Evolution of Drug Resistance in Tuberculosis: Recent Progress and Implications for Diagnosis and Therapy

Andrej Trauner · Sonia Borrell · Klaus Reither · Sebastien Gagneux

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Abstract Drug-resistant tuberculosis is a growing threat to global public health. Recent efforts to understand the evolution of drug resistance have shown that changes in drug–target interactions are only the first step in a longer adaptive process. The emergence of transmissible drug-resistant Mycobacterium tuberculosis is the result of a multitude of additional genetic mutations, many of which interact, a phenomenon known as epistasis. The varied effects of these epistatic interactions include compensating for the reduction of the biological cost associated with the development of drug resistance, increasing the level of resistance, and possibly accommodating broader changes in the physiology of resistant bacteria. Knowledge of these processes and our ability to detect them as they happen informs the development of diagnostic tools and better control strategies. In particular, the use of whole genome sequencing combined with surveillance efforts in the field could provide a powerful instrument to prevent future epidemics of drug-resistant tuberculosis.

1 Introduction

The burden of tuberculosis (TB) caused by drug-resistant Mycobacterium tuberculosis is increasing. As a result, the standard treatment—directly observed therapy, short course (DOTS), composed of 2 months of a four-drug regimen (isoniazid [INH], rifampicin [RIF], pyrazinamide [PZA], and ethambutol [EMB]) followed by 4 months of treatment with RIF and INH—is failing in many settings. While any degree of drug resistance might worsen the prognosis of a TB patient [1, 2], there are two definitions of M. tuberculosis drug resistance that are particularly relevant in the clinic. The first refers to strains resistant to both INH and RIF and is termed multidrug-resistant (MDR) TB. Treatment of patients with MDR-TB takes up to 2 years, and currently relies on fluoroquinolones (FQ) and injectable aminoglycosides (AG) to compensate for the loss of two of the most potent drugs. The acquisition of resistance to these classes of antibiotics defines extensively drug-resistant (XDR) TB. XDR-TB requires even longer treatment with drugs that are much more costly and show limited efficacy and increased side effects. Thus, XDR-TB is often associated with poor treatment outcomes [3].

The global genetic diversity of drug-resistant M. tuberculosis [4–10] indicates that drug resistance evolved on multiple occasions in geographical hotspots characterized by a high incidence of TB and inappropriate drug treatment. The latter is mostly driven by a lack of resources resulting in two important failures: an inability to detect drug resistance and a systemic failure to deploy effective treatments [11–13]. The ongoing evolution of M. tuberculosis in these settings [14, 15], provides an excellent opportunity to explore the genetic determinants of drug resistance in this microbe.

Unlike most other bacterial pathogens, resistance plasmids and horizontal gene transfer play no role in the acquisition of drug resistance in M. tuberculosis. Moreover, efflux mechanisms appear to serve only as a ‘stepping stone’ to high-level resistance. They allow the bacteria to tolerate higher levels of drug but do not per se result in clinically relevant levels of resistance to multiple antibiotics in M. tuberculosis [14, 16–18]. Instead, the evolution
of strains resistant to multiple antibiotics is driven by the sequential acquisition and accumulation of resistance-conferring mutations on the bacterial chromosome. These mutations primarily interfere with drug-target binding (e.g. RIF- *rpoB* [19], FQ-*gyrA* [20]), compromise prodrug activation (e.g. INH-*katG* [21], PA-824-*fgd* [22]), or cause over-expression of the target (INH/ETH—promoter region of *inhA*). The elucidation of the mechanisms of action for many antimycobacterials led to the identification of key determinants of resistance (see Zhang and Yew [23] and Almeida Da Silva and Palomino [24] for reviews), and the realization that the corresponding genetic mutations can be used as reliable molecular markers for drug susceptibility testing (DST) [25]. The application of this knowledge to the clinic has resulted in the development of diagnostic tools based on nucleic acid amplification (NAA) that overcome many of the shortcomings of phenotypic DST; these include a long turnaround time, outcome variability, and infrastructure requirements [26–29]. A comprehensive overview of these diagnostic tools has been given elsewhere [30]. Recently, one of these tools, known as Xpert MTB/RIF, has been the subject of a policy update published by the World Health Organization. Their recommendation for Xpert MTB/RIF to replace microscopy in HIV-positive patients, patients suspected of MDR-TB, and those suspected of TB meningitis follows its successful implementation in many countries [31, 32]. The instrument is designed to analyse sputum directly, hence bypassing the need for primary bacterial culture. It simultaneously tests for the presence of *M. tuberculosis* and RIF resistance. Even though this technology is having a positive impact on TB control by offering high sensitivity and reducing the time to TB diagnosis, the associated costs and infrastructural requirements (e.g. a constant power supply, machine maintenance) remain limiting for many high-burden countries [32, 33]. Hence, on-going efforts in product development focus on cheaper and simpler so-called ‘point-of-care’ diagnostics [34]. Nonetheless, DST without bacterial culturing will continue to require NAA. The choice of targets for NAA will determine the power of future diagnostics. It should be based on detailed knowledge of the relationship between strain genotype, drug resistance phenotype, and the patient treatment outcome in terms of relapse rate and treatment failure. Whole genome sequencing (WGS) of clinical drug-resistant strains of *M. tuberculosis* should be combined with the analysis of clinical information from patients infected by these strains to yield valuable new insights into the biology of drug resistance.

In this review, we first summarize key findings from recent WGS studies of mycobacterial drug resistance. We focus specifically on results that shed light on why the acquisition of individual drug resistance-conferring mutations is only part of the problem. We then use specific examples to illustrate how understanding more broadly the evolutionary mechanisms that drive drug resistance can inform the development of improved diagnostic tools as well as better strategies to preserve both existing and new treatment regimens.

2 New Genomic Insights into *Mycobacterium tuberculosis* Drug Resistance

The evolutionary path leading through drug resistance is strongly influenced by two factors: epistasis and bacterial fitness [35–38]. We define epistasis as a set of genetic interactions where the phenotypic effect of one mutation is determined by the presence of one or more other mutations. For example, resistant strains carrying the same resistance mutations vary in their capacity to transmit from patient to patient [39–42], showing that the strain genetic background can determine the course of evolution of drug resistance. Bacterial fitness, on the other hand, is a function of growth rate, virulence, and transmissibility [15, 43]. Any mutation that reduces it in relation to the wild-type strain is said to carry a ‘fitness cost’. The most immediate way to estimate the relative fitness is to measure the growth rate of bacteria in culture. On average, most drug-resistance mutations carry a fitness cost (see Fig. 3) [44] whose magnitude positively correlates with the frequency of different resistance mutations in the clinic; resistant strains with the smallest fitness defect are most abundant [45, 46]. Furthermore, the fitness of resistant mutants is not fixed; evolution is an ongoing process, and a comparison between clinical and laboratory strains carrying the same drug-resistance mutation shows that clinical strains often successfully bypass any fitness cost imposed by resistance [45]. The acquisition of such compensatory mutations is also an example of epistasis and is key in the evolution of transmissible drug-resistant strains that pose the greatest risk to public health [6, 10, 47–51]. Unfortunately, we do not currently have sufficient knowledge to predict epistatic interactions a priori, so we must rely on detecting them empirically by studying the genetics of drug resistance [52].

A number of recent studies used WGS to address the evolution of drug-resistant *M. tuberculosis* [7, 9, 51, 53, 54]. The authors of these studies used approaches based on phylogeny [7], molecular epidemiology [9, 51, 53], and mutation frequency analyses [54] to compare drug-susceptible and drug-resistant clinical strains of *M. tuberculosis*. The shared aim of these studies was the identification of bacterial genes under positive evolutionary selection by drug pressure. The detailed discussion of the merits and shortcomings of the analytical approaches used in these studies is beyond the scope of this review, but it is
important to note that there is considerable overlap in the findings of these studies. In addition to known drug targets, all studies identified novel bacterial genes and intergenic regions whose function may be ancillary to drug-resistance mutations (see Fig. 1). In particular, several genes involved in lipid metabolism, cell wall homeostasis, purine metabolism, and transcriptional control appear to be positively selected in presence of anti-TB drugs [7, 54]. These studies represent an important step towards an improved understanding of drug resistance. However, with the exception of just a few mutations (e.g. *ponA1*, and the promoters of *thyA* and *thyX* [7, 54]), the actual role of these genes remains unclear, essentially offering an extended list of genes of interest that require further investigation. Consequently, it is currently not possible to evaluate the role of these genes in the development of future diagnostics or therapies.

In addition to the above genes, there are a growing number of novel genes for which experimental evidence is available to support a role of epistasis in adaptation to resistance. These genes were identified through recent studies aimed at elucidating the evolutionary trajectory of drug-resistant *M. tuberculosis* in the clinic [6, 47] and include *rpoC*, which mediates the adaptation to RIF resistance, and Rv3806c, which appears to have a role in EMB resistance. RIF resistance is caused by mutations of the beta subunit of RNA polymerase encoded by *rpoB*. Mutated amino acids are normally involved in drug binding and are usually restricted within a short stretch of the protein termed the rifampicin resistance determining region (RRDR) [19]. Many of these mutations carry a fitness cost [45, 49] that appears to be negatively correlated with the activity of the mutant enzyme [55]. The importance of this
aspect of RNA polymerase physiology is clearly illustrated by the fact that one of the most frequent resistance mutations observed in the clinic, S450L, (equivalent to S531L in *Escherichia coli*) also carries the lowest fitness cost [45]. Moreover, this mutation appears to be almost ubiquitous among MDR strains of *M. tuberculosis* [6, 9, 10, 48, 51], and perhaps more importantly, is shown to be strongly associated with the acquisition of compensatory mutations within RNA polymerase genes (*rpoA, rpoB,* and *rpoC*). This combination of mutations is strongly associated with improved transmissibility of strains as evidenced by clonal expansion of *M. tuberculosis* strains carrying these particular mutations in patient populations [9, 48, 51]. The role of these compensatory mutations (see Koch et al. [56] for a more comprehensive review) appears to be to restore wild-type function of mutant RNA polymerase, probably on the level of enzymatic activity [49, 50]. Alternatively, these compensatory mutations may also restore the baseline gene expression profile of cells. Specifically, *rpoB* mutants were shown to have a modified lipid profile [57] as well as a modified expression of many proteins involved in lipid metabolism, particularly phthiocerol dimycocerosates (PDIMs) [58]. Interestingly, lipid metabolism genes, including those for PDIM metabolism, seem to be under positive selection during the evolution of drug resistance [7, 54]. Because PDIMs and other mycobacterial lipids play an important role in virulence [59, 60], this argues that global physiological consequences of drug-resistance mutations could provide a contextual framework, within which the compensatory mechanisms mediated by mutations in ancillary genes can be explored [7, 51, 54].

A recent set of experiments reported by Safi et al. [47] demonstrate the effect of epistasis in the progressive increase of minimal inhibitory concentration (MIC) for a drug. Focusing their study on EMB, they observed that acquisition of high-level resistance to EMB is a multistep process. In addition to the most frequently isolated resistance mutant—*embB* M306V—they identified nonsynonymous mutations in *Rv3806c* and a synonymous mutation in *Rv3792* as important contributors to EMB resistance in vitro. They proceeded to show that *Rv3806c* is involved in modulating the availability of EmbB substrates, while the synonymous single nucleotide polymorphism in *Rv3792* apparently stabilized *embC* RNA, leading to a de facto over-expression of the gene, resulting in reduced susceptibility to EMB [47].

Finally, epistasis is not limited to the physiology of a single drug. Investigations into the interaction between mutations resulting in resistance to disparate drugs have suggested that positive epistasis may drive multidrug resistance [61]. Our group recently published a report showing that specific resistance mutations in *rpoB* and *gyrA* can compensate for each other’s fitness defects, to the point that some strains carrying both mutations are fitter than either single mutant [46]. Moreover, the particular combinations of mutations conferring resistance to RIF and ofloxacin (OFX) associated with the highest overall fitness appear to be positively selected in high-burden settings.

The examples listed here are not designed to offer a comprehensive overview of all reported examples of epistasis in drug-resistant *M. tuberculosis*; we have tried to sketch a more complete picture in Fig. 1. Instead, they were chosen to illustrate important concepts brought to light by recent studies: in the first place, based on current data, it appears that compensatory mutations occur most frequently in strains that already harbor the least costly mutation [48]. Second, epistatic interactions occur between specific mutations [46, 47, 49], and in some cases these can be mutually exclusive; for example, a strain harboring an *rpoA* mutation does not then also acquire an *rpoC* or additional *rpoB* mutations [6, 50, 51, 62]. Continued exposure to a drug seems to impose constraints on evolution that facilitate the acquisition of compensatory mutations in strains that are already resistant [36, 50]. Once generated, these strains are more likely to be transmitted than strains carrying the resistance mutation alone [48, 51]. A further consequence of continued drug exposure is the stepwise accumulation of mutations that result in an increased level of resistance to a drug [47]; a factor that is already influencing INH resistance in XDR strains [63] and may contribute to higher levels of resistance to FQ [64, 65]. Moreover, epistasis can occur between drug-resistance mutations [46, 61, 66], implicating individual resistance determinants as drivers of polydrug resistance. Combined, these observations clearly indicate that continued inappropriate treatment, in part caused by misdiagnosis of resistance, drives the evolution of more transmissible, increasingly drug-resistant strains [10, 51].

A further set of factors, not considered hitherto, is involved in the generation of protein heterogeneity in the cell. The primary source of this is mutations, and mutation rates were shown to vary between different phylogenetic lineages of *M. tuberculosis*. The Beijing family of strains in particular seems to have a higher mutation rate [67–69]. The exact consequences of this remain to be determined, but differences in mutation rate have been used to explain the correlation between this genotype with a higher rate of drug-resistance acquisition. Mathematical models estimate that Beijing strains are 22-fold more likely to produce MDR strains [68]. Another intriguing possibility was recently put forward by Javid et al. [70], who argue that protein variability driven by errors in the central information-processing pathway (DNA-RNA-protein) may provide a phenotypic stepping-stone to resistance akin perhaps to other ancillary mechanisms shown in Fig. 1.
intellectually appealing, the clinical relevance of such a scenario remains to be substantiated.

3 Implications for Diagnosis and Therapy

The translational potential of the knowledge gained from the above studies lies in the implementation of molecular tools in TB control (see Table 1 for a summary). Wells et al. [71] offer an excellent review of current and future needs for the clinical management of TB, addressing diagnostics, surveillance, and programmatic approaches. It is useful to take advantage of their conceptual framework to discuss the impact of the themes that emerge from evolutionary studies. They place capabilities available to TB control programs onto a continuum that spans from community-based physicians to supranational reference laboratories (see Fig. 2). Accordingly, the available resources combined with the specific needs of each level will dictate the contribution that molecular approaches can offer.

In the clinic, the main goal is rapid and effective treatment of infected individuals. This relies on quick and reliable case detection, and, given the need for speed, is likely to depend on molecular diagnostics in the future [71]. In this setting, focusing on drug-resistance mutations offers high sensitivity and specificity for key first-line drugs [25]. Including compensatory mutations (e.g. in rpoC or rpoA, discussed above) as diagnostic markers is unlikely to provide additional clinical benefit, because mutations in rpoB already display a high sensitivity and specificity for detecting MDR-TB in individual patients [33, 72]. In contrast, a key area in which evolutionary lessons could be brought to bear in a significant manner is surveillance. Consider the reproductive number of a pathogen ($R_0$, see Fig. 3). Two dimensions are paramount to the outcome of transmission cycles; one is pathogen transmissibility ($t$) and the other is time to effective treatment ($c \times d$). Strain fitness plays a role in the former, while drug resistance considerably influences the latter. It is in the interest of public health to identify and eradicate transmissible drug-resistant strains quickly. We are currently failing on this front—in 2012, fewer than one-quarter of estimated MDR-TB cases were detected, and only 23% of those had DST results reported for PZA and FQ [73]. To this end, there is scope for the implementation of ‘molecular diagnostics for public health’. Screening for the emergence of compensatory mutations in a population, perhaps by

Table 1 Implications of understanding evolutionary mechanisms of drug resistance for tuberculosis control

| Experimental observation | Physiological consequence | Implications |
|--------------------------|--------------------------|--------------|
| Resistant strains gain compensatory mutations that change the basic physiology, e.g. RIF-rpoC | Increased transmissibility, increased propensity to acquire additional resistance | Focus surveillance on mutations that correlate with transmissible, highly resistant strains Use spent biosamples to establish wide catchment area for WGS-based surveillance |
| Continued exposure to RIF directs evolution towards the acquisition of compensatory mutations | More rapid evolution of compensatory mutations | Use molecular tools to probe the genotype of strains and stop administering ineffective drugs immediately |
| Epistasis exists between drug-resistance mutations for a single drug: e.g. EMB, FQ, INH, RIF | Multi-step acquisition of high level of drug resistance | Define clinical breakpoint concentrations based on specific susceptibility profiles for a drug |
| Positive epistasis between rpoB and gyrA mutations | Strains with specific combinations of resistance mutations (e.g. gyrA D94G and rpoB H445Y) are fitter than the wild type | Drug regimens containing both RIF and FQ may fail more quickly. Assess current clinical trials for evidence Evaluate if the order in which drugs are administered might enhance negative epistatic interactions |
| Some mutations conferring resistance to FQ and bedaquiline have no fitness costs attached | No need to compensate for specific resistance mutations | Enforce appropriate administration of drugs to avoid adding these antibiotics to failing regimens or use as monotherapy Explore the spectrum of resistance mutations, identify low cost and frequent mutations before releasing the drug into the market |
| Mutation rates vary between M. tuberculosis lineages | Beijing family strains acquire resistance to INH and RIF at a higher rate and are 22 times more likely to develop into MDR than the laboratory-adapted strain | Increase frequency of phylogeny-based surveillance and focus monitoring on areas with high rates of Beijing strains |

EMB ethambutol, FQ fluoroquinolones, INH isoniazid, RIF rifampicin, WGS whole genome sequencing

△ Adis
focusing on re-treatment cases [74] to pinpoint areas with evolved highly transmissible strains as they emerge [48, 51], would aid the public health official to prioritize resource allocation and interrupt the spread of these strains. Examples of such measures include targeted delivery of individualized, albeit expensive treatments, resource-intensive active case finding, and, in extreme cases, isolation [75, 76]. In line with the described differences in mutation rates between M. tuberculosis lineages [67, 68], the public health official may include phylogenetic markers to focus monitoring on areas with a high incidence of Beijing strains. Performing surveillance based on high-resistance mutations alone may be insufficient. Mutations conferring intermediate resistance, namely, below that of empirically determined resistance breakpoints, play a potentially pivotal role in the spread of resistance [47]. We have used the ‘fitness-MIC space’ to illustrate these concepts in Fig. 3. It is also important to stress at this point, that strains harboring mutations conferring low-level resistance can often be treated effectively with existing drugs [77], either by increasing the dosage of the same drug, or by using alternative drugs from the same class, as is the case of FQ [78]. Conversely, the use of ineffective drugs should be stopped immediately to avoid directing the evolution of a strain [36, 50] towards a more transmissible phenotype.

In addition to surveillance, knowledge of epistasis should be applied to the design and deployment of future drug regimens. Given the genetic interaction between mutations in rpoB and gyrA [46], it may be dangerous to administer RIF and FQ simultaneously, and should perhaps be avoided. A number of current clinical trials are testing an iteration of such a combination [79], and their outcomes should be carefully scrutinized by using WGS for evidence of epistasis-driven drug resistance [74, 80–82]. Protecting new drug classes is equally important. Bedaquiline is the first new antituberculosis drug to be approved by the US FDA for over 40 years [83, 84]. Given the availability of bedaquiline resistance mutations with no fitness cost, or even a fitness benefit (see Fig. 3) [85], it is key that the guidelines for the administration of the drug are adhered to [86, 87], especially in light of the fact that no standardized regimen exists for it yet. Most importantly, bedaquiline should not be used to rescue a failing regimen, and should
always be administered with other effective drugs to minimize the emergence of resistance.

### 4 Conclusion

Consensus is growing that biological parameters such as the frequency of resistance mutations, the occurrence of low- or no-cost drug-resistance mutations, epistasis between resistance mutations, as well as host genetics should be considered when deciding on future treatment protocols [88]. Efficient inclusion of all of these factors for drug-resistance surveillance requires broader implementation of WGS. The World Health Organization is currently analyzing the results of a surveillance effort looking for underlying resistance to PZA and FQ where phenotypic DST was paired with DNA sequencing (Zignol, unpublished). The timing of the study is crucial in view of the fact that many of the new treatment regimens under investigation rely on these two drugs, and underlying resistance may severely compromise their success. Applying WGS to clinical trials can also contribute important information on the success of a new treatment [81], as well as sorely needed data for new-in-class antibiotics such as PA-824, delamanide, bedaquiline, and SQ109 [89]. Together, this knowledge should be used to build an accurate picture of how the genetic makeup of a strain ultimately determines the success of treatment.

Due to high costs and logistical requirements, WGS technology might not find its way into many of the most affected and often resource-poor countries in the short term. However, applied at a supranational or national level with a wide-spread community-based catchment area, WGS would allow high-throughput analysis of known but also unknown mutations. Healthcare officials would thus obtain surveillance data and essential information for the development of new diagnostic tests adjusted to the prevailing resistance pattern [39, 40, 42, 90]. For example, one could imagine an approach in which used sputum-microscopy slides from primary TB-diagnostic centers are recycled as a source of *M. tuberculosis* DNA for pooled DNA sequencing to measure the frequency of drug-resistance alleles in a particular patient population. Similarly, spent DST samples, such as mycobacterial growth indicator tubes (MGIT) and Loewenstein–Jensen slopes, would provide an excellent source of DNA for WGS-based surveillance. The application of WGS and evolutionary principles to drug resistance in *M. tuberculosis* has been furthering our understanding of the challenges faced in the clinic as well as contributing key data for developing tools and strategies to control drug-resistant TB. As new treatment regimens containing new drugs are implemented, we will have to establish the spectrum of epidemiologically relevant mutations as soon as possible.

This will help us track the emergence of strains resistant to these drugs in real-time, thereby prolonging the life span of the new regimens—a fundamental concern given how precious new drugs are.

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