

Preferred routes to resistance of different *M. tuberculosis* genotypes

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Last year our laboratory received a grant from ESCMID which allowed us to investigate the accumulation of drug resistance mutations in different strains of *M. tuberculosis*. Our aim was to obtain an insight into the mechanisms by which drug resistant tuberculosis emerges and spreads.

Mycobacterium tuberculosis, the etiological agent of tuberculosis, is a clonal organism with no evidence of horizontal gene transfer and a low recombination rate. Antimicrobial resistance in *M. tuberculosis* is therefore almost exclusively due to the acquisition of point mutations or, very rarely, genetic rearrangement. Under non-selective conditions point mutations arise once every 10^6 - 10^8 replications on average. Due to the clonal nature of *M. tuberculosis*, these mutations are passed on to the progeny and mutations accumulate in the genome. Most resistance mutations confer a fitness cost when newly acquired, but provide a benefit under antibiotic treatment. Genetic characterization of drug-resistant clinical isolates has indicated that only a small range of mutations lead to drug resistance without compromising the viability of the bacteria; for rifampicin-resistance 3-5 mutations typically account for >80% of all clinical resistance.

Virtually the same mutations conferring resistance to rifampicin *in vivo* are also observed *in vitro* when spontaneous resistant mutants are selected on rifampicin-containing agar plates from non-selective liquid cultures. This implies that certain mutants are genuinely more likely to survive, even when there is no direct competition between mutants.

Multidrug-resistance (MDR), resistance to at least the two potent antibiotics rifampicin and isoniazid, develops by sequential acquisition of mutations at different loci. It is therefore possible the order of drug resistance acquisition is constrained, with certain mutations conferring a greater chance of further development to an MDR than others. Although the acquisition of resistance mutations is random, the genetic routes leading to the development of successful and virulent (multi)drug-resistant bacteria may be quite restricted and dependent on the genetic background of the strain in question.

The general assumption is that isoniazid resistance typically precedes rifampicin resistance in the route to MDR-TB, since isoniazid monoresistance among clinical isolates is much more widespread than rifampicin monoresistance. However, this assumption has not been confirmed. We hypothesized that the genetic background of the *M. tuberculosis* bacteria, such as the genotype or pre-existing drug resistance, partly determines the optimal evolutionary

route of the bacteria in question. To test this hypothesis, we focused on acquisition of rifampicin resistance as a model for genetic adaptation. The spectrum of resistance-conferring mutations was determined for strains representing two different genotypes and isoniazid resistance profiles. Spontaneous rifampicin-resistant mutants were selected from liquid, non-selective cultures of these strains and characterized by sequencing and Multiplex Ligation-dependent Probe Amplification (MLPA), a technique allowing the simultaneous detection of multiple mutations in one assay.

We found that the spectrum of mutations differed not only between the different strains, but also, and even more so, when different selection methods were used. With one method single resistant colonies derived from 10-ml cultures were selected on rifampicin-containing plates. A laboratory strain with the Haarlem genotype showed a remarkably high propensity for *rpoB*-H526Y mutations with this method, confirming earlier results obtained in our laboratory. However, this bias was no longer apparent when colonies were selected from multiple individual 1-ml cultures seeded with ~1000 cfu, the second method.

Moreover, for all strains tested the method using a single 10-ml culture resulted in a much lower range of mutations than with the method using multiple 1-ml cultures. The difference in the mutational spectra between these two selection methods strongly suggests that the relative fitness of the different mutations is the critical factor in their survival. We propose that the wide diversity of mutations seen when mutants are selected from multiple small cultures is a direct result of low competition between mutants (generally only 1 mutant per tube) and presumably more accurately reflects the relative mutation rate for individual genetic loci. In contrast, the differences in the mutation spectrum observed between strains when selected from larger cultures reflect the relative fitness of the individual mutations when present in different strains.

In summary, the mutations that are most likely to occur and the mutations that are most likely to persist are not necessarily the same and our data suggest that both are dependent on pre-existing drug resistance as well as the genotype of the bacteria.

If we assume these discrepancies are also true *in vivo*, this would have several implications. For instance, Beijing strains have often been associated with (multi)drug resistance. Initially it was suggested that these strains may have a higher mutation rate, thereby leading to a higher rate of drug resistance. An alternative hypothesis would be that certain drug-resistant Beijing strains have a higher survival rate (or lower fitness cost) compared to drug-resistant mutants of other genotypes carrying the same drug resistance mutations. In parallel, apparent clusters of drug resistant isolates could be generated by multiple, identical mutational events, rather than being the result of clonal expansion. Recent advances in whole genome analysis will hopefully be able to resolve this issue in the near future.

If the evolutionary path to multidrug resistance is not only constrained but also in large part strain-specific, this has implications for infection control and the design of treatment regimens.

On the basis of these tantalizing results we are in the process of confirming and expanding our observations to allow publication in the near future. We hope this work will stimulate discussion and more detailed investigations of the adaptation of *Mycobacterium tuberculosis* and thank ESCMID for the valuable support.