Drug Resistance in *Mycobacterium tuberculosis*: Molecular Mechanisms and Laboratory Susceptibility Testing

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Abstract

Evidence is accumulating that drug resistance in *Mycobacterium tuberculosis* is by no means a homogeneous biological entity. This perception implies limitations of current procedures for in vitro drug susceptibility testing, based on unique but often poorly understood techniques in diagnostic mycobacteriology. The aim of this paper is to briefly review the molecular mechanisms of mycobacterial drug resistance, to critically reflect the principles involved in drug susceptibility testing of *M. tuberculosis* in the diagnostic laboratory and to discuss possible implications for therapy.

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Die Philosophen haben die Welt nur verschieden interpretiert, es kommt darauf an, sie zu verändern.

*Karl Marx, Thesen über Feuerbach (1845)*

The treatment of tuberculosis (TB) faces 3 problems: (1) interruption of further transmission, (2) curing of the acute disease and (3) preventing relapse. Anti-TB drugs were first introduced for TB therapy in the 1940s [1, 2]. However, despite a positive initial clinical response, single-drug therapy resulted in the rapid emergence of drug-resistant strains [3]. By combining the 2 anti-TB drugs available at that time – streptomycin (STM) and para-aminosalicylic acid – the emergence of resistance was reduced to approximately 10% [4]. When isoniazid (INH) was introduced in the early 1950s, and combined with STM and para-aminosalicylic acid, this combination effectively prevented the emergence of resistance but required 18 months of treatment to ensure cure of the disease [5]. Subsequently, pyrazinamide (PZA) [6], rifampicin (RIF) [7] and ethambutol (EMB) [8] were introduced (for an overview of anti-TB agents and drug targets, see table 1). Extensive studies were carried out by the British Medical Research Council to define the optimal drug combination and the minimal duration of therapy [9, 10; for a review, see 11, 12]. The outcome consisted of a therapeutic regimen comprised of an initial 2-month treatment with INH, RIF and PZA, followed by a 4-month treatment with INH and RIF. Combination therapy is necessary for the successful treatment of disease and to avoid the emergence of resistance, while a minimum 6-month treatment is required to prevent relapse [13]. This protocol, further developed and now termed standard therapy short course, is still in use today and is recommended with slight modifications (including the addition of EMB or STM) by the International Union against Tuberculosis and Lung Disease, the World Health Organization and the American Thoracic Society. While drug-resistant TB was reported in the past before the emergence of HIV, the increased susceptibility of HIV patients to disease following infection with *Mycobacterium tuberculosis* has contributed greatly to the current situation.

Genetic Aspects of Drug Resistance

In contrast to other bacterial pathogens, plasmid-mediated mechanisms of resistance are absent in *M. tuberculosis*, but acquired drug resistance is exclusively due to chromosomal alterations such as mutations or deletions. These chromosomal alterations affect either the drug target itself or bacterial enzymes activating/modifying the drug. Drug resistance in *M. tuberculosis* occurs when resistant mutants
that are present naturally in the mycobacterial population are selected out by inadequate or interrupted treatment. Mutants resistant to a single drug occur approximately in every $10^{-6}$ to $10^{-8}$ cells [14]. In theory, the effective presence of a mutant which is resistant to 2 drugs would require a population of $10^{12}$–$10^{16}$ mycobacterial cells. This mathematical concept provides the basis for the successful use of combination drug therapy to prevent the emergence of resistance.

During the past 15 years, significant knowledge has been gained concerning the molecular mechanisms of mycobacterial drug resistance [for a review, see 15–17; see also http://www.tbdreamdb.com]. These studies have unequivocally established that the chromosomal loci responsible for the resistance to various drugs are not linked. Thus, clinical poly- or multidrug resistance in \textit{M. tuberculosis} is not due to a single genetic locus, such as upregulation of an efflux pump or induction of a transcriptional regulator, but rather due to an accumulation of multiple different mutations.

In general, there is a clear correlation between the genetic mechanism and the resistance phenotype. Thus, mutations in \textit{rpsL} (STM), \textit{ropB} (RIF) or 16S ribosomal RNA (KAN, AMK; 2-deoxystreptamine aminoglycosides) are associated with high-level drug resistance, and mutations in \textit{gldB} (STM), \textit{eis} (kanamycin, KAN), and \textit{inhA} (INH) confer a low-level resistance phenotype. Resistance-conferring chromosomal alterations in genes involved in prodrug conversion, for example \textit{pncA} and \textit{ethA}, often display a wide diversity, indicating that there is little functional constraint as a loss of gene function phenotype is apparently well tolerated [for a review, see 18, 19].

Intuitively, a strain’s background – that is, nucleic acid sequence polymorphisms and unknown genetic alterations – would be expected to affect the phenotype of a chromosomal resistance determinant. It is perhaps surprising that the resistance level associated with a defined resistance mutation is a rather stable characteristic. Significant levels of phenotypic heterogeneity for a given resistance mutation have been observed only rarely, for example the \textit{katG} S315T alteration and INH resistance (see below). In addition, resistance to a single drug may involve multiple genetic alterations locating to different genes, as well as multiple genetic alterations within a single gene. Presumably, this accumulation of various resistance mutations, all associated with resistance to a single drug, will either affect (increase) phenotypic resistance [20] or ameliorate the fitness cost associated with a defined resistance mutation [21, 22].

### Principles of Drug Susceptibility Testing in the Laboratory

During the 1950s, the establishment of laboratory methods for \textit{M. tuberculosis} drug susceptibility testing was a tremendous challenge. At that time, when diagnostic procedures for drug susceptibility testing of bacteria were largely unexplored, sensitivity and resistance in \textit{M. tuberculosis} were defined as follows: ‘sensitive’ strains are those that have never been exposed
to anti-TB drugs (‘wild’ strains); ‘resistant’ strains are those that differ from sensitive strains in their capacity to grow in the presence of higher concentrations of the drug [23]. Fortuitously, it was found that drug-susceptible strains of *M. tuberculosis* that have not been exposed to anti-TB drugs (wild-type strains) do not exhibit much variation in minimum inhibitory concentrations (MICs) to those drugs. Depending on which laboratory method was used for susceptibility testing, significant differences were found in the drug concentrations which discriminate most efficiently between susceptible wild-type strains and probably resistant strains. For example, with proportion testing of STM a maximum discrimination was achieved with a resistance proportion of 1% on 4 mg/l dihydro-STM, while with the absolute-concentration method maximum discrimination was at 16 μg/ml [23].

Current procedures for drug susceptibility testing of mycobacteria are characterized by 2 peculiarities: (1) the ‘critical concentration’ and (2) the ‘critical proportion’ [24]. The drug concentration which categorizes a clinical *M. tuberculosis* isolate as either susceptible or resistant is defined as the concentration that inhibits the growth of wild-type strains and probably resistant strains. For example, with proportion testing of STM a maximum discrimination was achieved with a resistance proportion of 1% on 4 mg/l dihydro-STM, while with the absolute-concentration method maximum discrimination was at 16 μg/ml [23].

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Fig. 1. Critical concentration and proportion testing: drug susceptibility of a wild-type bacterial population follows a gaussian distribution.

<table>
<thead>
<tr>
<th>Drug concentration (mg/l)</th>
<th>Number of bacilli in wild-type strains able to grow in the presence of indicated drug concentration</th>
<th>Critical proportion of resistance %</th>
</tr>
</thead>
<tbody>
<tr>
<td>INH 0.2</td>
<td>$1 \times 10^{-4}$</td>
<td>1</td>
</tr>
<tr>
<td>INH 1.0</td>
<td>$1 \times 10^{-5}$</td>
<td>0.1</td>
</tr>
<tr>
<td>STM 4.0</td>
<td>$1 \times 10^{-3}$</td>
<td>10</td>
</tr>
<tr>
<td>STM 8.0</td>
<td>$1 \times 10^{-5}$</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Fraction of population against drug concentration.

- in resistant strains the *proportion* of such bacilli is considerably higher than in sensitive strains’ [25]. The drug susceptibility of a bacterial wild-type population follows a gaussian distribution. Thus, depending on the drug concentration, a small fraction of the population will show phenotypic resistance (fig. 1). This observation forms the basis for combining proportion testing and ‘critical concentration’ (table 2).

Standardization of the ‘critical concentration’ has not been without controversy. For example, the recommended concentrations for EMB underwent adjustments over time [26, 27], not the least because of low interlaboratory reproducibility of EMB susceptibility testing [28]. Most likely, this is due to the very small differences between the concentration used for in vitro drug susceptibility testing and the natural drug susceptibility of wild-type isolates of *M. tuberculosis* (table 3). Thus, minute changes in drug susceptibility will have a major impact on interpretation of in vitro test results, with only a narrow range between the MICs of susceptible isolates and resistant isolates.

Among mycobacteriologists a misperception of ‘critical proportion’ and clinical resistance frequently prevails. While the critical proportion of cells (subpopulation) able to grow in the presence of the critical concentration is mostly defined as equal or greater than 1% of the population (1 in 100) [24], the frequency of mutational resistance is much lower, approximately $10^{-7}$ (i.e. 0.000001%, or 1 in ten million).
It is, however, the mutational resistance which is responsible for treatment failure and for the emergence of resistance following inappropriate drug regimens. The critical proportion of resistance is a technical term and should not be confused with mutational resistance. In combination with the critical concentration, the critical proportion is a laboratory term used in in vitro drug susceptibility testing to define the epidemiological cut-off.

### Isoniazid

Isonicotinic acid hydrazide (INH) is a highly specific antimycobacterial agent, being exquisitely potent against the *M. tuberculosis* complex. INH is a prodrug which needs to be converted into its active form by the bacterial enzyme KatG, a catalase-peroxidase [29]. Biochemical and genetic studies suggest that activated INH targets InhA [30], an enoyl-acyl carrier protein reductase, which takes part in fatty acid biosynthesis. INH resistance is genetically heterogeneous and may involve mutations in *inhA* and/or *katG* [31–34]. While mutational alterations in *inhA* mainly affect the gene’s promoter resulting in InhA overexpression and are associated with low-level resistance to INH, mutations in *katG* confer moderate- to high-level drug resistance (fig. 2). The clinically most prevalent moderate- to high-level resistance mutation is the serine → threonine exchange at amino acid position 315 of KatG; this single genetic alteration accounts for more than 60–80% of INH resistance due to *katG* mutations [35, 36]. Of note, the KatG S315T replacement is associated with a surprisingly heterogeneous phenotype of resistance [37, 38] – all clinical strains with this mutation are able to grow in the presence of at least 1 mg/l INH; however, the exact MIC values may range from 2 to >10 mg/l INH (fig. 3).

More recently, it has been suggested that the strain genetic background may impact on the type of INH resistance mutation associated with a particular isolate, explaining why different phylogenetic lineages of *M. tuberculosis* may be associated with different resistance mutations [39]. However, several words of caution are necessary here. (1)
Fig. 2. INH resistance: molecular mechanisms and phenotypic drug susceptibility. **a** Molecular mechanisms and phenotypic resistance level. **b** Schematized changes in drug susceptibility – exemplary gaussian distributions of a population’s drug susceptibility.

### Table: Genes involved in resistance

<table>
<thead>
<tr>
<th>Genes</th>
<th>Role in resistance</th>
<th>Phenotypic resistance</th>
<th>Frequency in clinical strains</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>katG</em></td>
<td>prodrug conversion</td>
<td>moderate- to high-level (&gt;1 mg/l)</td>
<td>70–80%</td>
</tr>
<tr>
<td><em>inhA</em></td>
<td>drug target</td>
<td>low-level (&lt;1 mg/l)</td>
<td>20–30%</td>
</tr>
</tbody>
</table>

**a** Frequency based on clinical strains categorized as resistant as per critical concentration testing.

![Image](image-url)

### Fig. 3. Phenotypic heterogeneity in INH-resistant clinical isolates with the *katG* S315 mutation. Drug susceptibility was determined using MGIT 960 instrumentation equipped with the EpiCenter TBeXiST software. As per proportion testing, the bacterial inoculum for the drug-free growth control is 1% of the inoculum used for the drug-containing vial. The drug-containing vial is incubated an additional 7 days following positivity of the drug-free growth control to facilitate quantitation of resistance, both at a drug concentration and at a population level. The term ‘intermediate’ indicates that a drug concentration significantly inhibits bacterial growth, although not completely. For example, as indicated in **d**, 3.0 mg/l INH inhibits the growth of more than 99.9% of the population of isolate 176291. Drug concentrations are represented by colours as follows: blue = 0.1 mg/l; green = 1.0 mg/l; yellow = 3.0 mg/l; red = 10.0 mg/l; black = drug-free growth control; GU = growth units. The figure is taken from Springer et al. [38], with permission of the publisher. **a** Isolate 177836: resistant at 0.1, 1.0, 3.0 and 10.0 mg/l. **b** Isolate 186137: resistant at 0.1, 1.0 and 3.0 mg/l and intermediate at 10.0 mg/l. **c** Isolate 186069: resistant at 0.1, 1.0 and 3.0 mg/l and susceptible at 10.0 mg/l. **d** Isolate 176291: resistant at 0.1 and 1.0 mg/l, intermediate at 3.0 mg/l and susceptible at 10.0 mg/l.
In vitro laboratory generation of INH-resistant mutants by selection for spontaneous resistance cannot be used to faithfully reconstruct clinical resistance, as the resistant mutants selected in vitro do not reflect the in vivo mechanism of INH resistance [40]. In addition, the unique strain-specific phenotypic heterogeneity for a given INH resistance mutation (see above) points to methodological problems in choosing the appropriate drug concentration for in vitro selection. (2) Strain-specific differences in resistance-associated mutations may reflect geographic-specific differences in the way drug susceptibility testing is performed in the diagnostic laboratory rather than differences in strain genetic background. For example, in one study from New York 20% of INH resistance was associated with mutations in \textit{inhA}, while this was less than 1% in a study from the Russian Federation [34, 41]. However, this difference does not necessarily reflect an impact of a strain genetic background, but more likely differences in drug susceptibility testing (table 4). Thus, in Europe and in North America the established procedure is proportion testing using INH at a concentration of 0.1 mg/l. However, in Russia the established procedure is absolute concentration testing using INH at a concentration of 1.0 mg/l. Limiting the New York study to strains with INH resistance >0.4 mg/l changes the results significantly. Now, the data between the two studies become virtually identical with the vast majority of INH resistance (>85%) due to mutations in \textit{katG}. Mutations in \textit{inhA} are not associated with INH resistance in Russia, presumably not because of differences in strain genetic background, but more likely because of the procedures used in the diagnostic laboratory to define resistance.

### Rifampicin

RIF is not specific for mycobacteria but affects many bacteria by interacting with the RNA polymerase β-subunit and preventing transcription. Clinical RIF resistance is mostly high-level resistance and associated with distinct mutations in \textit{rpoB} [42, 43] (fig. 4). Resistance mutations cluster in a region comprising amino acids 500–540 in \textit{RpoB}. While there is some heterogeneity in the type of replacement and the position of the amino acid affected, e.g. positions 531 and 526, the 531 serine → leucine replacement is the most prevalent alteration, accounting for more than 50% of clinical RIF resistance.

### Ethambutol

EMB is thought to act on the mycobacterial cell wall, with arabinan synthesis as the primary site of action [44, 45]. As discussed above, drug susceptibility testing for EMB is particularly problematic [27]. Several reasons may account for this, e.g. the bacteriostatic nature of EMB and the reduced activity of the drug in culture medium. Most important, however, is the small difference between the drug concentration used for in vitro drug susceptibility testing and the natural drug susceptibility of wild-type isolates of \textit{M. tuberculosis} (table 3). Thus, minute changes in drug susceptibility will have a major impact on the interpretation of the in vitro test result, with only a narrow range between MICs of susceptible and MICs of resistant isolates of \textit{M. tuberculosis}. Acquired EMB resistance is associated with mutations

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**Table 4. Are different phylogenetic lineages associated with different resistance mutations?**

<table>
<thead>
<tr>
<th>Study</th>
<th>INH-resistant isolates</th>
<th>Mutation \textit{katG}</th>
<th>Mutation \textit{inhA}</th>
<th>No mutation</th>
<th>Laboratory drug susceptibility testing</th>
</tr>
</thead>
<tbody>
<tr>
<td>New York State, USA [29]</td>
<td>92 (100)</td>
<td>51 (55)</td>
<td>20 (22)</td>
<td>21 (23)</td>
<td>proportion method 0.1 mg/l</td>
</tr>
<tr>
<td></td>
<td>35 (100)</td>
<td>2 (6)</td>
<td>18 (52)</td>
<td>15 (42)</td>
<td>proportion method &lt;0.4 mg/l</td>
</tr>
<tr>
<td></td>
<td>57 (100)</td>
<td>49 (86)</td>
<td>2 (4)</td>
<td>6 (10)</td>
<td>proportion method &gt;0.4 mg/l</td>
</tr>
<tr>
<td>Various geographical regions, Russian Federation [37]</td>
<td>319 (100)</td>
<td>287 (89)</td>
<td>3 (1)</td>
<td>29 (10)</td>
<td>absolute concentration method 1.0 mg/l</td>
</tr>
</tbody>
</table>

Data are expressed as numbers, with percentages in parentheses.

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in *embB*, in particular codon 306 [46–49]. However, these mutations confer only a small increase in MIC (2- to 4-fold; fig. 5). The clinical implications of laboratory-assessed phenotypic resistance are unclear at present, as is the finding of an *embB* mutation.

**Pyrazinamide**

PZA is a nicotinamide analogue, which is converted into its active form by the bacterial pyrazinamidase PncA. While the drug target is still unclear, clinical resistance is mostly associated with various mutations, including non-sense mutations, in *pncA* [50–54]. The testing of PZA is problematic. PZA is a unique and unconventional antibiotic that is not active in vitro under normal culture conditions [55]. A paradox limiting analysis of PZA resistance is that the critical concentration used for in vitro testing largely exceeds the drug concentrations present in vivo (table 3). While the conditions established for PZA testing in vitro are highly artificial, they allow for recognition of resistance – in particular in combination with a determination of pyrazinamidase activity. The quantitative analysis of resistance levels, for example low-level versus high-level resistance, is not possible with the procedures currently in place. However, as PZA is ineffective against *Mycobacterium bovis*, which is naturally *pncA*-, it has to be assumed that *pncA* negativity confers high-level drug resistance – at least in clinical terms.