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

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Abstract

The global burden of tuberculosis (TB) remains a major public health concern despite the availability of effective antibiotic treatment. *Mycobacterium tuberculosis* (Mtb), the causative agent of TB, infected over 10 million annually and killed close to two million people in 2019 alone. Unlike other bacterial infections, TB requires 2 to 4 months of combination antibiotic treatment to achieve a durable cure. Even with the extended treatment regimen, up to 5% of apparently successful treatment cases still relapse mostly due to the recovering antibiotic tolerant forms upon treatment withdrawal. Therefore, initial responses to tuberculosis treatment are poor predictors of final therapeutic outcomes in drug-susceptible disease suggesting that treatment success depends on features that are hidden within a minority of the overall infecting Mtb population. Understanding the clonal origin and survival dynamics of drug tolerant forms of Mtb and the pathway by which drug-tolerance progresses to drug resistance is essential in devising novel treatment strategies for shortening anti-TB regimen while preventing the emergence of drug resistance. However, the lack of *in vitro* methods for studying extended time-kill-regrowth kinetics limits our understanding on the drug tolerance and evolution of drug resistance in Mtb. To address this, we developed a multi-transwell robotic system to perform numerous parallel cultures of genetically barcoded Mtb exposed to steady-state concentrations of rifampicin to uncover these difficult to eliminate minority populations. We found that tolerance repeatedly emerged from a subpopulation of barcoded cells. This tolerant subpopulation reproducibly passed through a phase characterized by multiple unfixed resistance mutations followed by emergent drug resistance in some cultures. Barcodes associated with drug resistance identified an especially privileged subpopulation that was rarely eliminated despite 20 days of drug treatment even in cultures that did not contain any classically drug resistance cells. This work provides the first window into the origin and dynamics of bacterial subpopulations whose elimination may be critical to developing rapid and resistance free cures.

Background

The requirement for a prolonged antibiotic chemotherapy remains a major obstacle in the efforts toward global TB control. Combating antibiotic tolerance in tuberculosis is essential for shortening chemotherapy and preventing the emergence of drug resistance. Drug tolerance is often studied *in vitro* by exposing bacterial cultures to lethal concentrations of antibiotics followed by determining the kill-kinetics over time. However, the drug instability poses a problem when this approach is applied on slow growing bacteria such as Mtb. Declining drug levels in treated cultures may alter bacterial kill kinetics and allow the emergence of preexisting low-level drug resistant mutants in ways that simulate drug tolerance. Our integrated approach of *in vitro* steady-state antibiotic exposure using the Transwell-Tolerance-Resistance (TTR) system, combined with chromosomal barcoding enabled in tracing drug tolerance and the emergence of drug resistance in Mtb.

Materials and 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 DNA extraction and deep sequencing of the barcodes.

Results

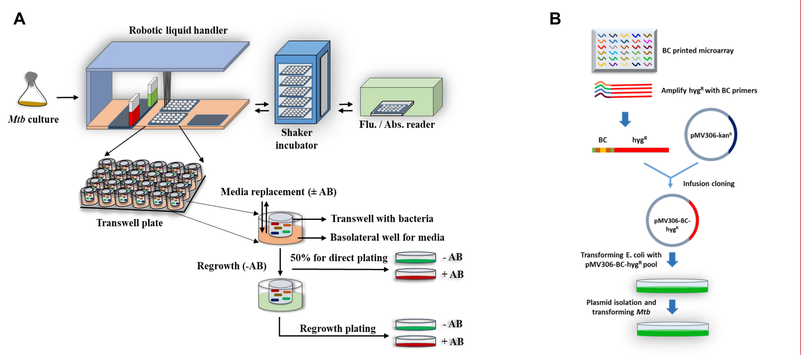

Fig. 1. (**A**) Transwell-Tolerance-Resistance (TTR) system with chromosomal barcoding for high resolution time-kill studies. In the TTR system actively growing *M. tuberculosis* is seeded into a 24 transwell plate, and a robotic system performs regular media exchanges providing controlled drug levels in each transwell culture. One-half of each well is directly plated onto drug-free and drug-containing media at defined time points while the other half is washed free of drug, regrown to an OD595nm of 0.2 and then similarity plated. (**B**) Construction of a barcoded *M. tuberculosis* library starting with barcode sequences printed on a microarray.

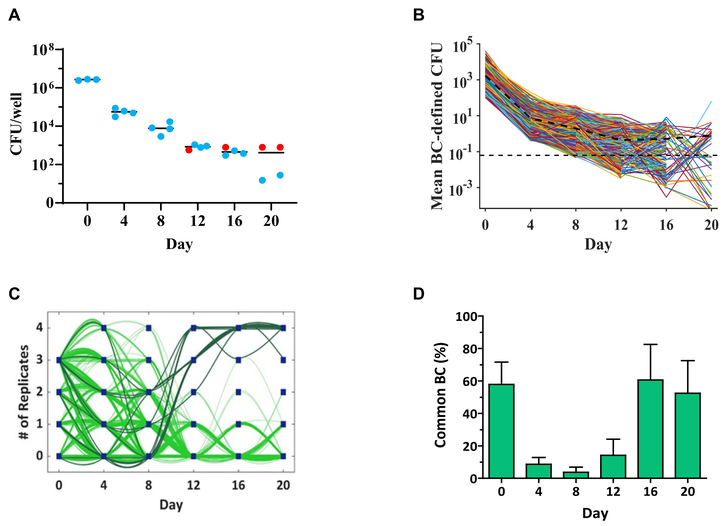

Fig. 2. (**A**) Time-kill kinetic studies using barcoded *M. tuberculosis* cultures showing the number of CFU observed during 10X MIC rifampicin exposure representing sensitive (blue dots) and rifampicin resistant (red) CFUs. (**B**) Barcode level time-kill kinetics. Individual barcode count kill curves, adjusted for the number of CFU in each assay well, denoted as mean BC defined CFU, were generated in all replicate culture wells at each time point. Each line represents the trajectory of a unique barcode. The bold dotted line represents the mean value of total barcodes at each time point. All barcode reads above the 10 barcode count per well cut off were included in the analysis; however, mean barcodes lower than one is reported (below the dotted line) when this is due to averaging barcode numbers across wells. (**C**) Line graph representing the presence of individual barcoded clones among the replicate cultures during rifampicin exposure. (**D**) Bar graph showing the percentage of common barcodes among the replicate cultures of different time points.

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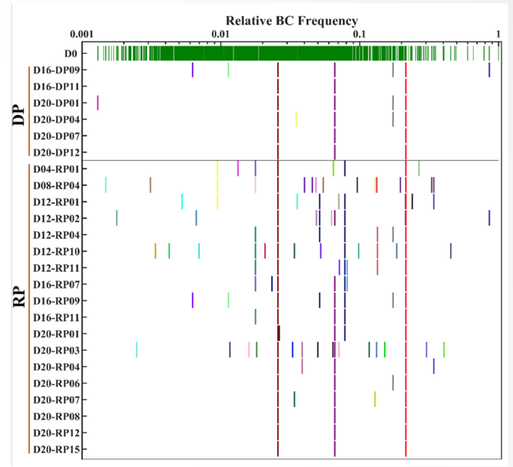

Fig. 3. Diversity and conservation of barcodes detected in drug resistant CFU. All barcodes detected on day 0 are distributed across the top row of the panel (green lines) according to their mean relative frequency at day 0 (from low to high frequency). All of the barcodes identified in resistant CFU are assigned a specific color and position that is aligned in the figure according to their mean frequency in drug susceptible day 0 wells. Barcodes found in resistant DP or RP cultures are indicated. Color and position denote a unique barcode. When resistance barcodes were detected in independent cultures they are shown in the same color and X axis position. The Y axis denotes cultures named with the day of treatment and replicate number.

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 from a specific subset of clones and the recovery after treatment enhanced the development of drug resistance.

Future Direction

Our study demonstrates the role of antibiotic tolerant subpopulations in Mtb cultures in sourcing drug resistance. This would aid in the discovery of molecular markers as well as the underlying mechanisms of drug tolerance and emergent drug resistance. Targeting these subpopulations with new therapeutics could shorten TB treatment and prevent resistance emergence, hastening the global eradication of this disease.

Acknowledgments

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