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Commentary

The devil is in the diversity: Exploring within-person evolution of *Mycobacterium tuberculosis*

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The study by Nimmo et al. published in this article of *EBioMedicine* used a whole genome sequencing (WGS) approach to explore the within-host *Mycobacterium tuberculosis (Mtb)* microevolution in response to antibiotic pressure, the impact of *Mtb* genetic diversity on treatment outcome, and the clinical implication of hetero-resistance in 331 patients with a single-strain *Mtb* infection [1]. What is unique and laudable about this study is its translational nature, where genetic diversity is linked with clinical data, resulting in important insights into how within-host diversity impacts treatment outcomes. Even though the investigators did not find an association between genetic diversity and clinical outcome, several interesting observations were made. Cavitary disease, not taking antiretroviral treatment if HIV positive, and lineage 2 strain infection were associated with *Mtb* diversity; within-host diversity increased temporarily in response to antibiotic pressure; and the presence of micro-hetero-resistance may not always be predictive of resistance emerging later on. Taken together, this work is important as it stands as one of the first examples of how clinical WGS cohort studies can explore the impact of the pathogen population's genetic make-up on disease progression and host adaptation.

The dogma has long been that *Mtb* is a clonal pathogen with no recombination, little between strain variation and little to no within-host evolution during infection [2]. The use of WGS has allowed us to question these long-held beliefs. WGS studies confirmed that little recombination occurs [3], but challenged the dogma of limited diversity. We now know that different *Mtb* lineages can have thousands of single nucleotide polymorphism differences between them [4], that the prevalence of mixed infections (infection with different strains in a single patient) is much higher than previously thought (over 20% in some settings) [5], and that the within-host population structure in single infection cases can vary between compartments, resulting in sub-clones and varying drug resistance patterns [6,7]. Within-host diversity can even be higher than the genetic distance observed between strains in different patients belonging to a single transmission cluster [6]. To date, most studies of within-host *Mtb* diversity have focused on mixed infections and demonstrated that mixed infections can result in worse clinical outcomes [8]. The few analyses of within-host diversity in single *Mtb* infections suggest that microevolution may be related to virulence, drug resistance or compensatory fitness mutations [7], but most genes for which nucleotide diversity was observed in one study were not confirmed in other studies. Similarly, the study by Nimmo et al. failed to identify clear patterns of diversity in gene functional categories.

Hetero-resistance, where drug-resistant and drug-susceptible strains or strains containing different combinations of resistance-associated variants coexist in a single patient, was also investigated in this study. While phenotypic hetero-resistance has been associated with poor treatment outcomes [9], the clinical implication of genotypic hetero-resistance remains unclear. Specifically, the conditions under which unfixed hetero-resistance precedes fixed resistance are not yet known. Traditionally, resistance is defined in a binary manner using culture-based methods where $< 1\%$ of colonies growing on drug-containing media is denoted as drug susceptible and any value between 1% and 100% is denoted as drug resistant. Using sequencing, hetero-resistance can now be evaluated as a continuum. Nimmo et al. found fluctuating levels of hetero-resistance over time and a high degree of noise due to large numbers of very low-frequency variants detected by deep sequencing. They also observed that $> 30\%$ hetero-resistance at baseline is clinically relevant whereas lower levels may not be.

Since WGS for *Mtb* is still a relatively new technology, many methodological limitations still hinder these studies: the potential culture or sub-culture bias, lack of representativeness of a single sputum sample of the within-host *Mtb* diversity, sequencing errors introduced by PCR, contamination, and varying sequencing depth [7,10]. Nimmo et al. eloquently tried to address these limitations by limiting sub-culturing steps, controlling coverage depth in the analysis and performing targeted deep sequencing to assess the presence of baseline variants in patients with resistance emerging during treatment. They demonstrated that, in their population, the number of PCR cycles, isolate contamination and type of culture media did not impact measures of *Mtb* within-host genetic diversity. Nevertheless, other important limitations

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remain to this study, especially the use of a single culture isolate, the focus on average genomic diversity knowing that a large proportion of the genome may not be under selection, and the limited number of follow up samples due to the secondary analysis design.

It is likely that there is pressure on the pathogen to diversify within the host environment, but whether this results in changes in transmission, virulence, lung destruction and cure rates remains to be seen. Analyzes mirroring those undertaken by Nimmo et al., where WGS is performed on carefully selected, well characterized clinical samples, are needed to further improve our understanding of the link between the within-host bacterial genetic landscape and clinically relevant outcomes.

Declaration of Competing Interest

Prof. Van Rie has nothing to disclose. Dr. Meehan has nothing to disclose.

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