Characterization of Mycobacterium orygis

To the Editor: We thank Gey van Pittius and colleagues for their addition to the markers that identify Mycobacterium orygis as a distinct subspecies in the M. tuberculosis complex (1). Its isolation from a wild buffalo broadens the host range of M. orygis. Gey van Pittius and colleagues raise 3 issues: the utility of the gyrB\textsuperscript{esx} single-nucleotide polymorphism (SNP) being equally specific as the reported SNP in Rv2042\textsuperscript{esx}, the presence of genomic regions RD701 and RD702 in M. orygis, and the addition of the sequence type (ST) 701 spoligotype to M. orygis-specific spoligotypes.

We agree that use of the gyrB\textsuperscript{esx} mutation is more practical for routine daily use because this gene helps identify several subspecies of the M. tuberculosis complex. However, use of the partial Rv2042 sequencing is similarly practical because it can be combined with sequencing of the adjacent pncA gene, which enables identification of several M. tuberculosis complex species and some subspecies (i.e., M. orygis, M. bovis, M. canetti) (2), to identify the CAS genotype of M. tuberculosis (J. van Ingen, unpub. data) and, to some degree, assess susceptibility to pyrazinamide (3).

With the added data, we can conclude that M. orygis is an M. tuberculosis complex subspecies defined by the presence of genomic regions RD1, RD2, RD4, RD5a, RD6, RD13–RD16, RD701, and RD702, by the C-to-G SNP in mmpL6\textsuperscript{esx}, and by the deletion of regions RD3, RD5b, RD7–RD12, RDoryx\_1, RDoryx\_4, and RDoryx_wag22. Subspecies-specific SNPs are present in gyrB and Rv2042. Spoligotypes ST587, ST701, and closely related types are characteristic of M. orygis, and this subspecies yields 17–20 copies of insertion sequence 6110 and a distinct 24-locus variable number tandem repeats pattern (4,5). Given the rapid progress in genome sequencing, additional markers specific for the different subspecies will further enrich this panel of differences.

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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 $10^{-9}$ 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 $10^{-6}$ 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 ($5 \times 10^{0}$ CFU/mL) used for time-kill kinetics experiments, although frequency of mutation to rifampin resistance was determined to be $10^{-1}$ 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 $10^{6}$ 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.

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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.