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The ins and outs of *Mycobacterium tuberculosis* drug susceptibility testing

E. C. Böttger

Institut für Medizinische Mikrobiologie, Universität Zürich, Zürich, Switzerland

Abstract

Drug susceptibility testing of *Mycobacterium tuberculosis* in the diagnostic laboratory classifies clinical isolates as either drug-'resistant' or drug-'susceptible', on the basis of their ability to grow in the presence of a 'critical concentration' of the test compound. From knowledge of the mechanisms that underlie drug resistance, it has become evident that drug resistance in *M. tuberculosis* is quite heterogeneous and involves low-level, moderate-level and high-level drug resistance phenotypes. Different mutations are associated with different levels of phenotypic resistance, and the acquisition of a genetic alteration leading to a decrease in drug susceptibility does not inevitably exclude the affected compound from treatment regimens. As a result, the simple categorization of clinical *M. tuberculosis* isolates as 'resistant' on the basis of susceptibility testing at 'critical concentrations' may need to be revised and supplemented by quantitative measures of resistance testing to reflect the biological complexity of drug resistance, with the view of optimally exploiting the compounds available for treatment.

Keywords: Molecular mechanisms, resistance, susceptibility testing, treatment, tuberculosis
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Corresponding author: E. C. Böttger, Institut für Medizinische Mikrobiologie, Universität Zürich, Gloriastrasse 30/32, 8006 Zürich, Switzerland
E-mail: boettger@imm.uzh.ch

> Our head is round to allow thought to change direction. *Francis Picabia*

As one of the leading causes of death from curable infectious diseases, tuberculosis (TB) is a serious global health issue. The high rates of TB incidence and prevalence in developing countries have a considerable impact on population-level morbidity and mortality, particularly in settings where human immunodeficiency virus incidence rates are high [1]. The current situation is characterized by an alarming emergence of drug resistance, and much attention has been focused on the burden of multidrug-resistant (MDR) and extended drug-resistant (XDR) TB [2].

In the diagnostic laboratory, drug susceptibility testing of *Mycobacterium tuberculosis* is notably different from standard procedures in clinical microbiology, where a series of drug dilutions is used to determine the minimal drug concentration (the MIC) required to inhibit bacterial growth *in vitro*. Currently established procedures for susceptibility testing of *M. tuberculosis* classify clinical isolates as either drug-'resistant' or drug-'susceptible', on the based of their ability to grow in the presence of a (mostly single) 'critical drug concentration' (Table 1). On the basis of a 1963 WHO document, the critical concentration is defined as the lowest concentration of drug that inhibits ≥95% of wild-type strains of bacilli that have not been exposed to the drug previously. 'Resistance is defined as a decrease in sensitivity of sufficient degree to be reasonably certain that the strain concerned is different from a sample of wild strains of human type that have never come into contact with the drug. This definition is based on laboratory testings; strains that are resistant in this sense do not necessarily fail to respond' [3]. Thus defined, the critical concentration is an epidemiological parameter used to distinguish 'wild-type' strains from 'non-wild-type' strains that are able to grow in the presence of higher drug concentrations; it corre-

TABLE 1. Mycobacterial drug susceptibility testing: the critical concentration

Antimicrobial agent	MIC (mg/L) of susceptible *Mycobacterium tuberculosis*[a]	Concentration (mg/L) in serum[b]	Concentration (mg/L) used for testing[c]	
			Low	High
Isoniazid	0.05–0.2	5–10	0.1	0.4
Rifampin	0.5	10	2	–
Pyrazinamide	20	40–50	100	–
Ethambutol	1–5	2–5	2.5	7.5
Ofloxacin	0.25–0.5	2–10	2	–
Ethionamide	0.5–2.5	2–20	1.25	–
Streptomycin	0.5–1.0	25–50	2	6
Amikacin	0.5–1.0	20–40	1	–
Capreomycin	2–5	10–30	5	–

[a]MIC of wild-type *M. tuberculosis*.
[b]Concentrations 1–4 h after usual dosage.
[c]Drug concentration ('critical concentration') used for testing in the diagnostic laboratory; these concentrations may differ slightly for different media, e.g. BACTEC broth, 7H10 medium, and 7H11 medium.

sponds to what is defined as the epidemiological cut-off [4]. The 'critical concentration' often bears little relationship to the drug concentrations present *in vivo* (Table 1), and its accuracy in predicting clinical failure may, in part, be limited [5–7].

During the past 20 years, significant knowledge has been gained concerning the molecular mechanisms of mycobacterial drug resistance (http://www.tbdreamdb.com). These studies have established, unequivocally, that the chromosomal loci responsible for resistance to various drugs are not linked. Thus, polydrug or multidrug resistance in *M. tuberculosis* is not caused by a single genetic locus, such as upregulation of an efflux pump or induction of a transcriptional regulator, but rather by an accumulation of multiple different mutations. These studies have also established that drug resistance in *M. tuberculosis* is by no means a homogeneous biological entity, but, on the contrary, is quite heterogeneous.

In particular, various levels of phenotypic drug resistance are found in *M. tuberculosis*—low-level, moderate-level and high-level drug resistance—and these different levels of phenotypic drug resistance are associated with distinct genetic mechanisms (Table 2). In general, there is a clear correlation between the genetic mechanism and the resistance phenotype. Thus, mutations in *rpsL* (streptomycin), *rpoB* (rifampin) or 16S rRNA (2-deoxystreptamine aminoglycosides) are associated with high-level drug resistance, whereas mutations in *gldB* (streptomycin), *eis* (kanamycin) and *inhA* (isoniazid) confer a low-level resistance phenotype (Fig. 1). In addition, depending on the specific mutation, an altered resistance

TABLE 2. Mechanisms of drug resistance in *Mycobacterium tuberculosis*

Drug	Gene(s) involved in resistance	Role in resistance	Phenotypic resistance
Isoniazid	*katG*	Prodrug conversion	Moderate to high level (always >1 mg/L) [11,29,30]
	inhA	Drug target	Mostly low level (<1 mg/L) [11,29,30]
Rifampin	*rpoB*	Drug target	Mostly high level, rarely low level (dependent on mutation) [8,9,11,31,32]
Pyrazinamide	*pncA*	Prodrug conversion	Mostly high level [14]
Ethambutol	*embB*	Drug target	Low to moderate level [11,33,34]
Streptomycin	*rpsL*	Drug target	High level [35,36]
	rrs	Drug target	Moderate level [35–37]
	gldB	Drug target	Low level [38]
Kanamycin	*rrs*	Drug target	Mostly high level (dependent on mutation) [39–44]
	eis	Drug	Low level [45,46]
Capreomycin	*rrs*	Drug target	Variable (dependent on mutation) [42,43,47] (F.A. Sirgel, M. Tait, R.M. Warren, E.M. Streicher, N.C. Gey van Pittius, G. Coetzee, P.D. van Helden, E.C. Böttger, E.Y. Hoosain, M. Chabula-Nxiweni, C. Hayes, T.C. Victor, A. Trollip, Unpublished data)
	tlyA	Drug target	Low level [48]
Fluoroquinolones	*gyrA*	Drug target	Low to moderate level (dependent on mutation) [16,17] (F. A. Sirgel, R. M. Warren, P. D. van Helden, E. C. Böttger, manuscript in preparation)
	gyrB		
Ethionamide	*inhA*	Drug target	Low to moderate level [49]
	ethA	Prodrug conversion	Moderate to high level [49]

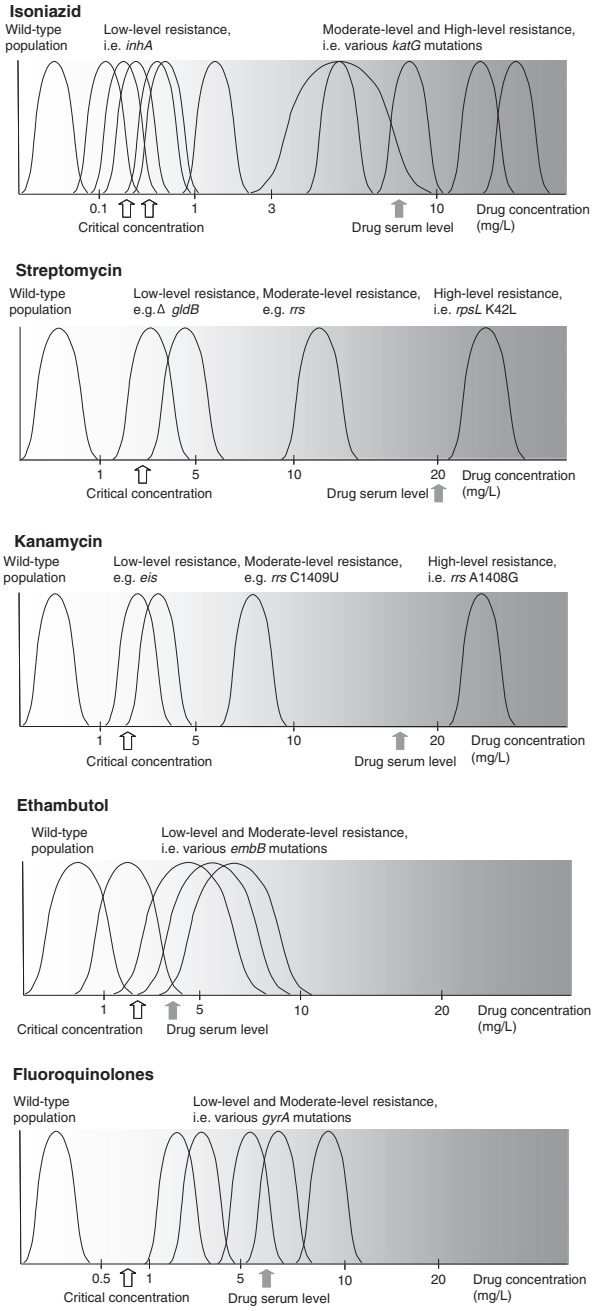

FIG. 1. Schematized changes in drug susceptibility upon mutational alterations—exemplary Gaussian distributions of population profiles. ⇧ = 'critical concentration'; ⬆ = drug serum level.

locus may result in different phenotypes; for example, most mutations in *rpoB* result in high-level rifampin resistance, whereas rare mutations also exist that confer a low-level resistance phenotype [8,9].

Clinical isolates may display a highly restricted number of resistance-conferring chromosomal alterations in a drug target gene. Presumably, this reflects the *in vivo* selection for resistance mutations that maintain gene function, readily explaining the predominance of certain resistance mutations, e.g. RpsL Lys42 → Arg (streptomycin), KatG Ser315 → Thr (isoniazid), and 16S rRNA 1408A → G (kanamycin and amikacin). In contrast, resistance-conferring chromosomal alterations in genes involved in prodrug conversion, e.g. *pncA* and *ethA*, often display a broad diversity, indicating that there is little functional constraint, as a loss of gene function phenotype is apparently well tolerated (for reviews, see [7,10]). Intuitively, a strain's overall genomic background—i.e. nucleic acid sequence polymorphisms and unknown alterations—would be expected to affect the phenotype of a chromosomal resistance determinant. It is therefore perhaps surprising that the resistance level associated with a defined resistance mutation is a rather stable characteristic. Phenotypic resistance heterogeneity reflects the different genetic resistance mechanisms—with each given mutational alteration being associated with a distinct phenotypic resistance level. Significant levels of phenotypic heterogeneity for a given resistance mutation have so far been observed only rarely, e.g. the *katG* Ser315 → T alteration and isoniazid resistance [7,11].

Depending on the Gaussian distribution of MIC levels associated with defined resistance mechanisms, separation into low-level, moderate-level and high-level resistance may be clear-cut with defined boundaries, or more reminiscent of a resistance continuum. Cumulative percentage diagrams can be used to describe the epidemiology of resistance to any specific compound. Such a cumulative percentage plot is the result of two factors: (i) the distribution of MIC levels associated with different resistance mechanisms; and (ii) the frequency of the different resistance mechanisms' presence in the population (Fig. 2). For several anti-TB compounds, including isoniazid, streptomycin, kanamycin, and capreomycin, a low-level resistance phenotype is both defined as a distinct genetic entity and clearly separated by quantitative measures of drug susceptibility from a high-level resistance phenotype. This was first recognized in the mid-1990s for streptomycin and isoniazd [12,13]. Drug concentrations that will overcome this phenotype of decreased drug susceptibility are readily obtained *in vivo* (Table 1), indicating that low-level drug resistance may not correspond to clinical resistance [14,15]. For other anti-TB compounds, such as ethambutol and quinolones, a clear-cut separation into distinct resistance levels, each associated with a distinct genetic resistance mechanism, is not observed in clinical strains. Here, resistance is apparently attributable mostly to a single genetic resistance mechanism affecting the drug target and associated with low to moderate levels of drug resistance—mutations in *embB* (ethambutol) or mutations in *gyrA*

(quinolones), respectively. The clinical implications of *embB* mutations are currently unclear [11]. On the other hand, mutations in *gyrA* typically result in low to moderate levels of quinolone resistance [16,17], and these are presumably sufficient to confer clinical resistance to ofloxacin. However, circumstantial evidence has been provided that, owing to more favourable MICs associated with the corresponding mutations, the later-generation fluoroquinolones, such as moxifloxacin, may still improve treatment outcome [18,19].

Unfortunately, despite the intricacies discussed above, these different levels of phenotypic resistance are not taken into account when 'critical concentrations' are used for mycobacterial *in vitro* drug susceptibility testing. As a result of this procedure, an isolate will be categorized uniformly as resistant in the diagnostic laboratory regardless of whether high-level, moderate-level or low-level drug resistance is present. However, the biological implications of low-level vs. high-level drug resistance are different, for a number of reasons, and not least because *in vivo* drug concentrations need to be taken into account. In other words, the resistance levels determined *in vitro* should be related to the drug concentrations that can be achieved *in vivo*. Peak serum levels of isoniazid, rifampin and aminoglycosides are much higher than the MIC. Conversely, peak serum levels of ofloxacin, ethambutol and ethionamide are close to MIC values. As a result, drug levels may remain subinhibitory for the latter compounds during much of treatment, in particular when mutations that further decrease drug susceptibility are present. Laboratory MIC data and pharmacokinetic (PK) modelling can be used to calculate PK/pharmacodynamic (PD) characteristics and to provide measures of drug bioavailability, such as area under the curve over MIC, the maximum concentration over MIC, and the time above MIC [4,20]. PK/PD characteristics allow us to tentatively suggest breakpoints, but they cannot be directly linked to clinical outcome. In addition, therapy for TB comprises a combination of three or four drugs administered simultaneously. Thus, whereas

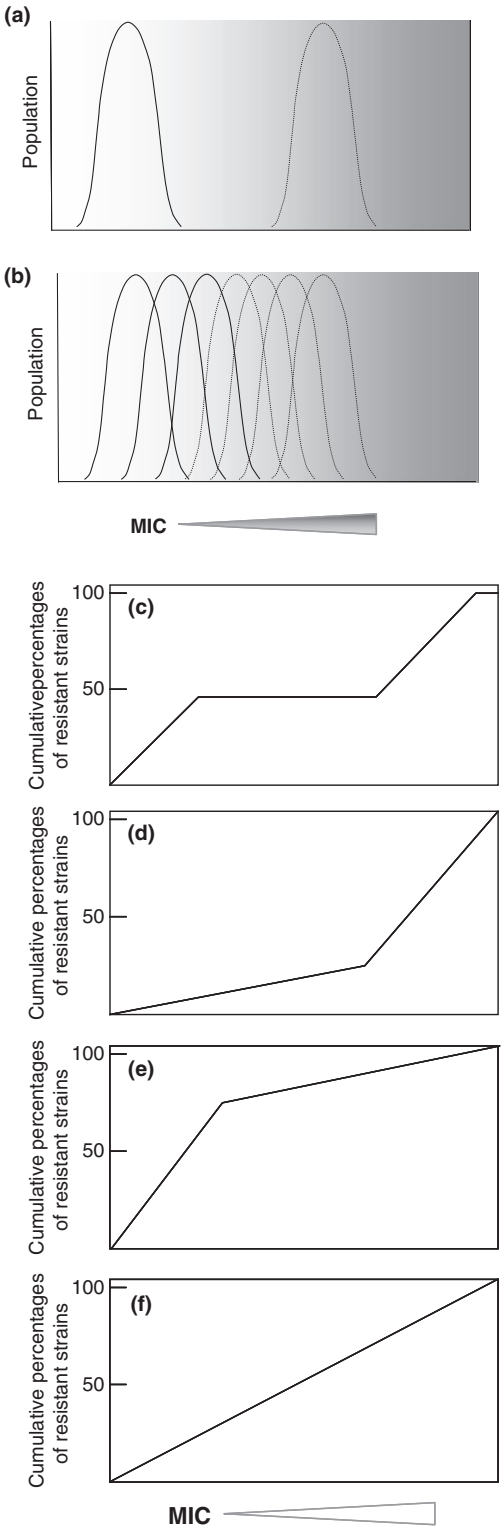

FIG. 2. Schematized drug-resistant populations and cumulative percentage plots of resistant strains. (a) Well-separated populations with low-level (——) and high-level (·····) drug resistance, each associated with a different genetic resistance mechanism. (b) Two different genetic resistance mechanisms (—— and ·····) are associated with different phenotypic resistance levels, but there is not a defined cut-off separating the two. (c–f) Cumulative percentage diagrams of strains with various levels of phenotypic resistance. Scenarios (c)–(f) assume two different mechanisms of resistance, which may (c) or may not (f) be well separated from each other. The cumulative percentage plot is a result of the distribution of MIC levels associated with each resistance mechanism and the frequency of the resistance mechanisms in the population.

in vitro drug susceptibility results apply to each of the drugs in the combination, and not to the entire regimen, the outcome of anti-TB therapy is a composite outcome, owing to the use of multiple drugs. The efficacies of non-antagonistic drugs in combination therapy will be at least additive, effectively reducing the MIC [21]. Thus, depending on PK/PD indices, sufficiently high drug concentrations may be achieved *in vivo*, despite low-level or moderate-level drug resistance *in vitro*.

Standardized treatment regimens for MDR TB are possible, and may result in good clinical outcome. However, treatment of XDR TB is much more problematic [22–26]. For both MDR TB and XDR TB, reliable and robust data on drug resistance are required for correct diagnosis and choice of the proper therapeutic regimen, so as not to result in therapeutic failure, further dissemination, and amplification of resistance [27]. With the global rise in MDR strains, there is an increasing need to determine susceptibility to first-line and second-line anti-TB agents precisely. Treatment of patients with drug-resistant TB should be based on reliable and quantitative measures of susceptibility testing, a cornerstone for preventing further amplification of resistance and for optimally exploiting the available compounds. However, even in the developed countries, only a limited panel of anti-TB drug concentrations is tested, leaving the exact resistance levels of clinical *M. tuberculosis* isolates in part unexplored. In principle, automated systems for cultural propagation of mycobacteria have the potential to meet the challenge of precise determination of drug resistance levels with reasonable labour input. For this purpose, we have recently adopted a fully automated platform by combining commercially available instrumentation (using a fluorescence-based oxygen sensor for growth detection) with software developed by the manufacturer according to our specifications [28]. This procedure provides a fully automated walk-away system for semiquantitative drug susceptibility testing of *M. tuberculosis*, equipped with an expert system for interpretation.

Limitations in mycobacterial drug susceptibility testing were noted as early as the early 1960s, when the principles of the procedures currently in place were first established. 'We consider that the best type of sensitivity test is a fully quantitative determination in which the organisms' capability of growth on medium containing a wide range of drug concentrations is known. This type of test would provide full information on the degree of resistance. However, since such a test requires large amounts of medium and is time-consuming, it cannot be recommended as a routine procedure' [3]. Nevertheless, achieving this aim is more relevant than ever; the emergence and rise of drug-resistant TB has created an urgent need to make optimal use of the available drugs. The consequences of possibly erratic drug susceptibility testing are particularly severe in terms of treatment options for apparent MDR or XDR TB. Following 40 years of proportion-based testing at critical concentrations, and in view of the techniques available today, it may be time to adapt mycobacterial drug susceptibility testing to standard bacteriology procedures. Although critical concentration testing is certainly appropriate for screening and for recognizing any changes in wild-type drug susceptibility, it should be supplemented by measures of quantitative drug susceptibility in cases of test results showing resistance, in particular for those drugs for which heterogeneity in phenotypic resistance is frequently present.

In conclusion, the term 'resistance' with regard to *M. tuberculosis* is by no means a simple homogeneous category, but is quite heterogeneous and frequently composed of low-level, moderate-level and high-level drug resistance. This presumably has important biological implications. It is conceivable that low-level drug resistance, in part, does not correspond to clinical resistance; conversely, in the presence of a high-level resistance phenotype, the drug is of little, if any, clinical benefit. The clinical implications of moderate levels of resistance are less clear, and need to be addressed more fully in future studies taking into account PK/PD parameters. However, changes in our methods for drug susceptibility testing are required to address these issues. Most important are standardized protocols for quantitative drug susceptibility testing of both first-line and second-line drugs as a prerequisite for prospective studies addressing the impact of resistance heterogeneity on treatment results, i.e. by correlating data from quantitative resistance testing with clinical outcome. Given the limited number of drugs available for the management of XDR TB, it is essential to take advantage of those that could possibly be used in a multidrug regimen to treat a significant proportion of corresponding cases.

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