The Surprising Diversity of *Mycobacterium tuberculosis*: Change You Can Believe In

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(See the Major Article by Sun et al, on pages 1724–33.)

Highly drug-resistant strains of *Mycobacterium tuberculosis* (*M. tuberculosis*) have surfaced in the news recently. Totally drug-resistant strains, unresponsive to any current therapies, were recently identified at several locations in India [1]. Although these strains have garnered international attention, similarly pan-resistant strains have been identified in several parts of the world [2, 3]. Where they have not been found, one cannot be confident that they do not exist, as many countries do not have the laboratory capacity to diagnose resistance to second-line tuberculosis drugs.

Globally, the rising rates of multidrug resistance (resistance to the 2 first-line antibiotics, isoniazid and rifampin) are alarming, suggestive of both the extent of the current problem and indicative of the future landscape of increasingly drug-resistant tuberculosis. In China, for example, nearly 6% of new tuberculosis cases are multidrug-resistant. The rates of multidrug resistance in new cases are >20% in many countries in Central Asia and Eastern Europe, where the incidence of multidrug resistance in previously treated patients soars to over 50% [4].

These sobering numbers emerge at a time of great promise in the tuberculosis field. A decade of investment in tuberculosis research is beginning to pay dividends. For the first time in nearly 40 years, several new antibiotics for tuberculosis are in late-stage clinical development [5]. The Global Alliance for TB Drug Development seeks to develop an entirely new first-line antibiotic regimen, one to which there is no circulating drug resistance [6]. In addition, several new molecular diagnostics for tuberculosis have been developed, giving us the potential capacity to rapidly diagnose both tuberculosis disease and resistance to at least 1 or 2 of the current first-line antibiotics (rifampin ± isoniazid) [7, 8].

With the arrival of new tuberculosis drugs and new diagnostic technologies, are there lessons to be learned about the emergence of drug resistance in tuberculosis that should guide future practice? A study by Gao et al [9] in this issue of the *Journal of Infectious Diseases* applies the power of whole genome sequencing technologies to this question. In this work, the authors performed deep sequencing of populations of *M. tuberculosis* bacilli from 3 patients at intervals during failed courses of antibiotic therapy. Instead of isolating and sequencing individual bacteria, the authors took full advantage of the quantitative capacity of new deep sequencing technologies; they sequenced the genomes and identified polymorphisms in any organism present in the sputum at a frequency of >5%. This approach allowed the authors to track the evolution of the bacterial population in these individuals over time at a whole genome level. Thus, they followed the emergence and fixation of the chromosomal mutations known to encode novel drug resistance, as well as other mutations that arose simultaneously. In *M. tuberculosis*, where all drug resistance is chromosomally encoded, this approach provides an unusually detailed picture of the within-host evolution of the pathogen.

Startlingly, the authors found a remarkable amount of genetic diversity in the bacterial populations within a given individual. Resistance to a given drug can arise repeatedly within a single patient. Because resistant strains emerge over and over again, the organism has multiple opportunities to optimize fitness, settling on the mutations that confer the highest level of antibiotic resistance and the lowest fitness cost. In addition, there is sufficient mutational capacity for compensatory mutations to emerge in the context of the most resistant bacilli. Compensatory mutations further improve the fitness of the resistant strain, essentially erasing any tax that a drug-resistance mutation might impose on the bacillus [10, 11].

These are, of course, familiar themes in the field of infectious diseases. In
many infections, there is significant genetic diversification of the pathogen within a host, which gives the organism the capacity to escape drug and immune pressure. What makes this mutational capacity so startling is that it occurs in *M. tuberculosis*. This is an organism that makes a mutational error when replicating its genome at only 2 bases in every 10,000 genomes copied [12] (a rate that is at the low end of the spectrum among bacteria) and, unlike other bacterial pathogens that can become hypermutable when subject to ongoing antibiotic selection, does not seem to modulate its mutation rate. In prokaryotes, mutation is predominately thought to occur during genome replication, yet *M. tuberculosis* replicates at most once every 20 hours and perhaps not at all during latent infection. Finally, *M. tuberculosis* has no capacity for horizontal gene transfer or other quick paths to rapid genetic variation. According to the prevailing assumptions about the mutational capacity of *M. tuberculosis* in vivo, the kind of diversity found by Gao and colleagues should be statistically impossible. Yet the authors quite convincingly show that it is not.

Given the depth of whole genome sequencing, it is also interesting to note the mutations that the authors did not identify in these patients. Although not a focus of this study, the treatment regimens for the patients in this study would have included multiple drugs to which the patients’ isolates were susceptible, and yet the patients failed therapy with strains that were resistant to only a subset of the administered drugs. This is, in fact, a common clinical observation that bears further consideration. *M. tuberculosis* bacilli that are resistant to only a subset of administered antibiotics survive in the face of combination drug therapy.

How does an organism that has not developed genetically encoded resistance to a given antibiotic escape clearance when that drug is administered? We typically blame this on nonadherence—we assume that the patient is not actually taking the drug. Yet new work suggests that the biology of drug responses is likely to be an important determinant of treatment outcome. Within a given individual on treatment, some bacilli might not be exposed to sufficient concentrations of the drug because of rapid drug clearance or uneven drug penetration [13]. In addition, recent studies suggest that a genetically susceptible bacillus can be fully exposed to an antibiotic and not killed by it—a state known as phenotypic drug tolerance. Phenotypically drug-tolerant bacilli can arise in a population of susceptible bacteria through the induction of efflux pumps [14] or through fundamental differences in the physiology of these cells [15]. Importantly, the rate of phenotypic drug tolerance is orders of magnitude higher than the genetic drug resistance rate and may also contribute to the success or failure of drug treatment.

Bacterial population heterogeneity, both genetic and phenotypic, has very practical implications for drug resistance detection. Genetic and phenotypic population heterogeneity poses challenges for both molecular- and culture-based diagnostics. Genotypic heterogeneity must be considered in scoring drug resistances molecularly. These assays will miss phenotypic differences in drug susceptibility entirely. However, culture-based drug susceptibility testing is insensitive to genetic heterogeneity and, as currently implemented, will also miss phenotypic drug tolerance. Culture-based drug resistance testing only yields a qualitative conclusion, that is, resistant or susceptible. Clinical diagnostics laboratories measure minimum inhibitory concentrations for other pathogens, but there are no accepted methods for such quantitative measures in *M. tuberculosis*. Determining both the magnitude of resistance and the underlying heterogeneity might allow us to better understand the outcome of therapy, at least in research settings.

The most important implication of this work is much simpler, however. Gao et al have described the extent to which the bacterial population diversifies and how the most drug-resistant, most fit *M. tuberculosis* bacilli emerge out of this diversity. Can we slow the seemingly inevitable march to totally drug-resistant organisms? It is important to recognize that the absolute number of bacterial variants in an infected individual—and the likelihood of a drug-resistant organism—is directly determined by the size of the bacterial population that the person harbors. Thus, a patient diagnosed and put on treatment who carries a smaller number of bacteria is at significantly lower risk of acquiring drug resistance than a patient diagnosed when he or she harbors a larger bacterial burden. Interventions that lead to earlier treatment should lead to a decrease in the development of resistance.

We can intervene now. Smear microscopy is the most common primary diagnostic technique for *M. tuberculosis* around the world and is mandated in the World Health Organization directly observed treatment short course program. However, smear microscopy is orders of magnitude less sensitive than both culture and the new molecular diagnostics [7, 16]. If the number of bacteria in the sputum reflects the number of bacteria in a patient, those diagnosed via smear are at a hundred-fold to a thousand-fold greater risk of harboring drug-resistant bacilli than those with access to more sensitive diagnostics. Thus, the simplest and most effective way to limit the emergence of novel drug resistances may be to widely implement a sensitive diagnostic at the point of care. We spend a tremendous amount of money and effort to ensure patient adherence to drug therapy. But there are surprisingly few data to support the idea that non-adherence leads to drug resistance [13, 17]. Investing in access to diagnostics may be a much more effective way to stop new drug resistance from developing.

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