Antibiotic Susceptibility Testing of Tuberculous Bacilli

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- **Laboratory drug susceptibility testing**
- **Clinical implications**

… some “complex” examples for illustration

"… 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."

Mitchison, 1962

☞ **Definition of an epidemiological cut-off (ECOFF)**

"… Strains that are resistant in this sense do not necessarily fail to respond."

Canetti, 1969

Pecularities of Mycobacterial Drug Susceptibility Testing (DST)

1. **Critical proportion**
   (proportion method)

2. **Critical concentration**
   (drug concentration)

The principles of M. tuberculosis drug susceptibility testing have been established in the early 60‘s – they have not changed much since and we need to ask whether these procedures are still adequate.

The Proportion Method

Principle of the method

"All strains of tuberculosis contain some bacilli that are resistant to antibacillary drugs - in resistant strains, the *proportion* of such bacilli is considerably higher than in sensitive strains."

Drug	Concentration (µg/ml)	Critical Proportion for Resistance %
Isoniazid	0.1	1
Rifampin	1.0	1
Pyrazinamid	100	10
Ethambutol	5.0	1
Streptomycin	2.0	1

Canetti et al. Bull. WHO 1969, 41: 21-43

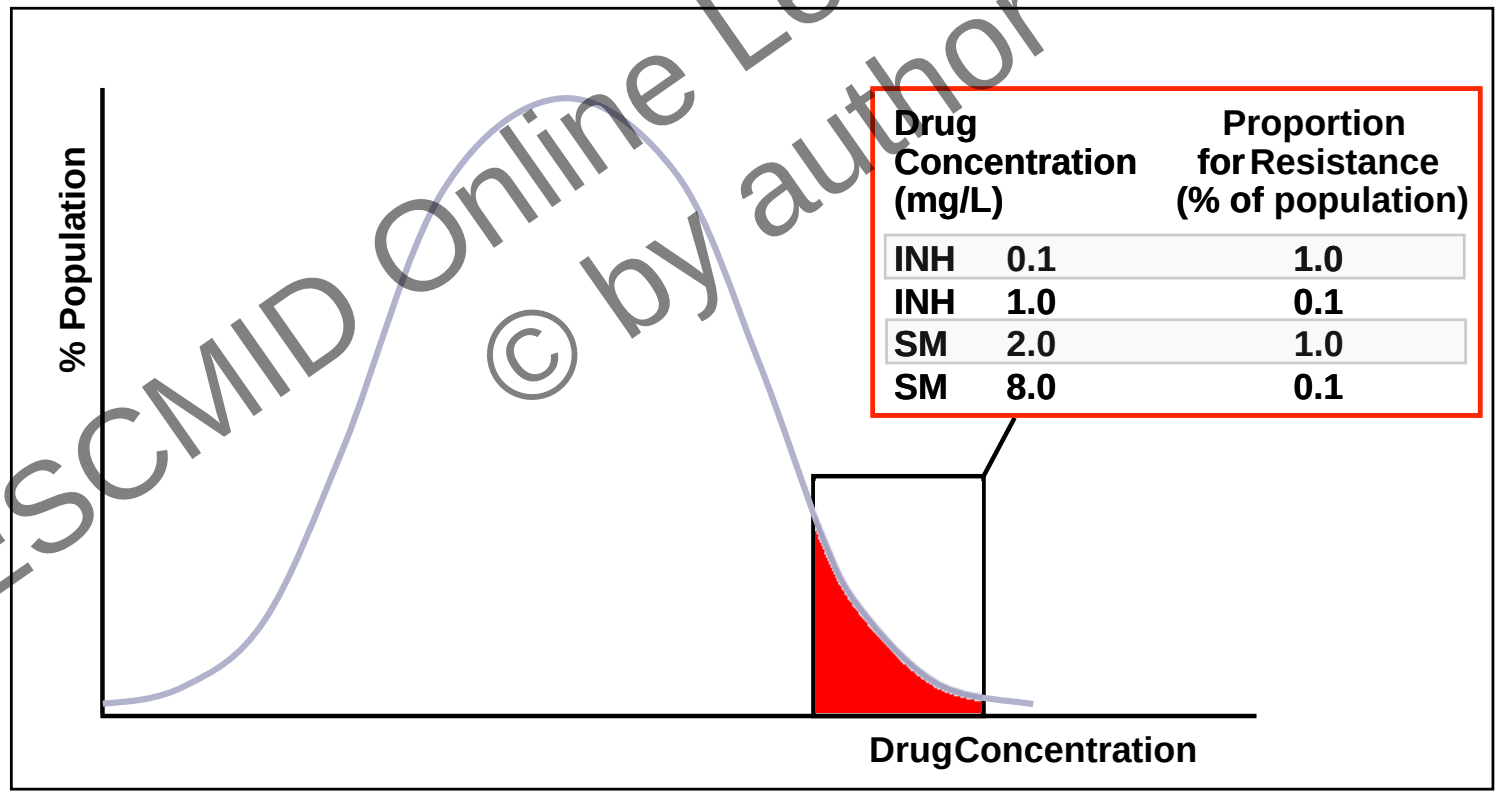
The Critical Concentration

☞ **mostly a single drug concentration (termed critical concentration) is used to define susceptibility vs. resistance**

Antimicrobial agent	MIC (µg/ml) of susceptible *M. tuberculosis*	Crit Con (µg/ml) MGIT	Conc (µg/ml) in serum
INH	0.05 - 0.1	0.1	7
RMP	0.5	1.0	10
PZA	20	100	45
EMB	1 - 5	5.0	2 - 5
SM	1	2.0	25 - 50

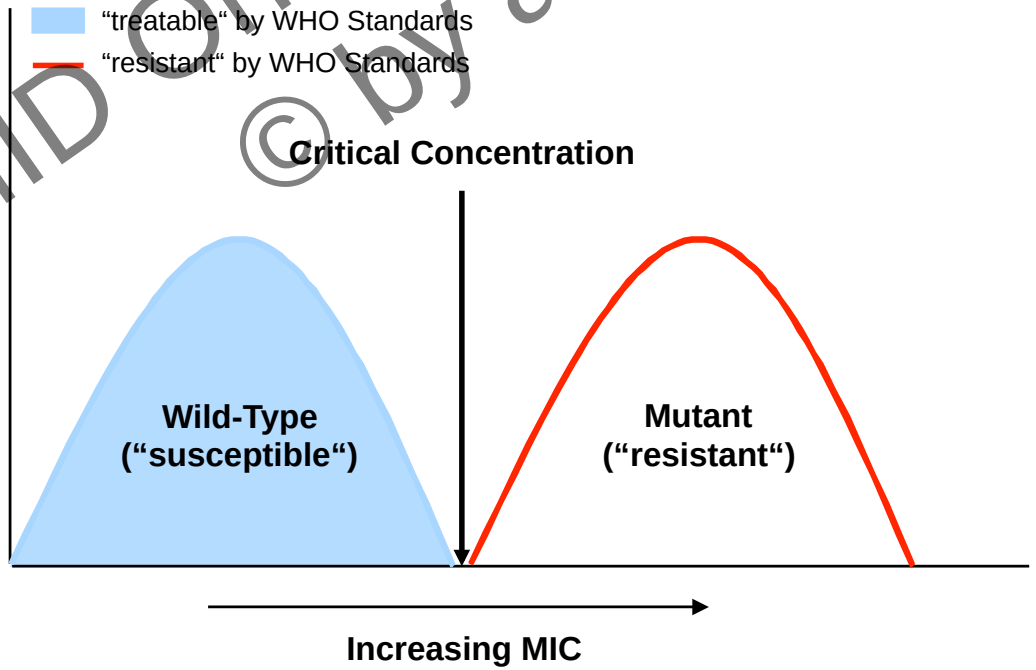
Proportion Method and Critical Drug Concentration

Wild Type Population

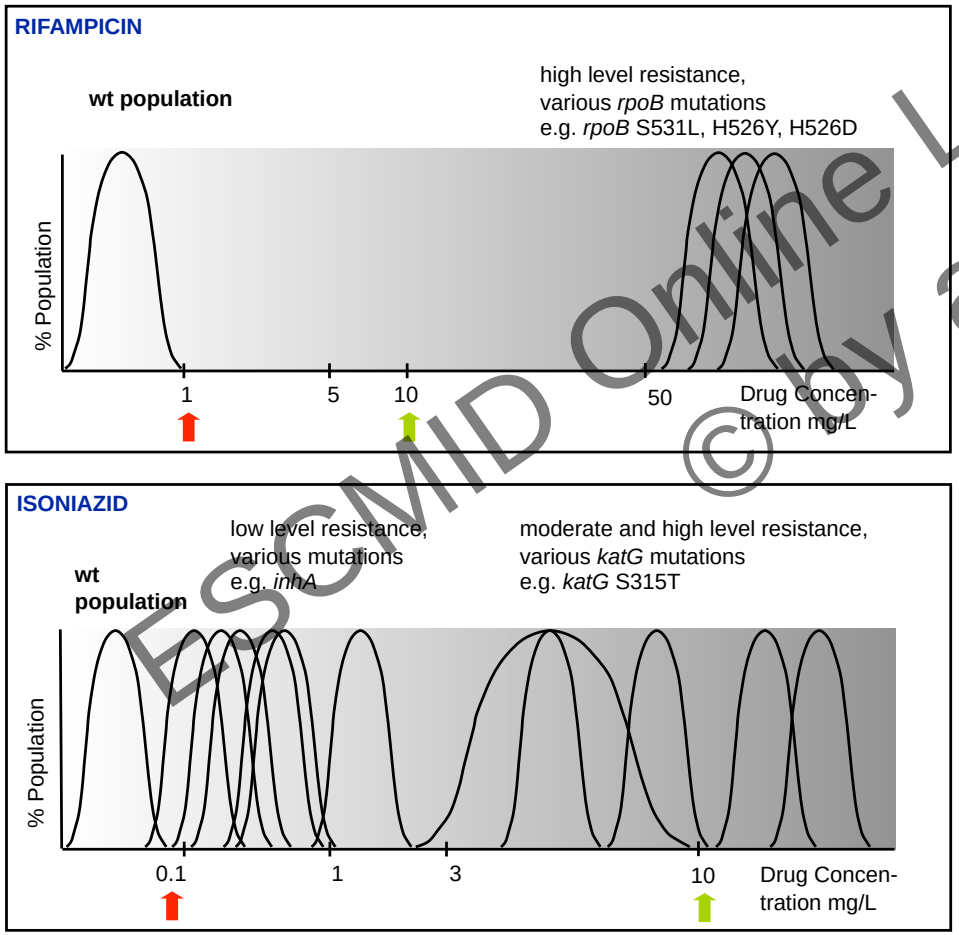


The Current Perception of *M. tuberculosis* Drug Resistance

Hypothetical distribution of MICs in wild-type and mutant *M. tuberculosis* isolates showing the proportion of isolates considered treatable and those determined to be resistant by critical concentration testing



Genetic alterations and phenotypic resistance



Schematized changes in drug susceptibility upon mutational alterations – exemplary Gaussian distributions of a population's drug susceptibility

Rifampicin: mostly high-level drug resistance associated with mutations in *rpoB*.

Isoniazid: various levels of phenotypic resistance – low-, moderate-, and high-level drug resistance; different phenotypic resistance levels are associated with distinct mutations in different chromosomal loci; in addition, a given resistance mutation may be associated with variable levels of phenotypic drug resistance.

⬆ = "critical concentration"
⬆ = drug serum level

from Böttger, E.C. and Springer, B. 2009, Mycobacterium tuberculosis: drug resistance and genetic mechanisms – facts, artifacts and fallacies. In: HIV and tuberculosis: a deadly liaison, eds. S.H.E. Kaufmann and B. Walker, Wiley VCH, p. 103-121

Low-Level Drug Resistance in *M. tuberculosis* is frequently observed in clinical isolates

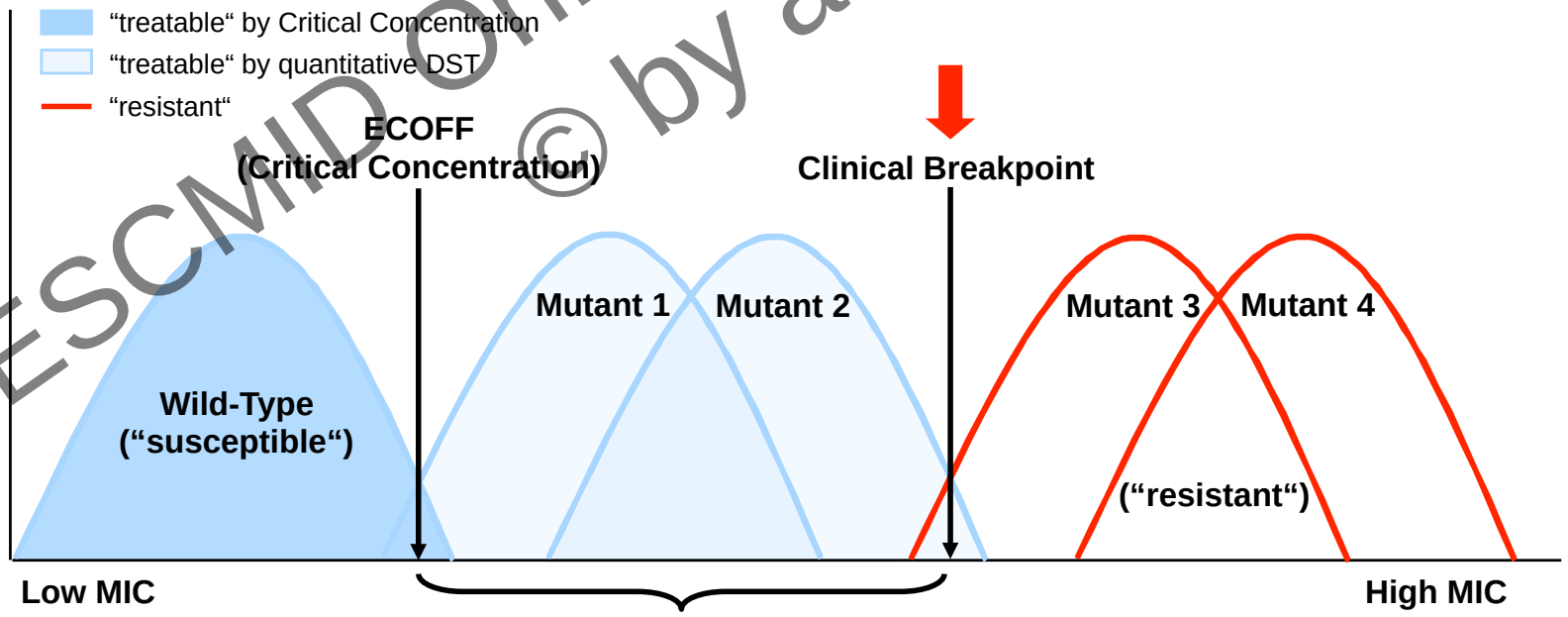
Drug	Resistance mechanism	Frequency in resistant clinical isolates
Isoniazid	inhA	20-30%
Streptomycin	gidB	20-30%
Capreomycin	tlyA, rrs	>50%
Kanamycin	eis	20-30%

☞ **Low-level drug resistance makes up a significant part of drug resistant *M. tuberculosis***

Banerjee et al. Science 1994, 263: 227
Meier et al. Antimicrob. Agents Chemother. 1996, 40:2452
Okamoto et al. Mol. Microbiol. 2007, 63: 1096
Johansen et al. Mol. Cell 2006, 23: 173
Zaunbrecher et al. Proc. Natl. Acad. Sci. USA 2009, 106: 20004
Telenti et al. Nat. Med. 1997, 3: 567

The Real World of *M. tuberculosis* Drug Resistance – different genetic mutations are associated with different resistance levels

Hypothetical distribution of MICs in wild-type and mutant *M. tuberculosis* isolates showing the proportion of clinical isolates considered clinically treatable (< clinical breakpoint) and those determined to be resistant by quantitative DST



Lost opportunity for treatment if using critical concentration only

… Some "Complex" Examples for Illustration

- "Known unknowns of drug susceptibility testing"
  - Ethambutol

- "Phenotypic heterogeneity of mutational alterations"
  - Rifampicin / Rifabutin

- "Pharmacokinetics, MICs and resistance development"
  - Fluoroquinolones

Ethambutol Drug Susceptibility Testing and Resistance Mechanisms – the known unknown

MIC wild-type	**1.25 – 5.0 mg/L**
Critical Concentration	**5.0 mg/L**
Drug Serum Levels	**2.0 – 5.0 mg/l**

- the only small difference between MIC of susceptible wild-type bacteria and the drug concentration used for in-vitro testing makes in-vitro test results in part unreliable, reliability of phenotypic test procedures is method dependent.

MIC *embB* mutants **5.0 – 12.5 mg/L**

- mutations in *embB* (aa 306) confer low-level drug resistance, i.e., a 3-4 fold increase in MIC.
  → dependent on the method used and the critical concentration chosen overlapping MIC distributions for wt and *embB* mutant strains, i.e., "susceptible" isolates may carry an *embB* mutation and "resistant" isolates may show a wild-type *embB.*

Starks et al. Antimicrob. Agents Chemother. 2009, 53: 1061-1066
Safi et al. Antimicrob. Agents Chemother. 2010, 54: 103-108
Plinke et al. Antimicrob. Agents Chemother. 2011, 2891-2896
Sirgel et al. Chemother. 2012, 58: 358-363

- **no data exist correlating in-vitro test results with in-vivo outcome**

RpoB Mutations in *M. tuberculosis*: MICs and Relative Resistance to Rifampicin and Rifabutin – phenotypic heterogeneity of mutational alterations

		Rifampicin CC 1.0 mg/L			Rifabutin CC 0.5 ug/ml		
Isolates (n)	**rpoB Mutation**	**MIC mg/L**	**RR**		**MIC mg/L**	**RR**	
26	wild-type	0.5	-	**S**	0.06	-	**S**
1	D516T	5.0	10	**R**	0.125	2	**S**
4	D516S	5.0-15.0	10-30	**R**	0.125-0.25	2-4	**S**
29	D516V	10.0-15.0	20-30	**R**	0.125-0.25	2-4	**S**
20	S531L	>10.0	>20	**R**	>1.0	>16	**R**

Cavusoglu et al. Clin. Microbiol. Infect. 2004, 10: 662-665
Lew et al. Tuberc. Respir. Dis. 2005, 59: 257-265
Zaczek et al. BMC Microbiol. 2009, 9:10, doi: 10.1186/1471-2180-9-10
van Ingen et al. Int. J. Tuberc. Lung Dis. 2011, 15: 990-992
Sirgel et al. PLoS One 2013, 8:e59414

Can we Explain how Quinolone Resistance Rapidly Developed in MDR, Despite DOTS Plus – pharmacokinetics, MICs and resistance development

- frequent observation in Eastern Europe and South Africa
- numerous hypotheses have been put forward

GyrA	MIC mg/L					C_{max} (mg/L)
	wt	A90V	D94A	D94N	D94G	
Ofloxacin	0.5-1.0	4.0-8.0	4.0-8.0	4.0-8.0	6.0-12.0	2.0-4.0
Moxifloxacin	0.125-0.25	1.0	1.0	1.0	2.0-4.0	3.0-5.0

Kam et al. Microb. Drug Res. 2000, 12: 7-11
Poissy et al. Antimicrob. Agents Chemother. 2010, 54: 4765-4771
Sirgel et al. J. Antimicrob. Chemother. 2012, 67: 1088-1093

☞ **Ofloxacin results in rapid resistance development**
- **MIC of wild-type is close to in-vivo drug concentrations**
- **Single-step mutational alterations confer clinical resistance**

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Quantitative Drug Susceptibility Testing of *Mycobacterium tuberculosis* by Use of MGIT 960 and EpiCenter Instrumentation▽

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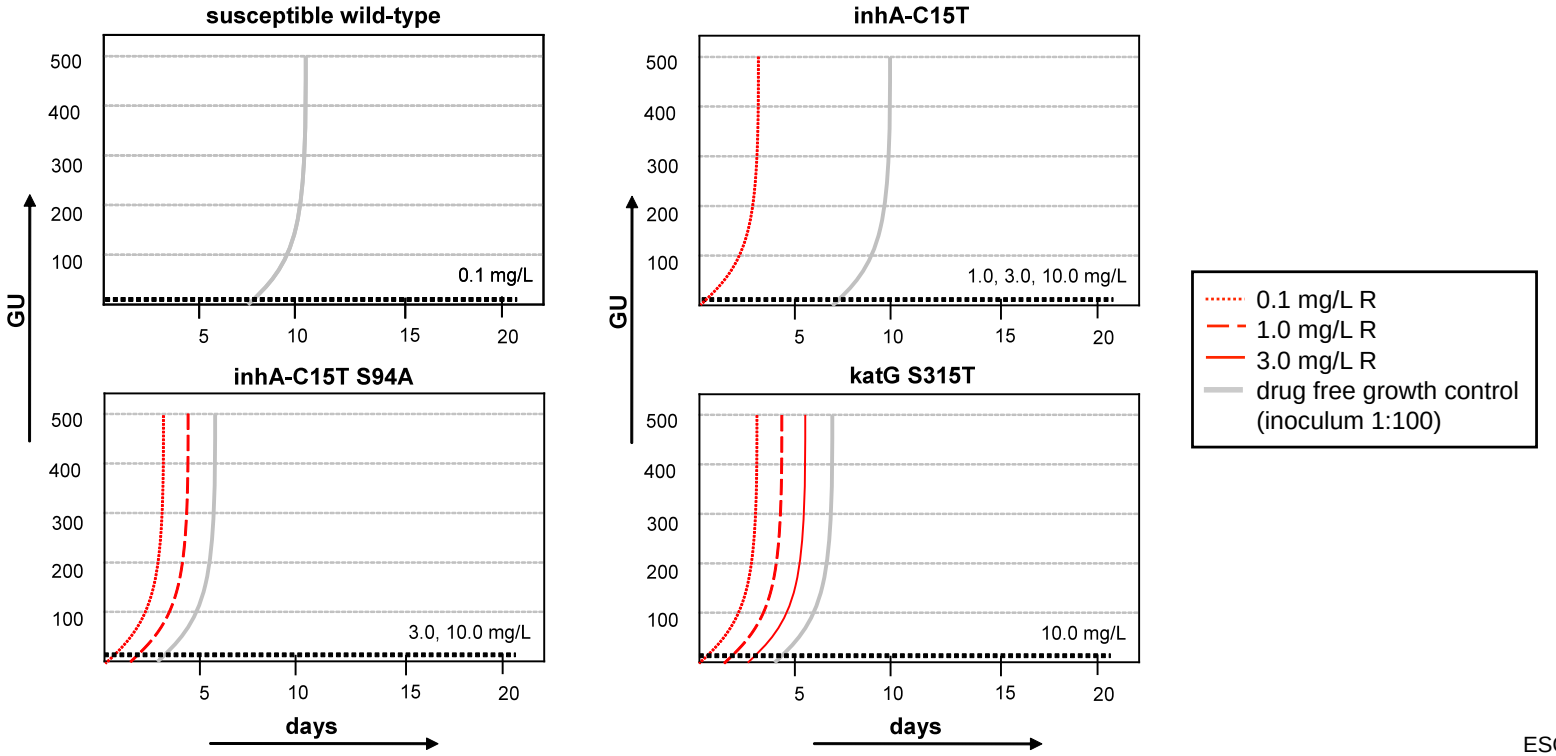
Since numbers of drug-resistant *Mycobacterium tuberculosis* strains are on the rise, the simple classification into "susceptible" and "resistant" strains based on susceptibility testing at "critical concentrations" has to be reconsidered. While future studies have to address the correlation of phenotypic resistance levels and treatment outcomes, a prerequisite for corresponding investigations is the ability to exactly determine levels of quantitative drug resistance in clinical *M. tuberculosis* isolates. Here we have established the conditions for quantitative drug susceptibility testing for first- and second-line agents using MGIT 960 instrumentation and EpiCenter software equipped with the TB eXiST module. In-depth comparative analysis of a range of well-characterized susceptible and resistant clinical isolates has allowed us to propose conditions for testing and to develop criteria for interpretation.

M. tuberculosis Semi-Quantitative Drug Susceptibility Testing – phenotypic heterogeneity of isoniazid resistance mutations

Molecular Resistance Mechanism	ECOFF / Critical concentration 0.1 mg/L	Semi-quantitative susceptibility testing 1.0 mg/L	3.0 mg/L	10.0 mg/L	Isoniazid
wt	**S**				**susceptible**
inhA-C15T	**R**	**S**	**S**	**S**	**low-level**
inhA-C15T S94A	**R**	**R**	**S**	**S**	**moderate-level**
katG S315T	**R**	**R**	**R**	**S/R**	**high-level**



European Study Group – EU-QDST

M. tuberculosis Quantitative Drug Susceptibility Testing

Scientific Objectives:

- Establish and standardize laboratory conditions for semi-quantitative drug susceptibility testing (qDST) of *Mycobacterium tuberculosis*, i.e., detection of resistance and assessment of susceptibility at a quantitative level for first- and second-line anti-tuberculous drugs
- Correlate results from qDST with molecular resistance mechanisms

Study Design:

- Mulit-center study of European expert labs

Methods:

- Consensus protocol for qDST using MGIT 960 / TBeXiST
- Molecular analyses as established in the individual participants laboratory, e.g. line-probe assay, sequencing

European Study Group – EU-QDST

M. tuberculosis Quantitative Drug Susceptibility Testing

MDR/XDR Isolates 2009/2010

Switzerland
France
Portugal
Germany
Italy
Sweden
Netherlands
Belgium
Spain

- **drugs tested R at screening concentration (ECOFF) are subjected to qDST**
- **for MDR test RBT, MOX, OFX, AMK, CAP, ETH, SM, PAS, LIN**

Study Protocol

Drug	qDST Concentrations mg/L	Molecular Target
INH	0.1*, 1.0, 3.0, 10.0	katG, inhA
RIF	1.0, 4.0, 20.0	rpoB
RBT	0.1, 0.4, 2.0	rpoB
EMB	2.5, 12.5, 50.0	embB
PZA	100	pncA
MOX	0.25, 0.5, 2.5, 7.5	gyrA
OFX	1.0, 2.0, 10.0	gyrA
AMK	1.0, 4.0, 20.0	rrs, (eis)
CAP	2.5, 5.0, 25.0	rrs, (tlyA)
ETH	5.0, 10.0, 25.0	inhA, ethA
SM	1.0, 4.0, 20.0	rpsL, rrs, (gidB)
PAS	4.0, 16.0, 64.0	-
LIN	1.0, 4.0, 16.0	rrl, rplV

*screening concentrations are underlined (critical concentration, ECOFF)

European Study Group – EU-QDST

M. tuberculosis Quantitative Drug Susceptibility Testing

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Mycobacterial Drug Susceptibility Testing – The Limitations of Current Procedures

Drug Resistance in *M. tuberculosis* is a mixed bag: significant hetero-geneity is present, i.e., low-, moderate- and high-level drug resistance.

- ☞ Regardless of low-, moderate-, or high-level drug resistance any corresponding clinical isolate is categorized as resistant in the diagnostic laboratory – due to the procedure of “critical concentration“ testing. However, the biological impact of low- versus high-level drug resistance is to be different.
  - drug concentrations present in-vivo
  - tuberculosis chemotherapy is a combination therapy of compounds which act in part in an additive / synergistic fashion and which includes agents targeting cell wall synthesis
- ☞ **The critical concentration corresponds to the ECOFF (epidemiological cut-off) value, rather than a clinical breakpoint.**

Mycobacterial Drug Resistance and Susceptibility Testing

- The clinical implications of any laboratory resistance presumably are to a large extent dependent on quantitative levels of resistance, i.e., low-level drug resistance may not correspond to clinical resistance.

- A working group of European expert laboratories has been established to standardize laboratory conditions for semi-quantitative drug susceptibility testing of *Mycobacterium tuberculosis.*

- qDST will provide an important means to guide chemotherapy of drug resistant *M. tuberculosis* infections.

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