Unveiling the evolution of antibiotic resistance in *M. tuberculosis* using Transwell Tolerance-Resistance system and chromosomal barcoding.

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Background: The requirement for prolonged antibiotic chemotherapy remains a major obstacle in the efforts toward global tuberculosis (TB) control. Combating the antibiotic tolerance in TB bacilli is essential for shortening chemotherapy and preventing the emergence of drug resistance. However, the lack of *in vitro* methods for studying extended time-kill-regrowth kinetics limits our understanding of the drug tolerance and evolution of drug resistance in *M. tuberculosis* (Mtb). We devised a novel integrated approach of *in vitro* steady-state antibiotic exposure called the Transwell-Tolerance-Resistance (TTR) system which is combined with chromosomal barcoding to trace drug tolerance and the emergence of drug resistance in Mtb.

Methods: The TTR system is a simplified version of the two-compartment hollow fiber system. Each transwell with bacterial culture (200 µl) is partially immersed into an individual media containing a basolateral well (1200 µl) separated by a 0.4 micron filter, allowing the diffusion of the antibiotic into the transwell while impermeable to cells. A pool of actively growing, chromosomally barcoded Mtb cells, each harboring a unique 11 base pair barcode was exposed to rifampicin (10 X MIC) for 20 days in the TTR system with regular replacement of the drug containing media using a robotic liquid handler. At 4 day intervals, 50% (100 µl) volume from replicate cultures were plated in equal halves over drug free and rifampicin containing agar medium (breakpoint MIC). The remaining 50% culture was allowed to regrow without drug and plated similarly. Colonies isolated from different time points were used for deep sequencing of the barcodes.

Results: The proportion of tolerant barcodes was 3.53% (98/2769) and a subset of 13 tolerant barcodes (0.47%) were common among the replicates during day 16 and day 20. Three out of the 13 tolerant barcodes gained rifampicin resistance between day 12 and day 20, indicating a strong overlap (23%) between tolerance and resistance. The absence of rifampicin resistant mutants at early time points excluded any preexisting mutants and suggested their *de novo* emergence during extended drug exposure. Distinctive tolerant (89.3 ± 7.39%) and resistant barcodes (none) between independent experiments suggested the stochastic formation of tolerant clones. Additional rifampicin resistant clones were observed in the regrowth cultures (37.5%, 9/24) compared to the direct exposure (16.7%, 4/24) (P value 0.0212, paired *t*-test).

Conclusion: We demonstrated a novel integrated approach for the simultaneous detection and tracing of antibiotic tolerant forms and their role in the emergence of drug resistance. Resistant mutants emerge during prolonged antibiotic exposure and the recovery after treatment enhanced the development of drug resistance.