Resistenz in Tuberkulose-bakterien und diagnostische Empfindlichkeitsprüfung: Fakten und Irrtümer

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Resistenz in Tuberkulosebakterien und diagnostische Empfindlichkeitsprüfung: Fakten und Irrtümer

- **Grundlagen und Mechanismen der Antibiotikaresistenz in Tuberkulosebakterien**
- **Antibiotische Empfindlichkeitsprüfung im Labor**
- **Klinische Implikationen**

… einige Beispiele zur Veranschaulichung

Pecularities of Mycobacterial Drug Susceptibility Testing (DST)

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

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

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	2.5	1
Streptomycin	2.0	1

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

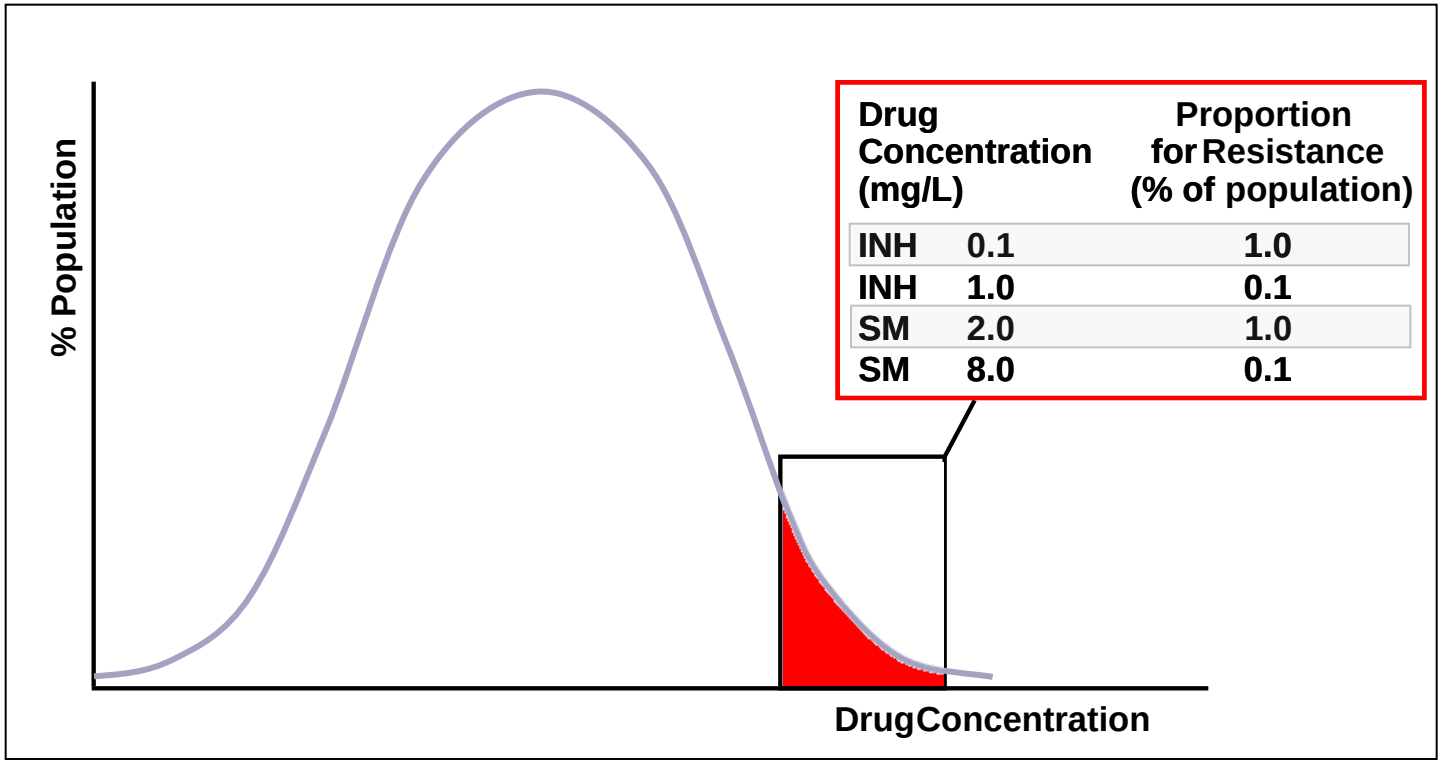
The Critical Concentration

☞ **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 - 0.2	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



Drug	Concentration (mg/L)	Proportion for Resistance (% of population)
INH	0.1	1.0
INH	1.0	0.1
SM	2.0	1.0
SM	8.0	0.1

… 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

Mycobacterial Drug Resistance Mechanisms

- Plasmid- or transposon-mediated mechanisms of resistance are absent.
- Drug resistance in *Mycobacterium tuberculosis* is exclusively due to chromosomal alterations, e.g. mutations, deletions.

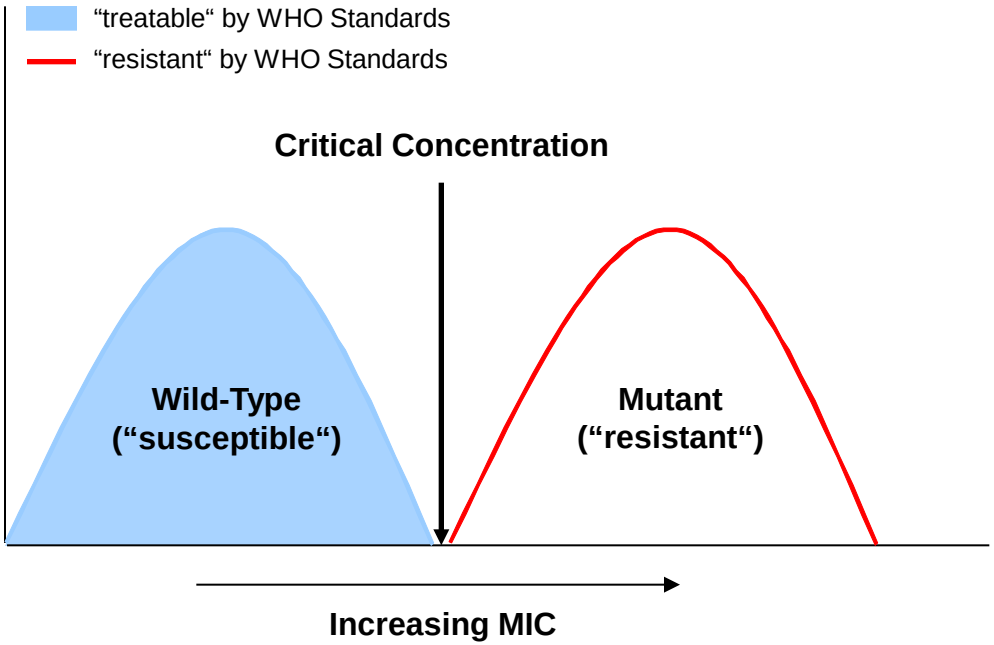
Drug	Resistance Mechanism
Isoniazid	katG
	inhA (target)
Rifampicin	rpoB (target)
Ethambutol	embB (target)
Pyrazinamid	pncA
Streptomycin	rpsL (target)
	rrs (target)
Aminoglycosides	rrs (target)
Quinolones	gyrA (target)
Ethionamid	ethA
	inhA (target)

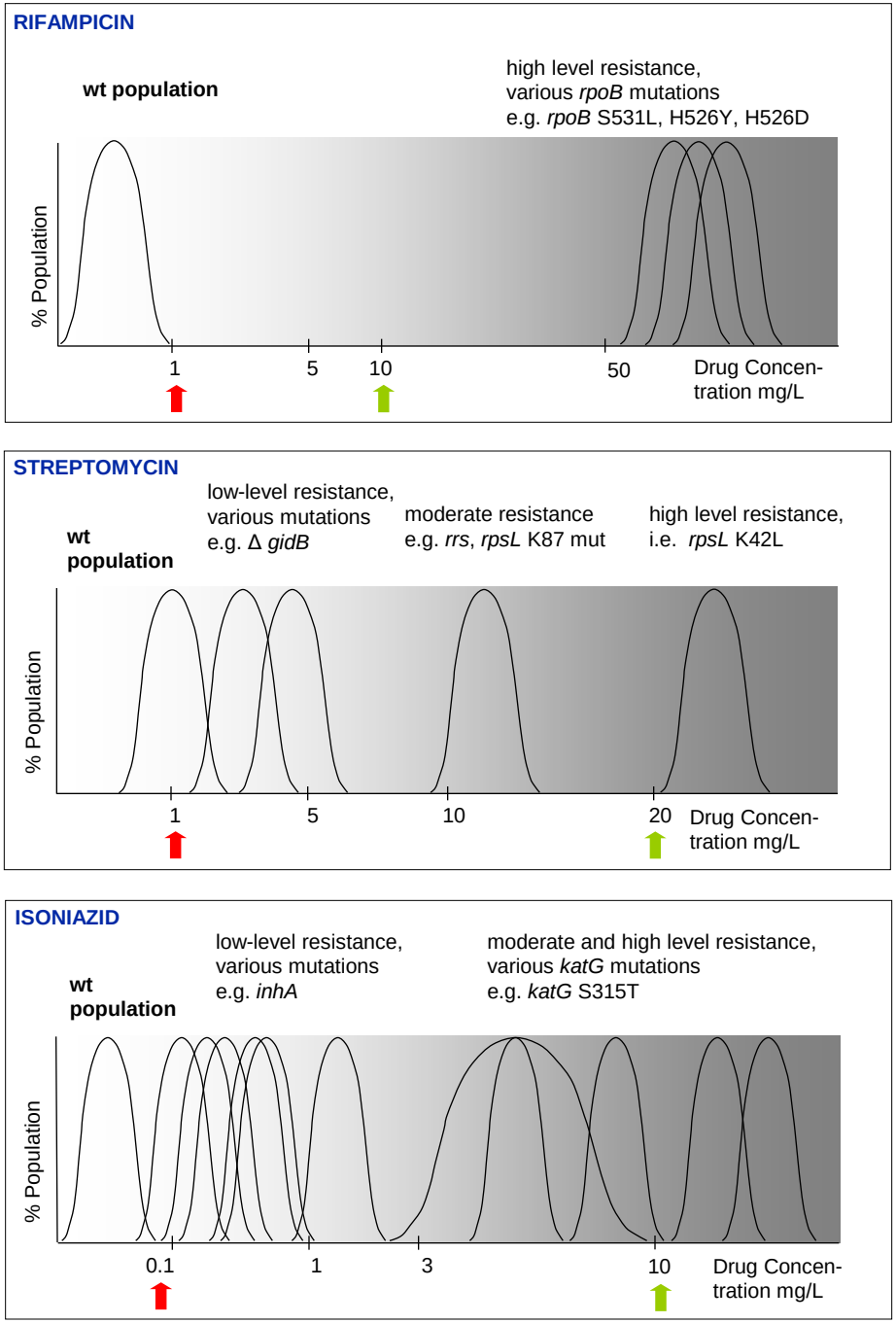
- drug target
- drug activating enzymes

- Multi-drug resistance is due to accumulation of chromosomal alterations.

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 conventional DST





“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*.

Streptomycin: 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.

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*

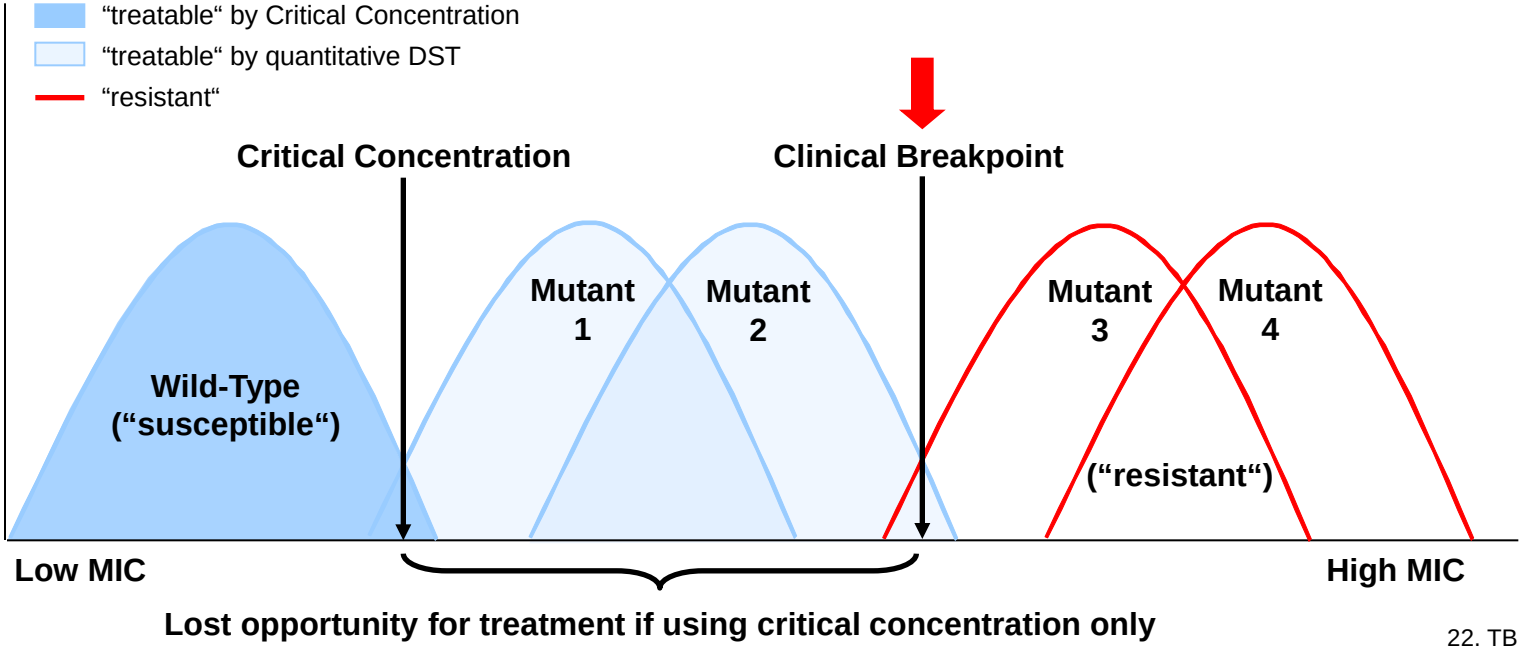
Drug	Genetic locus	Frequency in resistant clinical isolates
Isoniazid	*inhA*	20-30%
Streptomycin	*gidB*	20-30%
Capreomycin	*tlyA, rrs*	>50%
Kanamycin	*eis*	20-30%
Ethambutol	*embB*	>50%

☞ **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



… Some “Complex“ Examples for Illustration

- “Known unknowns of drug susceptibility testing“
  - Ethambutol
- “Phenotypic heterogeneity of mutational alterations“
  - Capreomycin / Amikacin
  - 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.

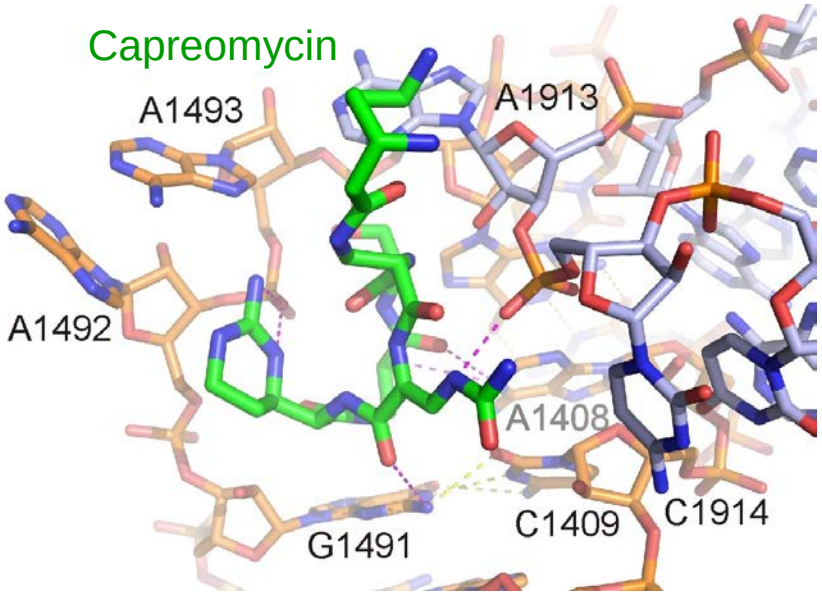
→ dependent on the method used and the critical concentration chosen overlapping MIC distributions for wt and *embB* mutant strains, i.e., "susceptible" isolates may carry an *embB* mutation and "resistant" isolates may show a wild-type *embB.*

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

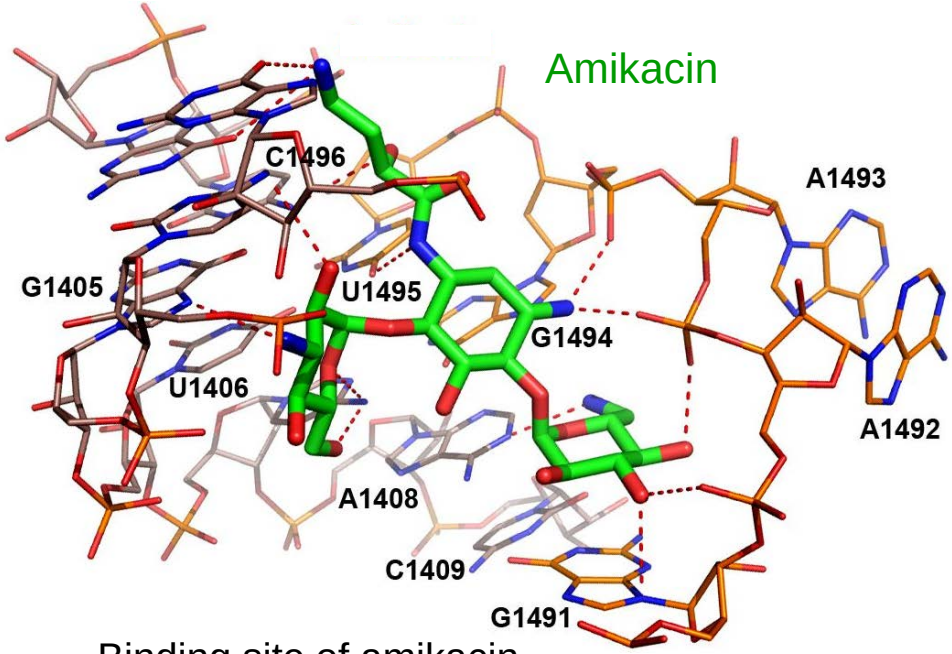
☞ **no data exist correlating in-vitro test results with in-vivo outcome**

Ribosomal Binding Sites: Tuberactinomycin Capreomycin and Aminoglycoside Amikacin – phenotypic heterogeneity of mutational alterations

Capreomycin



Amikacin



Binding site of capreomycin.
Capreomycin binds in the ribosomal interface of the small and the large ribosomal subunit and forms hydrogen-bond interactions with h44 of the 16S rRNA (orange) and H69 of the 23S rRNA (blue).

Binding site of amikacin.
Amikacin binds to the small ribosomal subunit and makes hydrogen-bond interactions with h44 of the 16S rRNA.

Mutations in 16S rRNA A1408G and C1409U and Resistance to Amikacin and Capreomycin in *M. tuberculosis* – phenotypic heterogeneity of mutational alterations

- **Amikacin: MIC wt ~1.0 mg/L**
  Critical concentration: 2 mg/L
  Drug serum concentrations: 10-25 mg/L
  Mutation A1408G confers high-level resistance: MIC >512 mg/L
  Mutation C1409U confers low-level resistance: MIC 2-4 mg/L
  Sander et al. Mol. Microbiol. 1996, 22:841
  Shcherbakov et al. Mol. Microbiol. 2010, 77:830

- **Capreomycin: MIC wt ~2.5 mg/L**
  Critical concentration: 5 mg/L
  Drug serum concentrations: 20-40 mg/L
  Mutation A1408G confers low-level resistance: MIC 10-15 mg/L
  Mutation C1409U confers high-level resistance: MIC >128 mg/L
  Maur et al. Antimicrob. Agents Chemother. 2005, 49:3192
  Feuerriegel et al. Antimicrob. Agents Chemother. 2009, 53:3356
  Jugheli et al. Antimicrob. Agents Chemother. 2009, 53:5064
  Akbergenov et al. Antimicrob. Agents Chemother. 2011, 55:4712
  Sirgel et al. Microb. Drug Res. 2012, 18:193

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
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, in press

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**

☞ **Moxifloxacin should be used for treatment of MDR-TB**

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

Burkhard Springer, Katja Lucke, Romana Calligaris-Maibach, Claudia Ritter and Erik C. Böttger

For clinical isolates of *Mycobacterium tuberculosis* 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.

Journal of Clinical Microbiology 2009, 47: 1773-1780

M. tuberculosis – semi-quantitative drug susceptibility testing

Molecular Mechanisms	**Critical concentration** 1.0 mg/L	**Semi-quantitative susceptibility testing** 4.0 mg/L	10.0 mg/L	**Amikacin**
wt	**S**			susceptible
eis	**R**	**S**	**S**	low-level
rrs C1409U	**R**	**S/R**	**S**	low-/moderate-level
rrs A1408G	**R**	**R**	**R**	high-level

Molecular Mechanisms	**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	**R**	**S**	**S**	**S**	low-level
katG	**R**	**R**	**S/R**	**S/R**	moderate-/high-level

Clinical Case

S.R., 29.01.1945, Schweiz (TI), HIV

	mg/L		Mutation
Isoniazid	0.1	R	katG S315 mut
	1.0	R	inhA wt
	3.0	R	
	10.0	S	
Rifampicin	1.0	S	rpoB L533 mut
Rifabutin	0.1	R	
	0.4	S	
Ethambutol	5.0	S	embB wt
Pyrazinamid	10.0	S	pncA wt

Laborbemerkung: Der Stamm zeigt eine katG Ser315Thr Mutation und eine rpoB Leu 533 Pro Mutation: funktionell Isoniazid monoresistent, empfindlich gegenüber Rifampicin.

Therapieempfehlung: MOX, RIF, EMB, PZA

Clinical Case

O.O., 02.12.1984, Uzbekistan

	mg/L		Mutation
Isoniazid	0.1	R	katG S315 mut
	1.0	R	inhA promoter mut
	3.0	R	
	10.0	R	
Rifampicin	1.0	R	rpoB 531mut
	4.0	R	
	20.0	R	
Rifabutin	0.1	R	
	0.4	R	
	2.0	S	
Ethambutol	5.0	S	embB 306 mut
Pyrazinamid	10.0	S	pncA wt

	mg/L		Mutation
Ethionamid	5.0	R	inhA promoter mut
	10.0	R	
	25.0	R	
Streptomycin	1.0	R	rrs A523 mut
	4.0	R	rspL wt
	20.0	S	
Amikacin	1.0	S	rrs wt
Capreomycin	5.0	S	rrs wt
Ofloxacin	1.0	S	gyrA wt
Moxifloxacin	0.25	S	
Cycloserin	50.0	S	-
Linezolid	1.0	S	rrl wt, rplC wt

Laborbemerkung: MDR Stamm: Resistenz gegenüber Isoniazid, Rifampicin, Rifabutin, Ethionamid, Streptomycin.

Therapieempfehlung: Moxifloxacin, PZA, Amikacin, Ethambutol, Cycloserin

European Study Group
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.

Study Design:

- Mulit-center study of 9 European expert labs.

Methods:

- qDST using MGIT 960 / TBeXiST.
- Molecular genetic analyses.
- Correlate results from qDST with molecular resistance mechanisms.

Mycobacterial Drug Susceptibility Testing – The Limitations of Current Procedures

Drug Resistance in *M. tuberculosis* is a mixed bag: significant heterogeneity 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.
- ☞ Prospective/retrospective studies are required to correlate the results of semi-quantitative DST with clinical outcome.

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Mycobacterial Drug Resistance and Susceptibility Testing

Drug Resistance in *M. tuberculosis* is heterogeneous: significant heterogeneity is present, i.e., low-, moderate- and high-level drug resistance.

Clinical implications

- low-level drug resistance
  - ☞ most likely compound still has clinical activity
  - ☞ may be maintained as part of combination therapy
- high-level drug resistance
  - ☞ no activity, only adds to toxicity (e.g. isoniazid-polyneuropathy)
  - ☞ compound should not be part of combination therapy

Ethambutol MICs and *embB*306 Mutations in *M. tuberculosis* Clinical Isolates

Ethambutol MIC Values (µg/ml)	Isolates with *embB*306 Mutations (n = 77)				Wt Isolates (n =26)
	Met306Ile (n = 56)		Met306Val	Met306Leu	
	ATG→ATC (n = 32)	ATG→ATA (n = 24)	ATG→GTG (n = 18)	ATG→CTG (n = 3)	
1.25 – ≤2.5	0	0	0	0	26
5.0	6	3	1	0	0
>5.0 – ≤12.5	22	21	3	0	0
>12.5 – ≤25.0	4	0	13	3	0
>25.0 – ≤50.0	0	0	1	0	0

Sirgel et al. Chemother. 2012, 58: 358-363