten Kate, G.J. de Knecht, H.A. Verbrugh, I.A.J.M. Bakker-Woudenberg); National Institute of Public Health and the Environment Center for Infectious Disease Control (RIVM), Bilthoven, the Netherlands (K. Kremer, D. van Soolingen); World Health Organization Regional Office for Europe, Copenhagen, Denmark (K. Kremer); and Radboud University Nijmegen Medical Center, Nijmegen, the Netherlands (R.E. Aarnoutse, M.J. Boeree, D. van Soolingen)

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Table 1. Mutation frequency of *Mycobacterium tuberculosis* genotype strains originating from Vietnam, by antituberculosis drug

Genotype	Frequency of mutation among strains*			
	Isoniazid	Rifampin	Moxifloxacin	Amikacin
Beijing				
1585	5.7×10^{-6} , 6.2×10^{-6}	3.0×10^{-3} , 4.3×10^{-3}	4.3×10^{-8} , 6.1×10^{-8}	2.3×10^{-8} , 3.2×10^{-8}
1607	8.6×10^{-6} , 1.4×10^{-5}	1.5×10^{-3} , 5.4×10^{-3}	6.9×10^{-8} , 2.4×10^{-7}	8.6×10^{-8} , 3.0×10^{-7}
2115	7.3×10^{-6} , 1.1×10^{-5}	1.0×10^{-3} , 9.2×10^{-5}	1.0×10^{-8} , 4.3×10^{-8}	1.4×10^{-8} , 2.8×10^{-8}
2121	6.8×10^{-5} , 2.9×10^{-4}	2.9×10^{-5} , 1.9×10^{-4}	1.1×10^{-7} , 1.6×10^{-7}	9.3×10^{-8} , 1.1×10^{-7}
2145	9.1×10^{-4} , 5.0×10^{-4}	1.6×10^{-5} , 5.5×10^{-5}	7.9×10^{-8} , 1.0×10^{-7}	7.6×10^{-7} , 1.1×10^{-6}
East-African/Indian				
1627	3.7×10^{-6} , 6.5×10^{-6}	4.1×10^{-6} , 2.8×10^{-6}	9.3×10^{-9} , 1.5×10^{-7}	5.6×10^{-8} , 4.5×10^{-9}
1606	8.7×10^{-6} , 1.6×10^{-4}	3.8×10^{-4} , 2.7×10^{-5}	3.2×10^{-8} , 1.0×10^{-7}	7.5×10^{-9} , 1.5×10^{-9}
1592	1.8×10^{-5} , 2.6×10^{-5}	3.0×10^{-4} , 2.4×10^{-5}	9.9×10^{-8} , 4.5×10^{-8}	9.4×10^{-8} , 1.5×10^{-9}
1596	3.9×10^{-5} , 2.8×10^{-5}	1.4×10^{-5} , 3.9×10^{-6}	1.7×10^{-7} , 2.0×10^{-7}	3.7×10^{-8} , 3.2×10^{-7}
2113	1.3×10^{-5} , 4.1×10^{-5}	6.7×10^{-8} , 6.3×10^{-8}	1.5×10^{-8} , 1.0×10^{-7}	4.4×10^{-8} , 3.3×10^{-7}

*Determined in duplicate.

MXF and 5 mg/L for AMK (11,12) (for details, see the online Technical Appendix). The mutation frequencies of the Beijing and EAI genotype strains were similar for INH, MXF, and AMK, but they were significantly different for RIF (1.6×10^{-5} to 5.4×10^{-3} for Beijing strains vs. 6.3×10^{-8} to 3.8×10^{-4} for EAI strains; $p = 0.003$, unpaired Mann-Whitney test) (Table 1; Figure 1). Because rifamycin drugs are widely used to treat TB, the difference in the mutation frequencies of Beijing and EAI genotype strains for RIF is a major finding.

For Beijing genotype strains, the increase in mutation frequency during exposure to RIF could be due to described missense mutations in the *mut* genes (13). Such mutations in the *mut* genes can change the DNA repair mechanism; as a consequence, the frequency of resistant mutant formation might increase. However, a direct correlation between the occurrence of particular mutations in *mut* genes and altered mutation frequency has not been proven. Furthermore, Werngren and Hoffner (14) found an equal mutation frequency for Beijing (3.6×10^{-8}) and non-Beijing (4.4×10^{-8}) genotypes. A possible explanation for the discrepancy in findings might be the concentration of RIF used in the subculture plates. In our study, the critical concentration of 1 mg/L RIF was used (11), whereas Werngren and Hoffner used a concentration of 2 mg/L RIF. In addition, Werngren and Hoffner compared the Beijing and non-Beijing genotypes of several genotype families, whereas we compared Beijing and EAI genotype strains that were selected from the same tuberculosis-endemic area and during the same period.

We determined the time-kill kinetics of RIF toward 2 strains with significantly different mutation frequencies: Beijing-1585 (3.7×10^{-3} [3.0×10^{-3} and 4.3×10^{-3} , duplicates]) and EAI-1627 (3.5×10^{-6} [2.8×10^{-6} and 4.1×10^{-6} , duplicates]). Cultures with low and high densities of Beijing-1585 and EAI-1627 were investigated as described (15). RIF showed strong time- and concentration-dependent activity toward low-density cultures of the 2 strains (Figure 2). Low concentrations of RIF were needed to achieve $\geq 99\%$

mycobacterial killing; differences between Beijing-1585 and EAI-1627 were minor (Table 2). However, to achieve 100% killing, especially for Beijing-1585, RIF concentrations had to be increased substantially (Table 2). Compared with the low-density culture for Beijing-1585, a substantial increase in RIF concentrations was needed to achieve 100% killing of the high-density culture (Table 2). This finding may be relevant in the clinical context because high-density mycobacteria populations are expected to exist in infected tissues of TB patients.

RIF-resistant mutants did not emerge in low-density cultures of Beijing-1585 and EAI-1627. However, RIF-resistant mutants were selected at relatively high numbers from high-density Beijing-1585 cultures compared with high-density EAI-1627 cultures. In Beijing-1585 cultures,

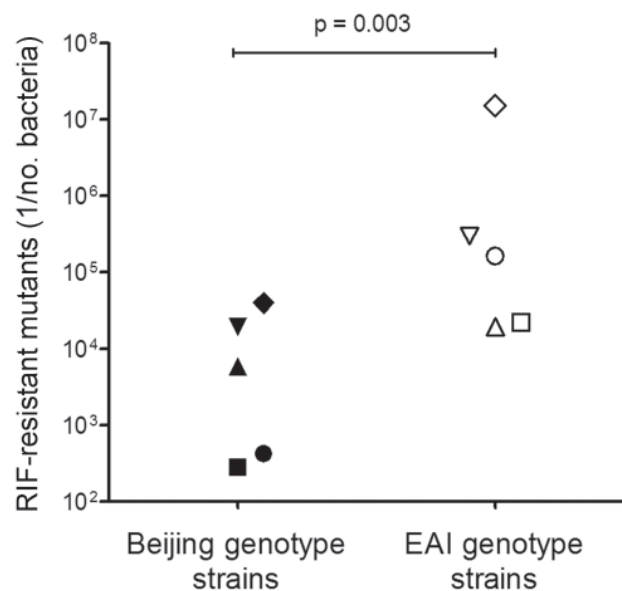


Figure 1. Frequency of rifampin (RIF)-resistant mutants in *Mycobacterium tuberculosis* Beijing and East-African/Indian (EAI) genotype strains (5 strains each) originating from Vietnam. Mutation frequencies were determined in duplicate. Statistical analysis was performed by using an unpaired Mann-Whitney test.

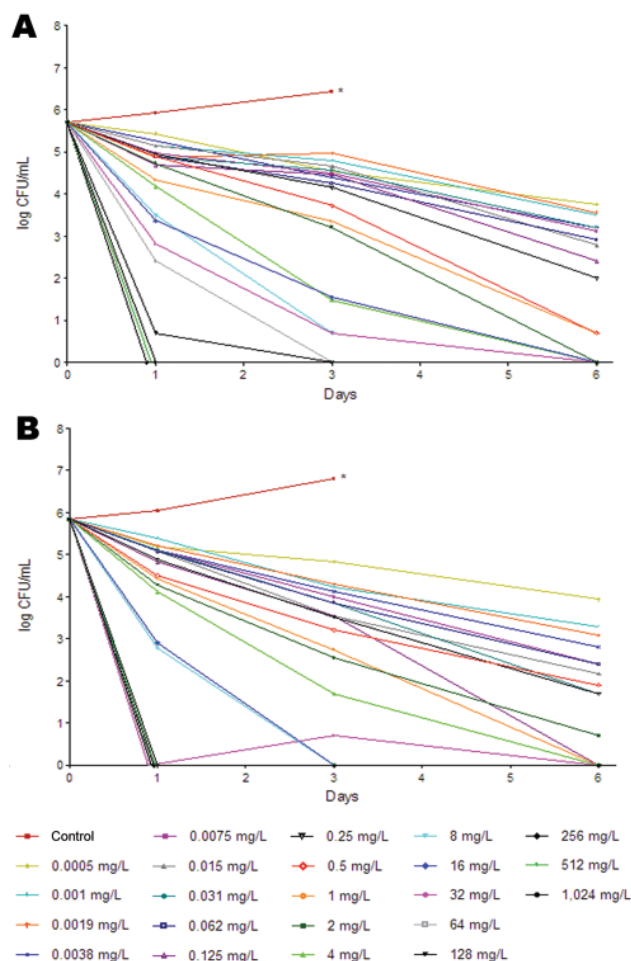


Figure 2. Concentration- and time-dependent bactericidal effect of rifampin (RIF) toward low-density cultures of *Mycobacterium tuberculosis* BE-1585 (5.1×10^5 CFU/mL) (A) and *M. tuberculosis* EAI-1627 (6.8×10^5 CFU/mL) (B). Cultures were exposed to RIF at 2-fold increasing concentrations for 6 days at 37°C. After 1, 3, or 6 days of exposure, subcultures were performed on solid media to count CFUs. *Accurate CFU counting could not be performed because complete outgrowth of mycobacteria occurred on the sixth day of RIF exposure, leading to aggregation.

exposure to RIF concentrations of 2–32 mg/L selected resistant mutants only; this was not observed in EAI-1627 cultures. Analysis of RIF-resistant Beijing mutants showed the following altered *rpoB* gene sequences: CAC→GAC (H526D), CAC→TAC (H526Y), and TCG→TTG (S531L), as assessed by using the GenoType MTBDR*plus* (Hain Lifescience, Nehren, Germany) assay (for details, see the online Technical Appendix).

For 3 of the 4 anti-TB drugs, the difference in the range of mutant prevention concentrations for the Beijing and EAI genotype strains was small: INH, 128–256 mg/L; RIF, 256–1,024 mg/L; and MXF, 2–8 mg/L. The mutant prevention concentration for AMK was >1,024 mg/L for all strains tested.

Conclusions

We showed that the currently used anti-TB drug susceptibility assays do not discriminate between the in vitro susceptibility, as determined by the methods used in this study, of the *M. tuberculosis* Beijing and EAI genotype strains. We also showed that the determination of mutation frequencies might be more informative than results of anti-TB drug susceptibility assays. For RIF, mutation frequencies in Beijing genotype strains were high compared with those in EAI genotype strains, and the selection of RIF-resistant mutants among Beijing strains, but not EAI strains, occurred within a wide range of RIF concentrations. In addition, the killing capacity of RIF toward the Beijing genotype is dependent on the density of mycobacteria: high concentrations of RIF are required to achieve 100% killing of high-density Beijing genotype populations but not of high-density EAI genotype populations. These in vitro characteristics might contribute to the less favorable treatment outcome of Beijing genotype TB infections and their significant association with drug resistance. Our findings demonstrate the need for anti-TB drug treatments that will prevent resistance among *M. tuberculosis* Beijing genotype TB cases, and they suggest that the development of genotype-specific TB therapy might be justified.

Table 2. Concentration- and time-dependent bactericidal effect of rifampin toward *Mycobacterium tuberculosis* genotypes in low- and high-density cultures*

Day	Lowest RIF concentration resulting in killing of <i>M. tuberculosis</i> , mg/L							
	Beijing-1585 genotype				EAI-1627 genotype			
	≥99% killing		100% killing		≥99% killing		100% killing	
	Low†	High‡	Low†	High‡	Low§	High¶	Low§	High¶
1	8	ND	256	ND	8	ND	32	ND
3	1	0.008	64	1024	0.125	0.03	8	32
6	0.001	0.008	2	64	0.001	0.06	1	2

*Cultures were exposed to RIF at 2-fold increasing concentrations for 6 days at 37°C; at indicated time-points, subcultures were performed on solid media for counting. Low, low-density culture; high, high-density culture; ND, not determined.

†Density of 5.1×10^5 CFU/mL.

‡Density of 4.4×10^5 CFU/mL.

§Density of 6.8×10^5 CFU/mL.

¶Density of 3.0×10^6 CFU/mL.

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Dr de Steenwinkel is a medical doctor, resident in training for medical microbiologist, and a PhD student in clinical microbiology and antimicrobial therapy at Erasmus University Medical Center. His interests include research on improving therapy for TB and fundamental exploration of resistance formation.

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Address for correspondence: Jurriaan E.M. de Steenwinkel, Department of Medical Microbiology and Infectious Diseases, Erasmus University Medical Center, PO Box 2040, 3000 CA Rotterdam, the Netherlands; email: j.desteenwinkel@erasmusmc.nl

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Technical Appendix

Bacteria

The 10 *M. tuberculosis* strains used in this study were all clinical isolates from Vietnam of which five represented the Beijing genotype and five the East-African-Indian (EAI) genotype. Strains were stored at the National Tuberculosis Reference Laboratory (RIVM, Bilthoven, the Netherlands) as Beijing VN 2002-1585 (Beijing-1585), VN 2002-1607, VN 1998-2115, VN 1998-2121 and VN 1998-2145, and EAI VN 2002-1627 (EAI-1627), VN 2002-1606, VN 2002-1592, VN 2002-1596 and VN 1998-2113. A subculture of strain Beijing-1585 (known as strain VN+) was previously subjected to genome sequencing described by Schürch *et al.* (*Infection, Genetics and Evolution* 2011; 11:587–597). The *M. tuberculosis* strains in this study were selected on basis of their diverse genotyping results, as determined by using spoligotyping and IS6110 restriction fragment length polymorphism (RFLP) typing according to the internationally standardized methods (Kamerbeek *et al.* *J Clin Microbiol.* 1997; 35(4):907-14 and van Embden *et al.* *J Clin Microbiol.* 1993; 31(2):406-9). The genotypes of the strains were defined as either Beijing or EAI according to their characteristic spoligotypes, following the well-accepted genotype definitions described by Kremer *et al.* (*J. Clin Microbiol.* 2004; 42(9):4040-9) and Brudey *et al.* (*BMC Microbiol.* 2006; 6:6-23). Comparison of the five different RFLP patterns of the five Beijing strains of this study to those of Beijing strains that were subjected to single nucleotide polymorphism (SNP) typing previously performed by Schürch *et al.* (submitted for publication) showed that strains VN 2002-1585 and VN 1998-2121 represent the typical Beijing SNP type and that strains VN 2002-1607, VN 1998-2115, and VN 1998-2145 represent an intermediate Beijing SNP type. The EAI strains showed RFLP patterns with either two (VN 2002-1596) or one copy of IS6110. However, the four 1-copy strains could be discriminated on basis of the molecular weight of the IS6110 containing RFLP fragment and/or their spoligo pattern.

Cultures

M. tuberculosis suspensions were cultured in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA), supplemented with 10% oleic acid-albumin-dextrose-catalase enrichment (OADC, Baltimore Biological Laboratories, Baltimore, MD, USA), 0.5% glycerol (Scharlau Chemie S.A, Sentmenat, Spain) and 0.05% Tween 20 (Sigma Chemical Co, St. Louis, MO, USA), under shaking conditions at 96 rpm at 37°C. Vials with *M. tuberculosis* suspensions were stored at -80°C. Cultures on solid media were grown on Middlebrook 7H10 agar (Difco), supplemented with 10% OADC and 0.5% glycerol for 28-35 days at 37°C with 5% CO₂. The exact incubation time was dependent on the growth rate of the *M. tuberculosis* genotype strain investigated.

Anti-TB Drugs

The anti-TB drugs assayed were isoniazid (INH, Hospital Pharmacy; Rotterdam, The Netherlands), rifampicin (RIF, Rifadin®, Aventis Pharma B.V, Hoevelaken, The Netherlands), moxifloxacin (MXF, Avelox®, Bayer Schering Pharma A.G, Berlin, Germany) and amikacin (AMK, Hospira Benelux BVBA, Brussels, Belgium). Dilutions of the drugs were prepared according to the recommendations of the manufacturers.

Susceptibility Testing (MGIT)

The *M. tuberculosis* genotype strains were subjected to the MGIT drug susceptibility testing at the National Tuberculosis Reference Laboratory using the BD BACTEC MGIT 960 System (BD Diagnostics, 61 Sparks, MD, US) (for details, see the online Technical Appendix, wwwnc.cdc.gov/EID/pdfs/11-0912-62_Techapp.pdf) for INH, RIF, AMK and MXF susceptibility (Woods *et al.* National Committee for Clinical Laboratory Standards. CLSI document M24-A; 2011).

Minimal Inhibitory Concentration (MIC)

To determine the MIC of the *M. tuberculosis* genotype strains we used the agar proportion method as described by the Clinical and Laboratory Standards Institute (CLSI) (Woods *et al.* National Committee for Clinical Laboratory Standards. CLSI document M24-A; 2011). Colonies grown on solid media were suspended in broth using glass beads and vortexing during 4 min. The suspension was left for 30 minutes, after which the supernatant was taken and

set to an optical density of McFarland standard 1. Using broth, a 1:10 dilution of this *M. tuberculosis* suspension was plated onto solid media containing serial, twofold dilution concentrations of anti-TB drug. After incubation the degree of growth was assessed. The MIC was defined as the lowest concentration of anti-TB drug that resulted in >99% growth inhibition. MIC determinations were performed in duplicate.

Mutation Frequency (MF)

Determination of the MF of the *M. tuberculosis* genotype strains was performed using the “critical concentrations” of the anti-TB drug, being 1 mg/L for isoniazid, 1 mg/L for rifampin, 5 mg/L for amikacin and 1 mg/L for moxifloxacin, as defined by the CLSI (Woods *et al.* National Committee for Clinical Laboratory Standards. CLSI document M24-A; 2011) and Gumbo *et al.* (Antimicrob Agents Chemother. 2010; 54(4):1484-91). Starting from a high density (concentrated) *M. tuberculosis* culture ($\sim 1 \times 10^{10}$ cfu), serial dilutions of the *M. tuberculosis* suspension were plated onto solid media without drugs and onto solid media containing the “critical concentration” of the respective anti-TB drugs. After incubation, the total numbers of colony forming units (cfu) and numbers of resistant mycobacteria were counted and the MF was calculated. MFs were determined in duplicate. In order to assess the stability of the resistant mutants isolated from the anti-TB drug-containing solid media, 10 colonies were randomly picked and plated onto solid media without anti-TB drug. After incubation, these colonies were plated to check for re-growth on solid media containing the “critical concentration” of the same anti-TB drug.

Time-kill Kinetics

For Beijing-1585 and EAI-1627 a time-kill kinetic assay for rifampin was performed. The concentration- and time-dependent bactericidal activity of the anti-TB drug was determined as described previously (de Steenwinkel *et al.* J Antimicrob Chemother. 2010; 65(12):2582-9). In short, *M. tuberculosis* cultures at low density or at high density were exposed to rifampin at 2-fold increasing concentrations, ranging from 0,5 µg/L to 256 mg/L for six days at 37°C. On days 1, 3 and 6, samples were taken for cfu counting provided that the *M. tuberculosis* suspension did not show visible aggregation.

Selection of Drug-Resistant *M. tuberculosis*

During the time-kill kinetic assay for Beijing-1585 and EAI-1627 the selection of resistant mutants was performed. In order to detect drug-resistant *M. tuberculosis* in the low density or the high density cultures, the samples taken after six days of exposure to rifampin were cultured on rifampin-containing solid media. The concentration of rifampin in the solid media was 4-fold the “critical concentration” (4 mg/L rifampin). Resistant *M. tuberculosis* colonies, able to grow on this medium, were characterized using the GenoType® MTBDRplus assay (Hain Lifescience GmbH, Nehren, Germany), to detect the most common mutations in *rpoB* gene conferring rifampin-resistance (J Clin Microbiol. 2007; 45(8):2635-40).

Mutant Prevention Concentration (MPC)

The MPC of the *M. tuberculosis* genotype strains was determined using the protocol as described by Drlica *et al.* (J Antimicrob Chemother. 2003; 52(1):11-7) and Goessens *et al.* (J Antimicrob Chemother. 2007; 59(3):507-16). In short, from a high density (concentrated) *M. tuberculosis* culture approximately 10^{10} cfu was plated onto solid medium containing 2-fold increasing concentrations of anti-TB drug, ranging from 64 mg/L to 1024 mg/L for isoniazid, rifampin or amikacin and from 1 mg/L to 32 mg/L for moxifloxacin. The MPC was defined as the lowest concentration of anti-TB drug in the solid medium, which prevented growth of *M. tuberculosis*.