4. van Ingen J, Rahim Z, Mulder A, Boeree MJ, Simeone R, Brosch R, et al. Characterization of *Mycobacterium orygis* as *M. tuberculosis* complex subspecies. Emerg Infect Dis. 2012;18:653–5. http://dx.doi.org/10.3201/eid1804.110888
5. Gey van Pittius NC, Perrett KD, Michel AL, Keet DF, Hlokwe T, Streicher EM, et al. Infection of African buffalo (*Syncerus caffer*) by oryx bacillus, a rare member of the antelope clade of the *Mycobacterium tuberculosis* complex. J Wild Dis. 2012;48:849–57. doi: 10.7589/2010-07-178.

Address for correspondence: Jakko van Ingen, Medical Microbiology, Radboud University Nijmegen Medical Center, PO Box 9101, Nijmegen 6500 HB, the Netherlands; email: vaningen.jakko@gmail.com

Mycobacterium tuberculosis Beijing Type Mutation Frequency

To the Editor: A striking finding in the study by de Steenwinkel et al. (*1*) is the high frequency of mutation to rifampin resistance by 2 *Mycobacterium tuberculosis* Beijing strains, which might play a role in the association between the Beijing strains and multidrug-resistant tuberculosis. Earlier reported frequency of mutation to rifampin resistance by *M. tuberculosis* has been 10^{-8} CFU (*2,3*), including the Beijing genotype (*3,4*). Of note, the Beijing 2002–1585 strain, for which frequency of mutation to rifampin resistance is 10^{-3} CFU (1 mutant/1,000 CFU), showed a moderate frequency of 10^{-8} CFU in another study (*4*). We think that a mutation frequency increase of 100,000× is remarkably high. In contrast, rifampin-resistant mutants of the Beijing 1585 strain did not emerge in low-density cultures (5×10^5 CFU/mL) used for time-kill kinetics experiments, although frequency of mutation to rifampin resistance was determined to be 10^{-3} CFU.

Mutation frequency is determined by fluctuation assays. To exclude preexisting mutants, which would bias the mutation frequency by so-called jackpots, a series of low-inoculum cultures is typically used (*5*). However, for unknown reasons, de Steenwinkel et al. used only 1 high-density culture of 10^{10} CFU of each strain to determine mutation frequency. This strategy is not recommended because mutations can occur early or late, resulting in substantial mutation frequency fluctuation between test episodes. A strain with known mutation rates should preferably be included to rule out possible technical errors.

We propose the following explanations for the remarkable results: 1) the rifampin concentration for selecting mutants might have been too low, enabling growth of some colonies of drug-susceptible bacteria; 2) rifampin mutants arose early or preexisted in the cultivation of Beijing strains 1585 and 1607, producing jackpots; or 3) the 2 Beijing isolates might contain rifampin-resistant subpopulations (heteroresistance). The capacity of the Beijing strain to develop and, especially, transmit multidrug-resistant tuberculosis remains to be further analyzed.

Jim Werngren

Author affiliation: Swedish Institute for Communicable Disease Control, Solna, Sweden

DOI: http://dx.doi.org/10.3201/eid1903.121001

References

1. de Steenwinkel JEM, ten Kate MT, de Knegt GJ, Kremer K, Aarnoutse RE, Boeree MJ, et al. Drug susceptibility of *Mycobacterium tuberculosis* Beijing genotype, association with MDR TB. Emerg Infect Dis. 2012;4:660–3.
2. David, HL. Probability distribution of drug-resistant mutants in unselected populations of *Mycobacterium tuberculosis*. Appl Microbiol. 1970;20:810–4.
3. Werngren J, Hoffner SE. Drug-susceptible *Mycobacterium tuberculosis* Beijing genotype does not develop mutation-conferred resistance to rifampin at an elevated rate. J Clin Microbiol. 2003;41:1520–4. http://dx.doi.org/10.1128/JCM.41.4.1520-1524.2003
4. Bergval I, Kwok B, Schuitema K, Kremer K, van Soolingen D, Klatser P, Anthony R. Pre-existing isoniazid resistance, but not the genotype of *Mycobacterium tuberculosis* drives rifampicin resistance codon preference in vitro. PLoS ONE. 2012;7:e29108. http://dx.doi.org/10.1371/journal.po ne.0029108
5. Gillespie SH. Evolution of drug resistance in *Mycobacterium tuberculosis*: clinical and molecular perspective. Antimicrob Agents Chemother. 2002;46:267–74. http://dx.doi.org/10.1128/AAC.46.2.267-274.2002

Address for correspondence: Jim Werngren, Unit of Highly Pathogenic Microorganisms, Dept of Preparedness, Swedish Institute for Communicable Disease Control, Nobels väg 18 S-17182, Solna, Stockholm S 17182, Sweden; email: jim.werngren@smi.se

In Response: We explain the differing frequencies of mutation to rifampin resistance mentioned by Werngren (*1*). First, the strains of *Mycobacterium tuberculosis* that we tested differed from those previously tested (*2*). Second, we used different rifampin concentrations in subculture plates. For Beijing strain 2002–1585, Bergval et al. (*3*) found a mutation frequency of $4–24 \times 10^{-8}$ at a subculture concentration of 8 mg/L, whereas we found a mutation frequency of $3–4 \times 10^{-3}$ at a subculture concentration of 1 mg/L and a lower mutation frequency at 2 mg/L. Thus, the concentration of drugs in subculture plates is crucial to mutation frequency assays. Absent a subculture concentration standard, we applied rifampin at 1 mg/L (*4*) because bacteria growing at this concentration are considered resistant to rifampin. Our mutation frequency and time-kill kinetics assay results are not contradictory

because in the time-kill kinetics assays, the subculture rifampin concentration was 4 mg/L.

We performed no classical fluctuation assays. We compared the Beijing genotype with the East African/Indian genotype to learn how M. tuberculosis strains differed in their capacity to withstand antituberculosis drug treatment. For reference strain H37Rv, mutation frequency was 1.5 × 10-6, higher than that found with higher subculture concentrations.

With regard to the 3 other issues, our drug-susceptibility testing of mutants showed a stable rifampin-resistant phenotype. We agree that these bacteria might represent preexisting mutants selected during drug exposure in a certain drug concentration window. By using different concentrations in subculture plates in our mutation frequency assay, we detected such preexisting mutants. Heteroresistance probably does not explain our observations because in our time-kill kinetics experiments, the whole mycobacterial population decreased over time in a drug concentration-dependent way, and regrowth of a drug-resistant subpopulation was not observed.

By not sticking to the fixed test conditions as used in the classical drug-susceptibility assays, research leads to highly interesting findings. One can conclude that serendipity flourishes with variation.

Jurriaan E.M. de Steenwinkel, Dick van Soolingen, and Irma A.J.M. Bakker-Woudenberg

Author affiliations: Erasmus University Medical Center, Rotterdam, the Netherlands (J.E.M. de Steenwinkel, I.A.J.M. Bakker-Woudenberg), National Institute of Public Health and the Environment (RIVM), Bilthoven, the Netherlands (D. van Soolingen); and Radboud University Medical Center, Nijmegen, the Netherlands (D. van Soolingen)

DOI: http://dx.doi.org/10.3201/eid1903.121849

References

1. Werngren J. *Mycobacterium tuberculosis* Beijing type mutation frequency [letter]. Emerg Infect Dis. 2013:19:522.
2. Werngren J, Hoffner SE. Drug-susceptible *Mycobacterium tuberculosis* Beijing genotype does not develop mutation-conferred resistance to rifampin at an elevated rate. J Clin Microbiol. 2003;41:1520–4. http://dx.doi.org/10.1128/JCM.41.4.1520-1524.2003
3. Bergval IL, Schuitema AR, Klatser PR, Anthony RM. Resistant mutants of *Mycobacterium tuberculosis* selected in vitro do not reflect the in vivo mechanism of isoniazid resistance. J Antimicrob Chemother. 2009;64:515–23. http://dx.doi.org/10.1093/jac/dkp237
4. Clinical and Laboratory Standards Institute. Susceptibility testing of mycobacteria, nocardiae, and other aerobic actinomycetes. Approved standard M24–A2. 2nd ed. Wayne (PA): The Institute; 2011.

Address for correspondence: J.E.M. de Steenwinkel, Erasmus MC, Room L-327, PO Box 2040, 3000 CA, Rotterdam, the Netherlands; email: j.desteenwinkel@erasmusmc.nl

Correction: Vol. 18, No. 8

The name of author Arina Zanuzdana was misspelled in the article Vaccination of Health Care Workers to Protect Patients at Increased Risk for Acute Respiratory Disease. The article has been corrected online (http://wwwnc.cdc.gov/eid/article/18/8/11-1355_intro.htm).