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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 $gyrB^{oryx}$ single-nucleotide polymorphism (SNP) being equally specific as the reported SNP in $Rv2042^{38}$, 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 $gyrB^{oryx}$ 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. canettii*) (*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 $mmpL6^{551}$, 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^{-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.

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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