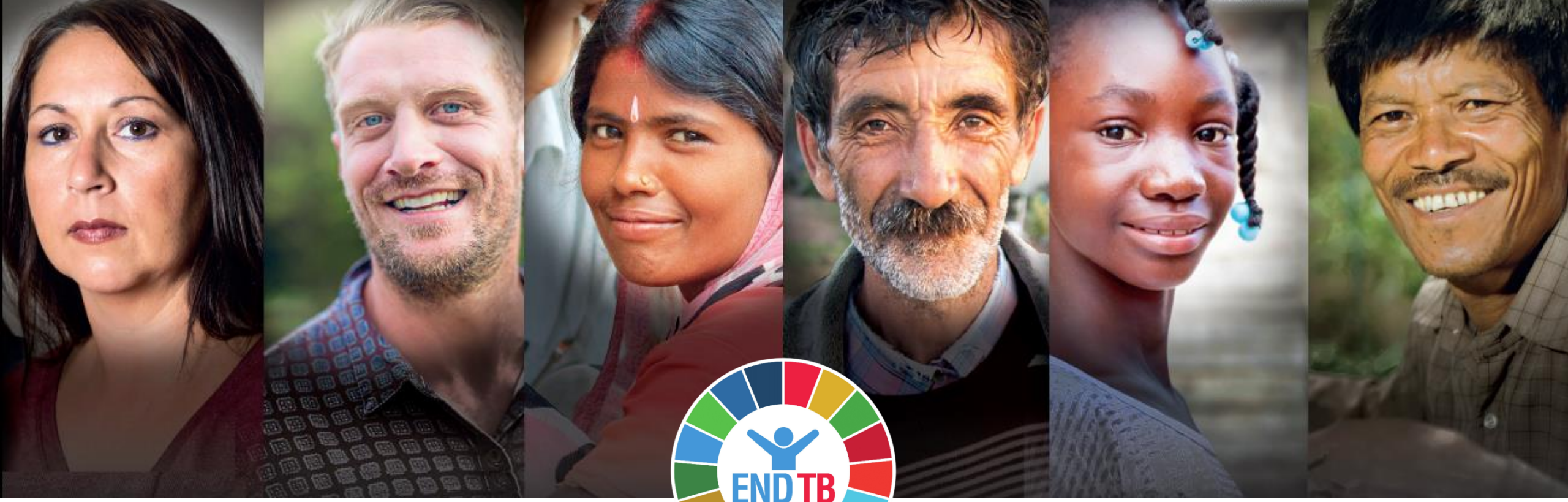


END TB

gli
global laboratory initiative
advancing TB diagnosis

Phenotypic drug-susceptibility testing
General concepts

Stop TB Partnership

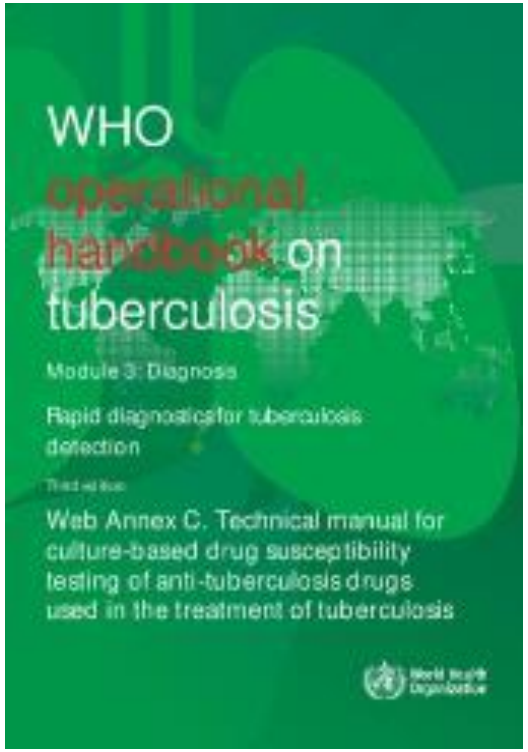
World Health Organization

What we will address

- Drug-resistance development and occurence
- Phenotypic drug-susceptibility testing concepts
  - DST versus MIC testing
  - Critical concentration & ECOFF
  - Clinical breakpoint
  - Proportion method
- Good practice and quality assurance
- Trouble shooting
- Hetero-resistance
- Cross resistance

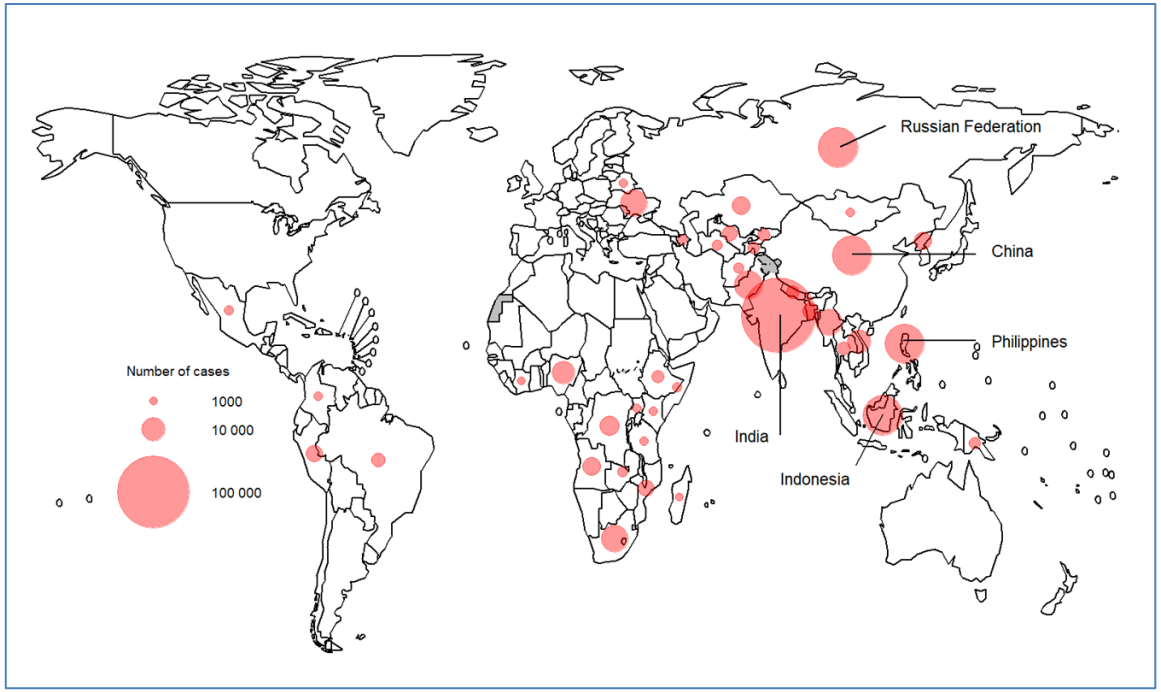
Based on

World Health Organization. (2024). WHO operational handbook on tuberculosis: module 3: diagnosis: rapid diagnostics for tuberculosis detection: web annex C: technical manual for culture-based drug susceptibility testing of anti-tuberculosis drugs used in the treatment of tuberculosis, 3rd ed. World Health Organization. https://iris.who.int/handle/10665/376286. License: CC BY-NC-SA 3.0 IGO

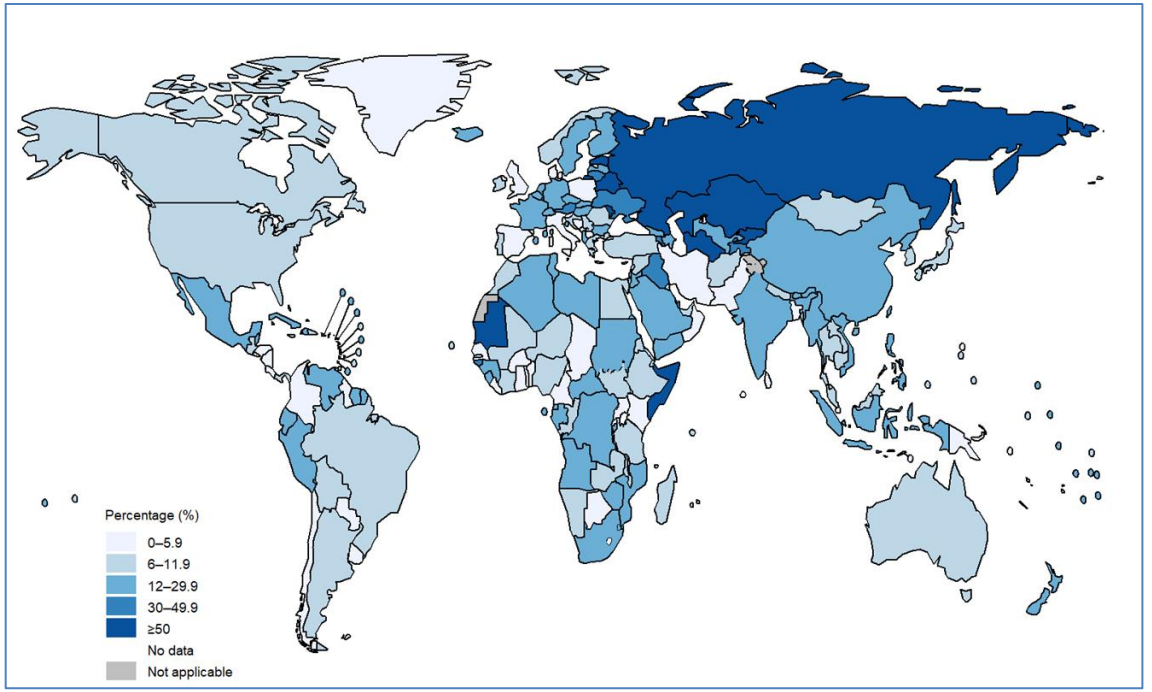


Drug-resistant TB development and occurence

Drug-resistant tuberculosis (DR-TB) is present world-wide wherever treatment is available



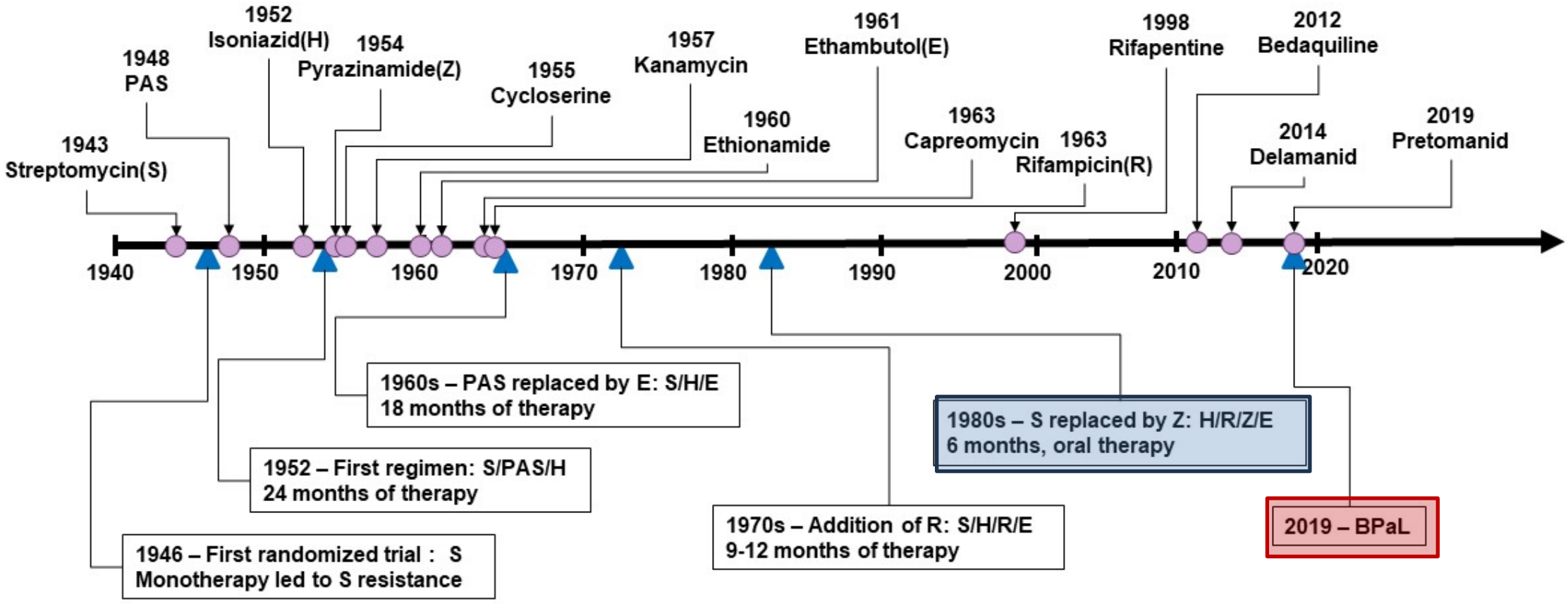
Estimated number of incident cases of MDR/RR-TB in 2023, for countries with at least 1000 incident cases (WHO)



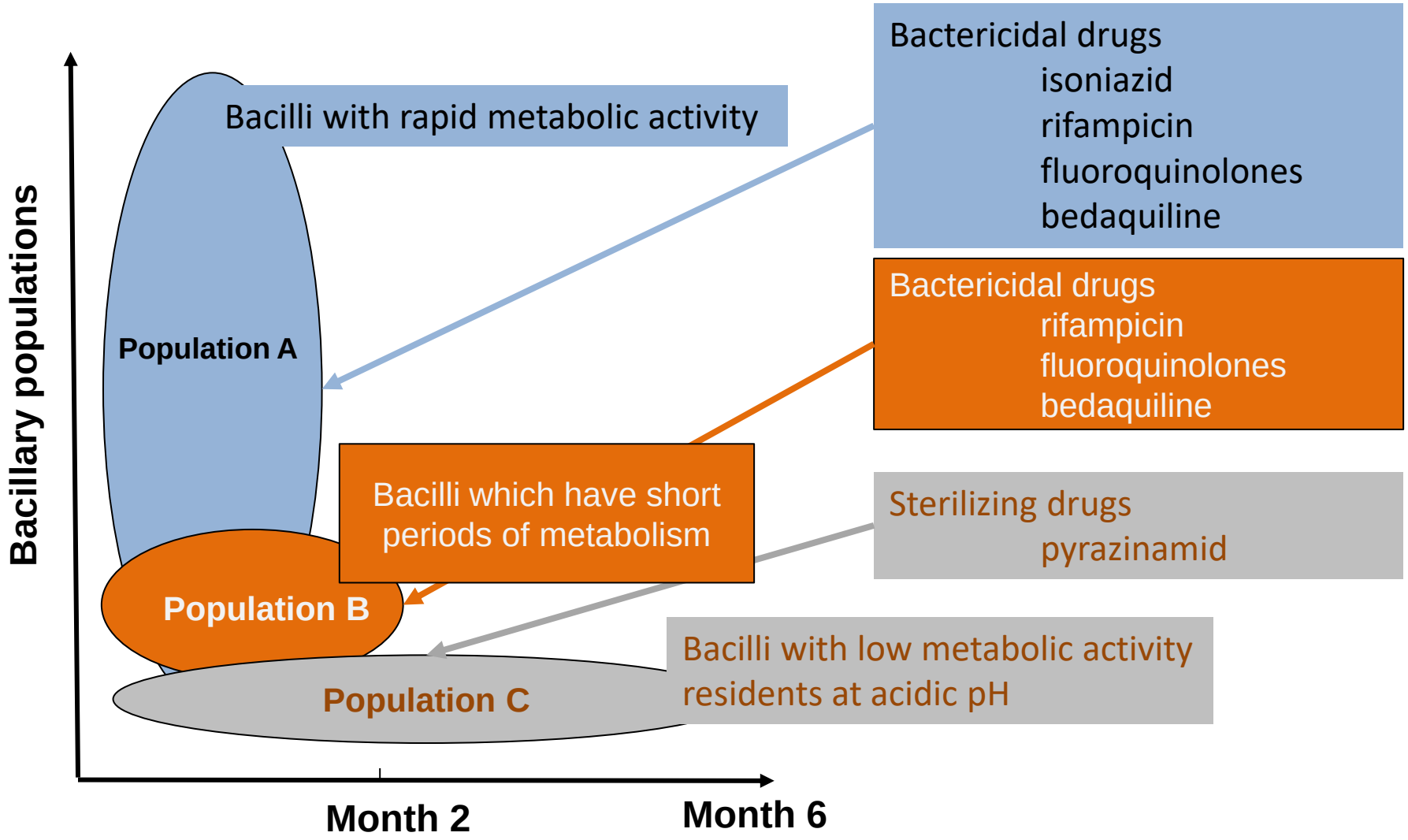
Percentage of previously treated TB patients with multi-drug/rifampicin-resistant (MDR/RR) TB in 2023 (WHO)

Evolution of TB therapy

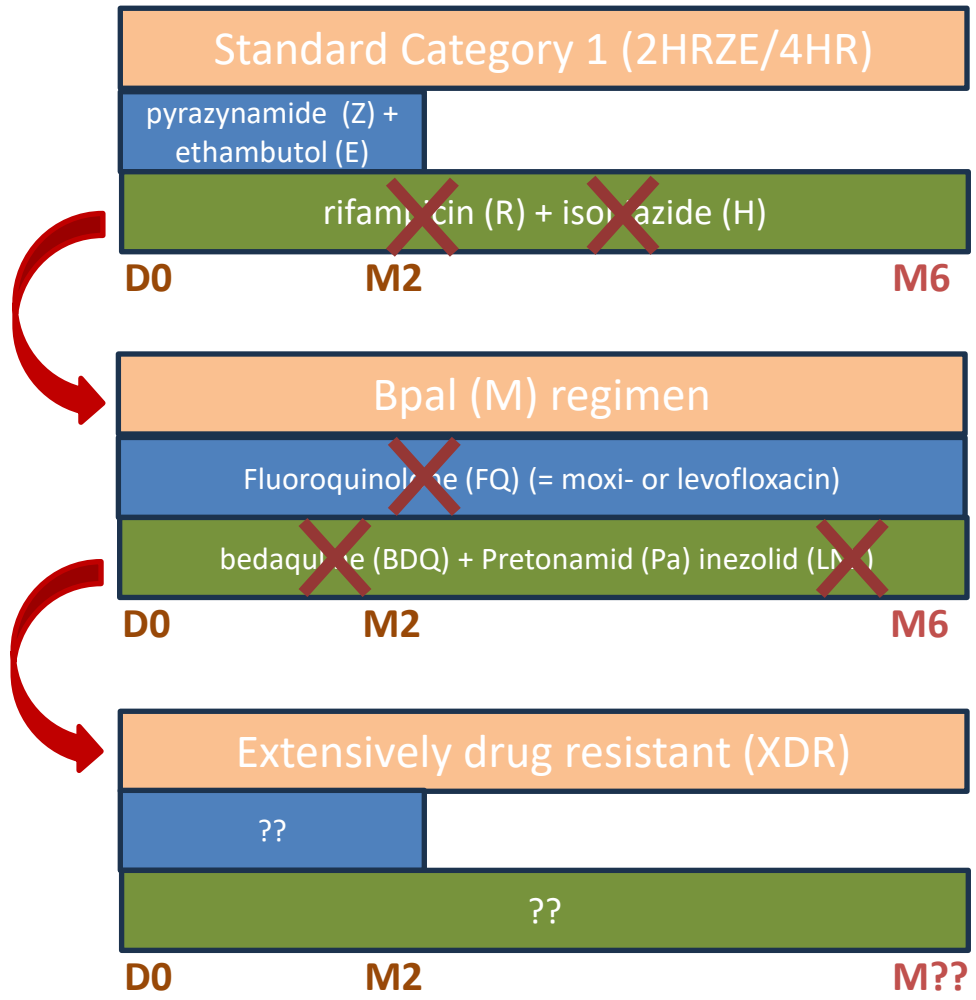
(source https://www.tballiance.org/content/drugs-regimens-transforming-tb-drug-development6)



Anti-TB drugs act on different populations during a regimen



Combination therapy regimen adapted to drug-resistance profile



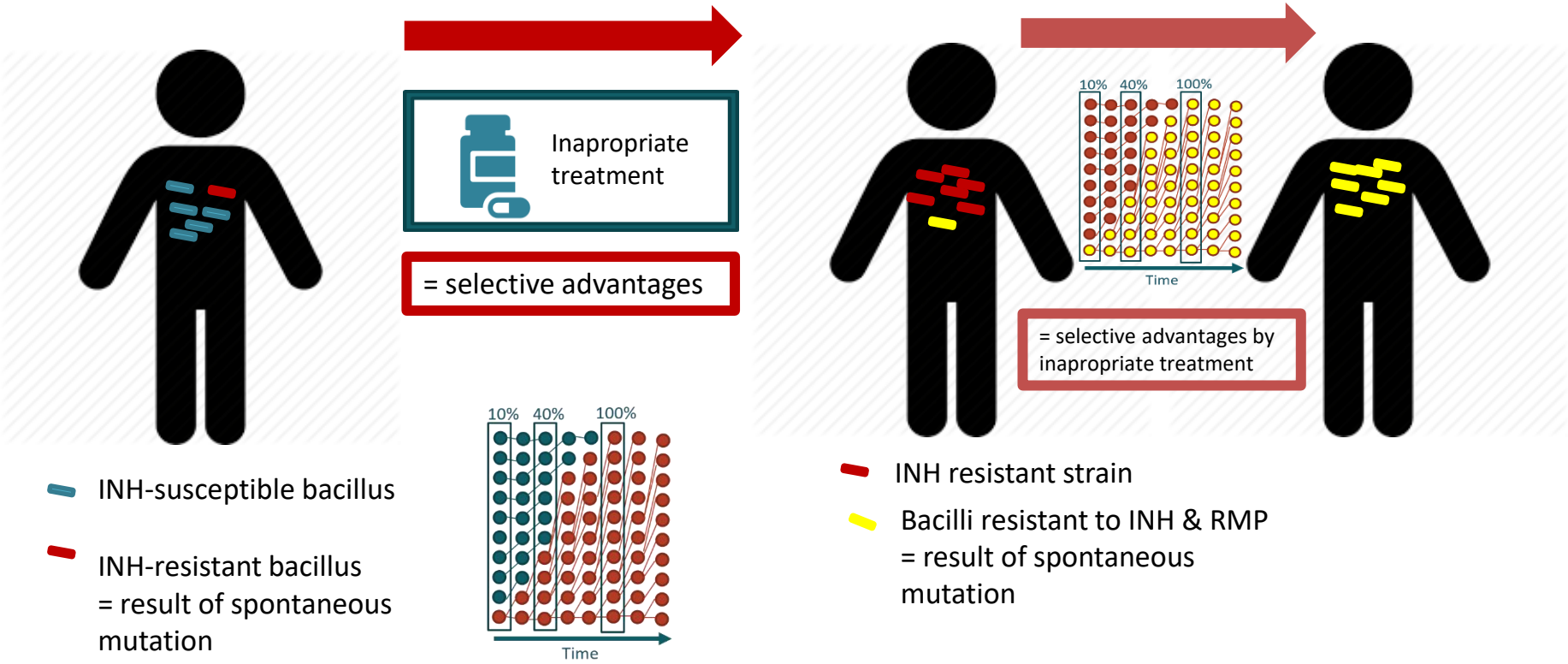
Initial treatment;
no indication for R-resistance

MDR = resistant to at least R and H

Pre-XDR = MDR + resistant to FQ

XDR = MDR + resistant to FQ & BDQ/LNZ

Acquisition of resistance during inapropriate treatment

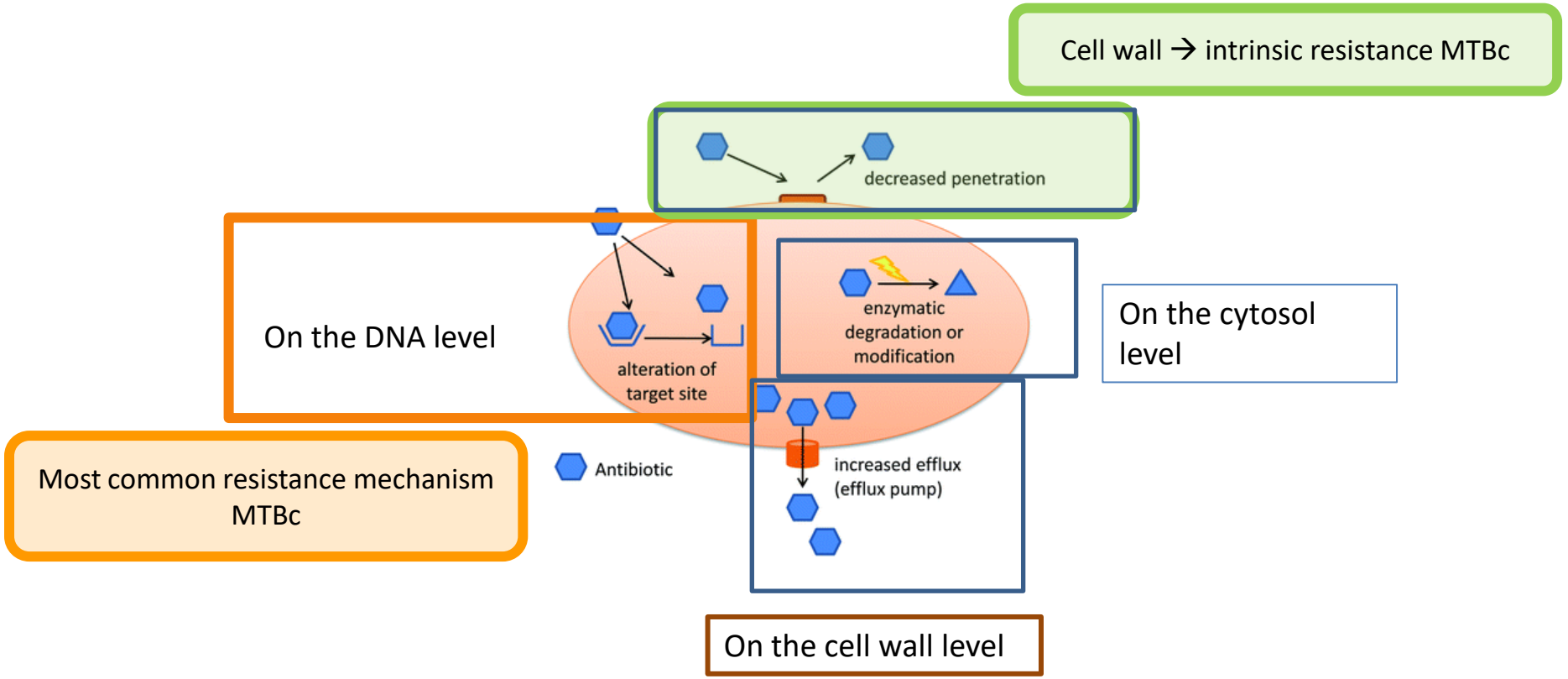


Cummulative process

Factors influencing the development of drug resistance

- Treatment with inappropriate drugs, combinations or dosages
  - Interruption or irregular treatment
  - Incomplete treatment
    - required number of doses not taken (patient non-compliant)
    - duration
- Metabolism of bacilli shifted to dormancy
  - Impaired/decreased drug uptake by *M. tuberculosis* cells
- Differential penetration of drugs to various body sites
- Suboptimal concentration of drugs at some sites
- Impaired drug absorption due to underlying host conditions such as HIV/AIDS

Drug resistance: molecular mechanisms



Phenotypic DST concepts

DST versus MIC testing

- Solid medium: counting colonies
- Liquid medium: mostly measuring metabolic activity

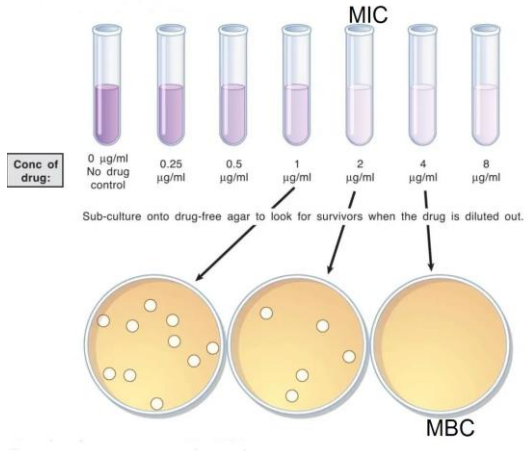
- Testing a single concentration = critical concentration
  - Separates susceptible (S) from resistant (R) bacilli
  - Wild type (WT) versus mutant (MUT)

  - Proportion method = most commonly used
  - Absolute concentration method

- Testing multiple two-fold diluted concentrations

  →Determine minimal inhibitory concentrations (MIC)

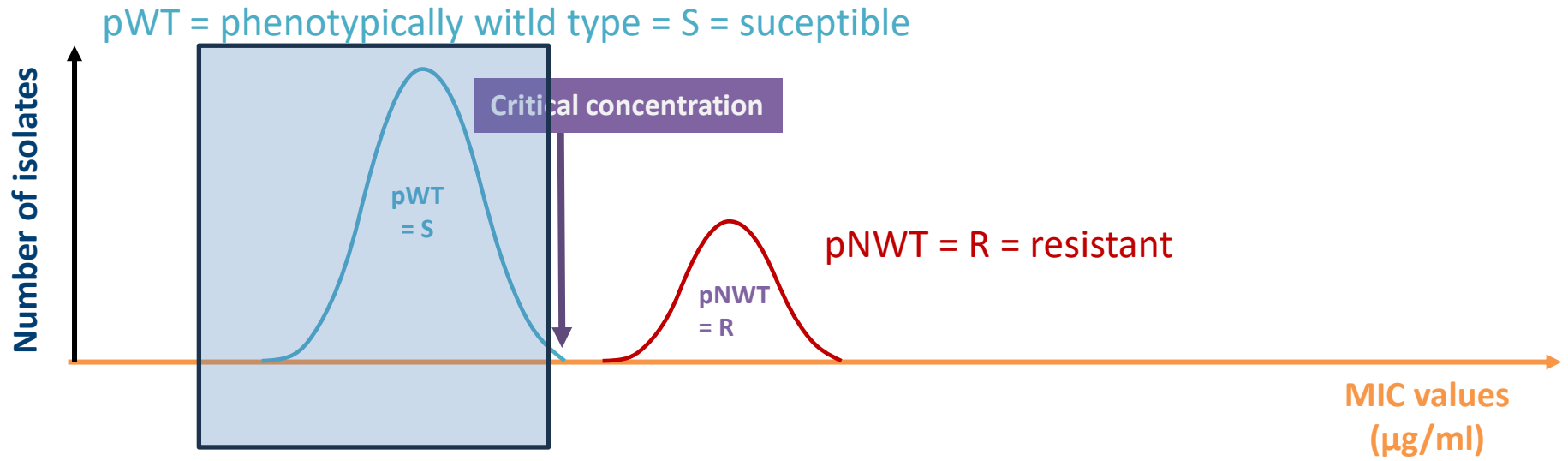
Minimal inhibitory concentration



Lowest concentration of anti-TB agent that prevents growth of 99% of a microorganism (in a sample/strain)

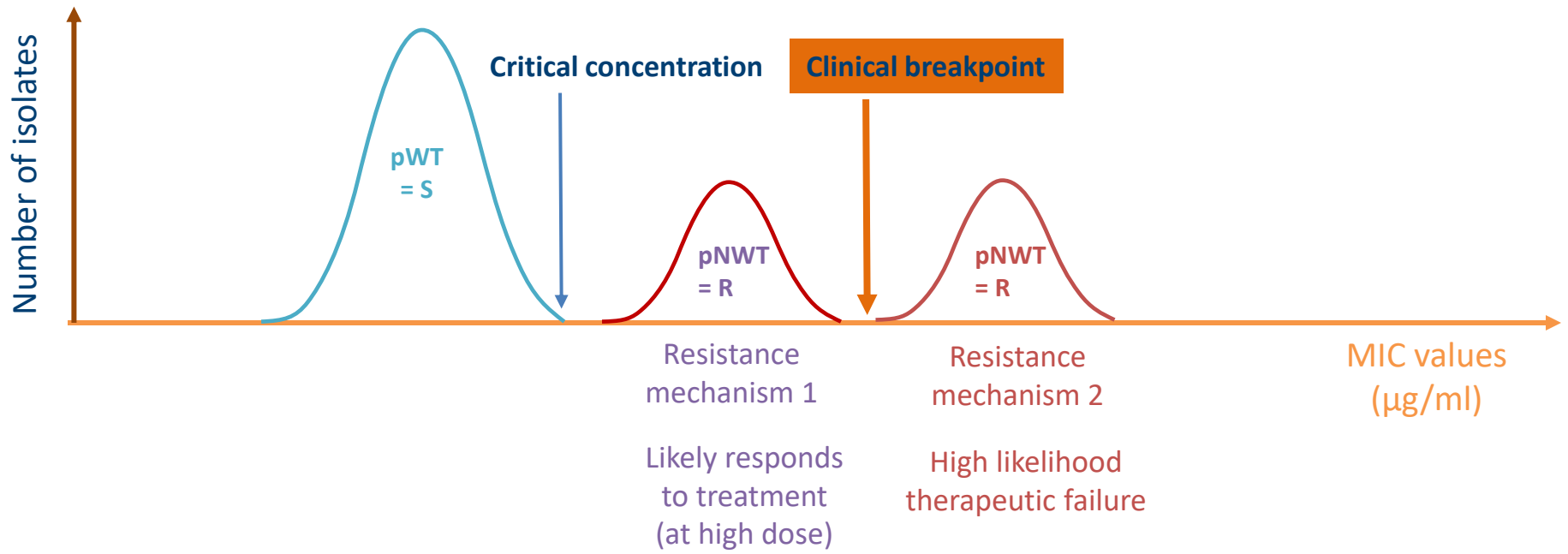
When testing many strains → typical normal distribution of MIC values

Critical concentration



Lowest concentration of anti-TB agent that will inhibit in vitro growth of 99% of pWT *M. tuberculosis* complex strains

Clinical breakpoint



- Concentration(s) of anti-TB agent which defines a MIC above the critical concentration that separates strains that will likely respond to treatment from those which will likely not respond to treatment
- Only defined for **moxifloxacin**
  - Low- versus high-level FQ resistance (for example *gyrA*_Ala90Val versus *gyrA*_Asp94Gly)
  - Linked to pharmacokinetic and – dynamic data

Critical proportion

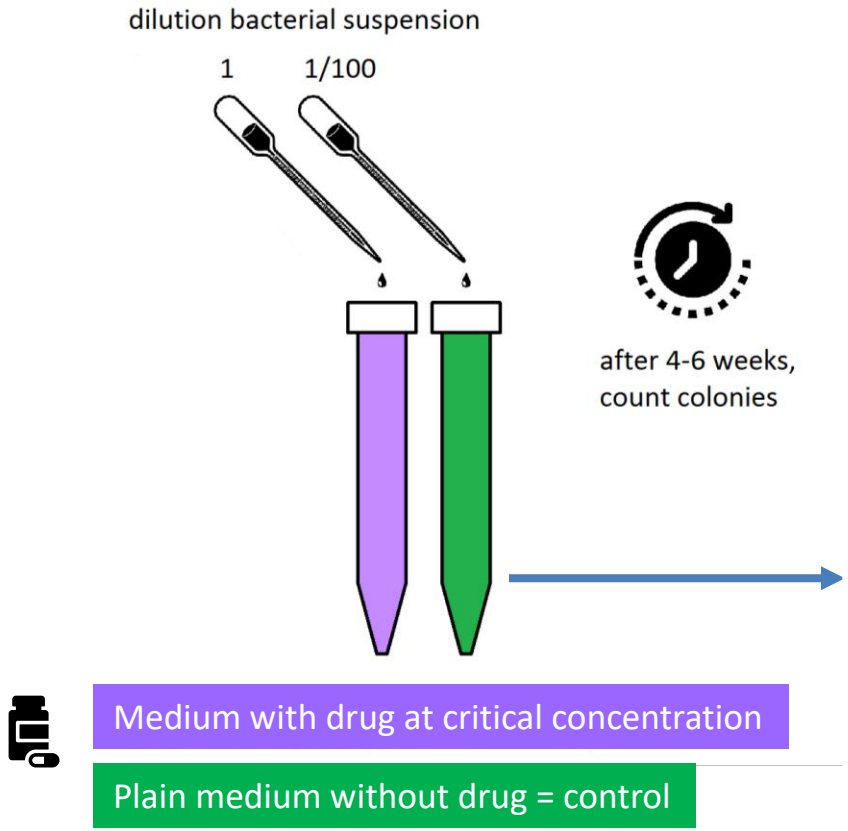
- Proportion of resistant bacilli within a particular cultured isolate that is used to determine resistance to a particular drug

→ % of bacilli that may grow in presence of an anti-TB drug before declaring the strain resistant

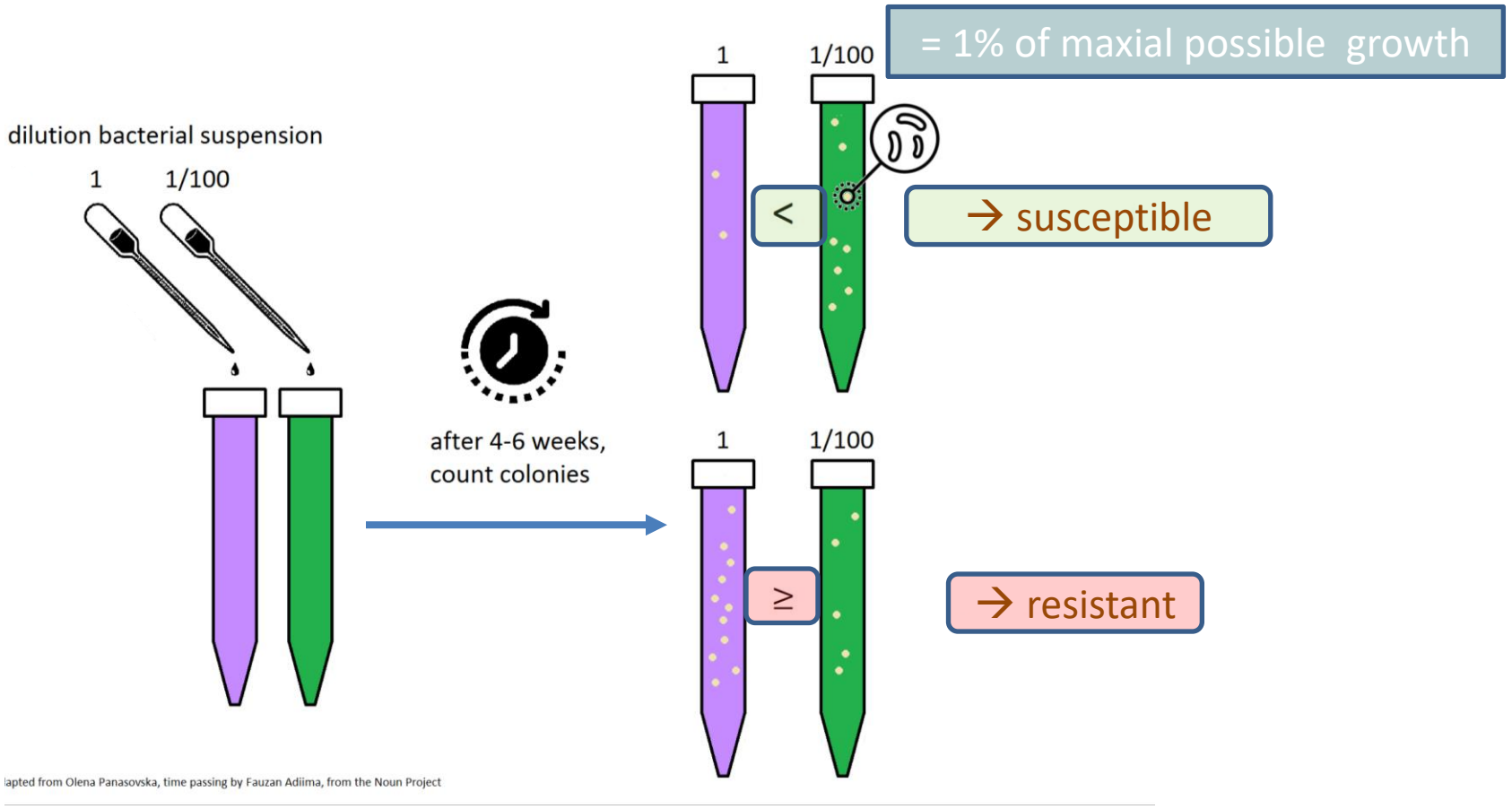
- 1% applies to first- and second-line drugs
- 10% for pyrazinamide

Proportion method on solid medium

Allowing <1% growth at critical concentration to classify as susceptible

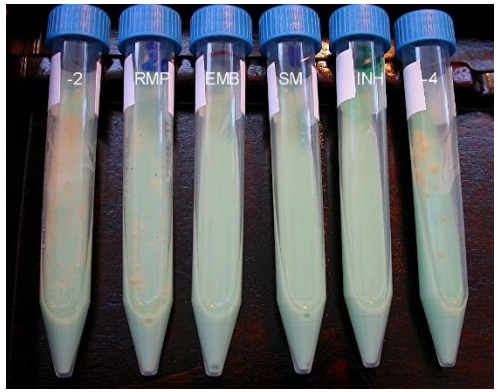


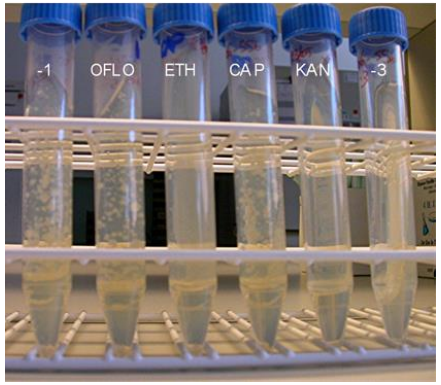
Proportion method on solid medium (2)

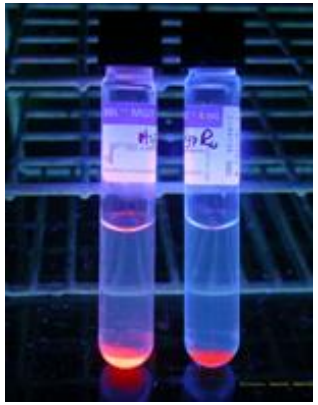


The most commonly used pDST media/systems

Medium	Solid	Liquid
Example	Löwenstein-Jensen Middlebrook agar	MGIT
Equipment	Incubator (5-10% CO_2)	Automated incubator (MGIT960)
Growth detection	Visible colonies; **manual reading**	Metabolic activity; **automated reading** ↘O_2 results in ↗ fluorescence
DST duration	**4-6 weeks**	Control = 400 growth units (GU) **(up to 13 days)**







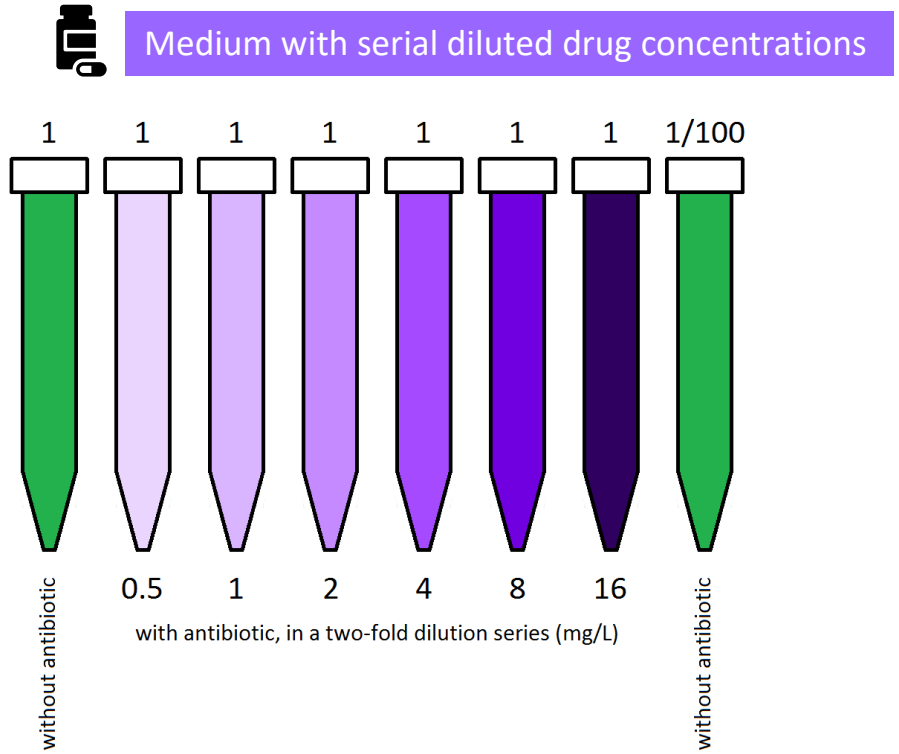
Drug-tube < 100 GU = S
Drug tube ≥ 100 GU = R

MIC value of an isolate can be translated in binary R/S result

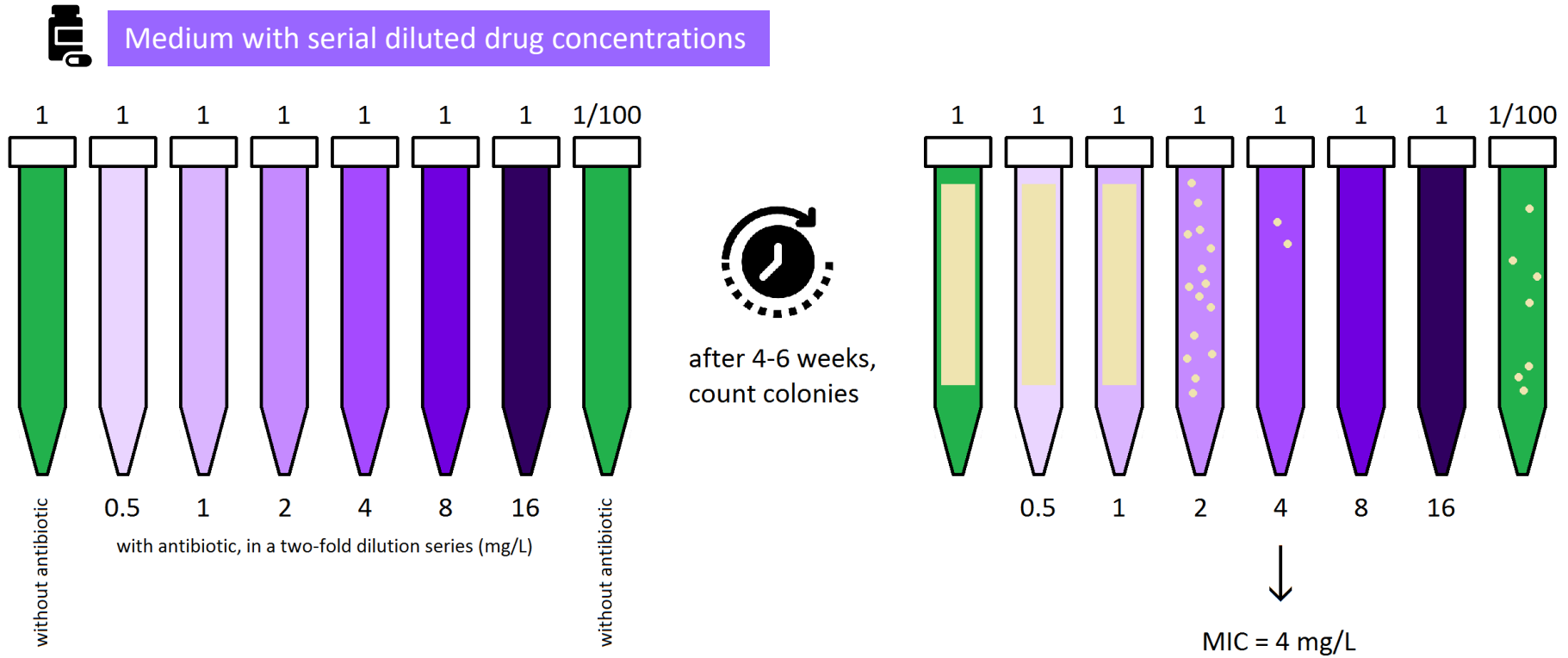
- MIC value < critical concentration = S
- MIC value = critical concentration = S
- MIC value > critical concentration = R

Minimum inhibitory concentration (MIC) provides more information on level of resistance

- ≥ 5 concentrations with **2-fold dilutions**



Minimum inhibitory concentration (MIC) provides more information on level of resistance



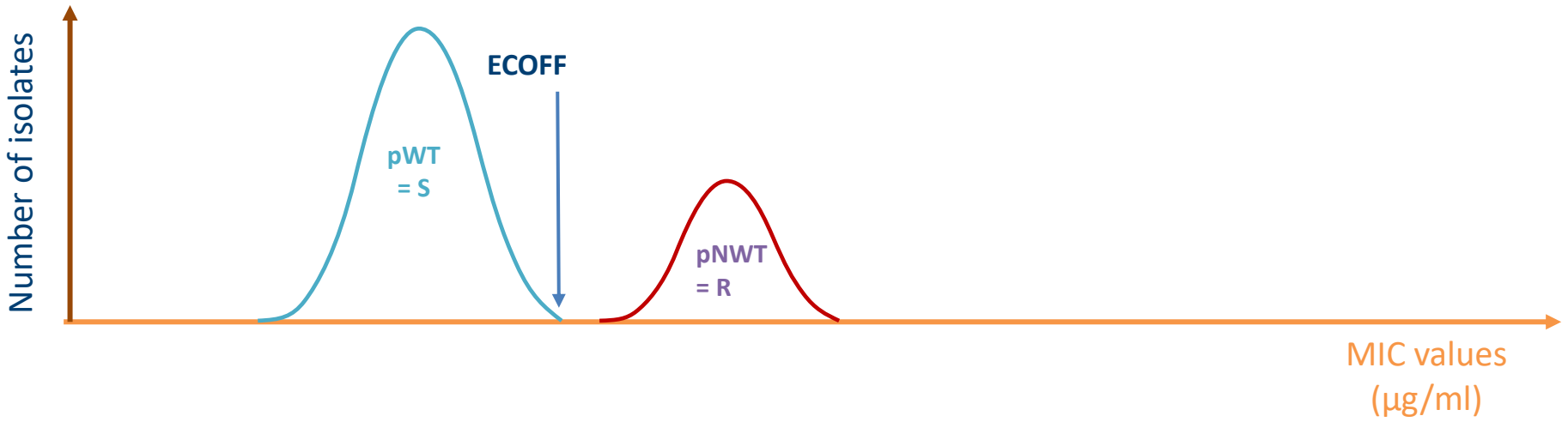
MIC values may vary with the medium used (e.g. protein binding)

Example rifampicin (RMP)

- MIC of 10 µg/ml on LJ medium → S (CC = 40 µg/ml)
- MIC of 10 µg/ml in MGIT → R (CC = 0.5 µg/ml)

→ MIC data should always come with info on medium used

Epidemiological cut-off (ECOFF)



- Upper end of the pWT distribution
  - Typically, MIC value for 99% of pWT strains fall below ECOFF
  - Data come from at least 100 strains (from 5 different laboratories)

- ECOFF often used as surrogate for critical concentration
  - In absence of knowledge on drug-resistance mechanism

ECOFF (critical concentration) versus clinical breakpoint

- ECOFF does not change by sampling time, source (human, animal, environmental), geographical origin

- ECOFF may change by testing medium (MGIT, LJ, agar) or procedure
  - 100 – 1000 isolates, multiple labs, per medium/method
  - Standardization

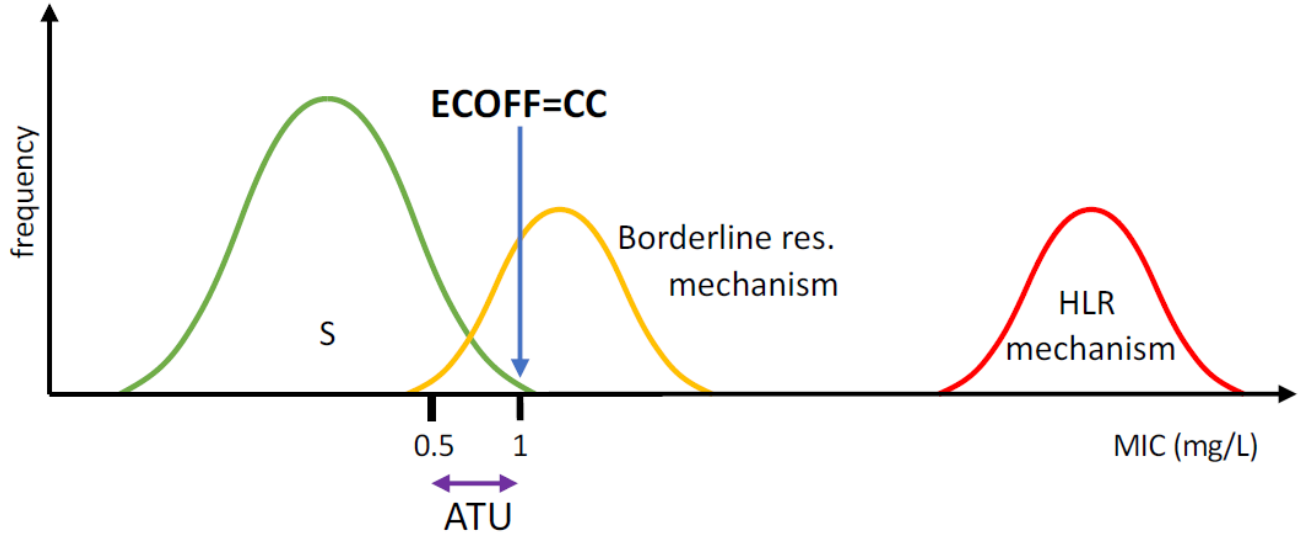
  → There is no “true MIC” – always mention medium

- Clinical breakpoint may change
  - New resistance mechanisms occurred
  - Altered dosing & indications for drug use

Rationale for MIC testing vs CC

- For the majority of TB drugs, some resistance mechanisms result in MIC distributions overlapping with the MIC distribution of susceptible strains, with the CC intersecting the MIC distribution of these mechanisms > poor reproducibility of categorical pDST
- The definition of the area of technical uncertainty (ATU), which typically encompasses one dilution, would delimit an "inconclusive" or "uncertain" interpretation

Figure 1. ATU for hypothetical MIC distributions of susceptible and resistant strains.



Source Optimized broth microdilution plate methodology for drug susceptibility testing of Mycobacterium tuberculosis complex. Geneva: World Health Organization; 2022

Rationale for MIC testing vs CC

- Providing more nuanced phenotypic information

  → Improved investigation on association with genotypic results

- The ECOFF encompasses approx. 99% of phenotypically wild-type strains

  → the positive predictive value of pDST will be poor in settings with a true rate of resistance that is close to 1%

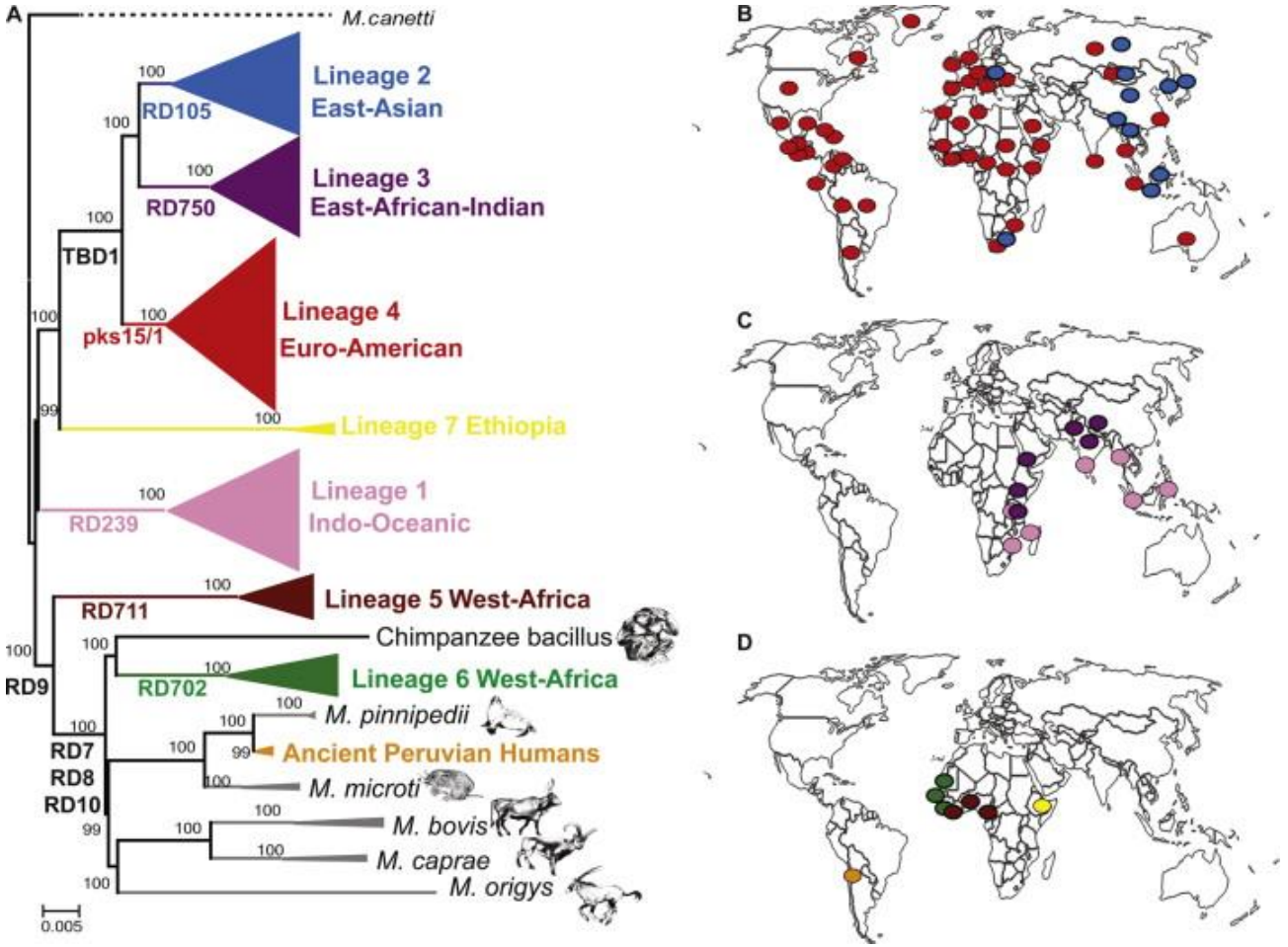
  To some extent, MIC testing would enable for such random false resistance results to be identified

- It could be investigated whether modest MIC increases may be treatable with either the standard or increased exposure of a drug

Source Optimized broth microdilution plate methodology for drug susceptibility testing of Mycobacterium tuberculosis complex. Geneva: World Health Organization; 2022

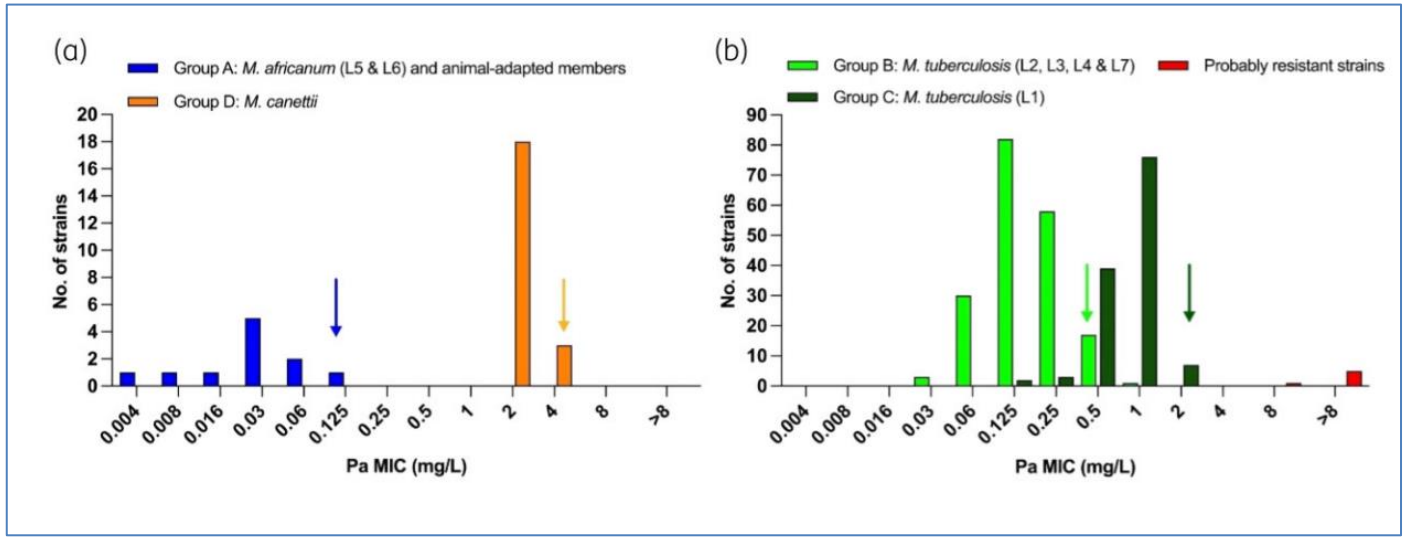
MIC distribution/ECOFF may vary accross MTBc lineages: example pretomanid (PA)

World-wide variety of *M. tuberculosis* complex lineages (L)

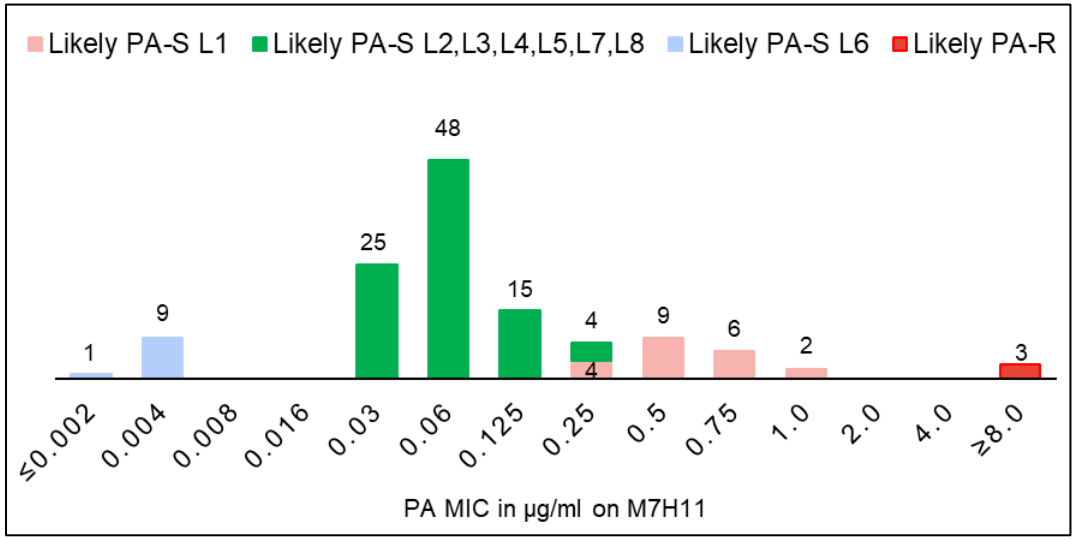


Source: Coscolla & Gangeux, 2014

Pretomanid (PA): lower MIC for L6 ↔ higher MIC for L1



Bateson et al, 2022
MGIT960
95% percentile indicated by arrows



Rupasingha et al, 2024
Solid medium = 7H11 agar

Not observed for most anti-TB drugs, but to better documented, especially for new drugs

EUCAST reference method for MIC determination of *M. tuberculosis*

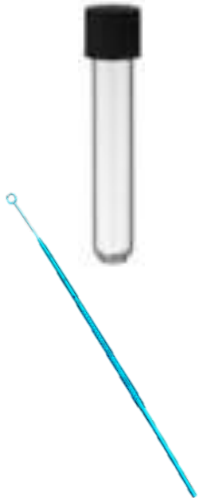
- EUCAST = European Committee on Antimicrobial Susceptibility Testing
- Reference method for the MTB complex is **broth microdilution**
  - Middlebrook 7H9
  - Microtiter plate
- To be used to calibrate new pDST/MIC methods against

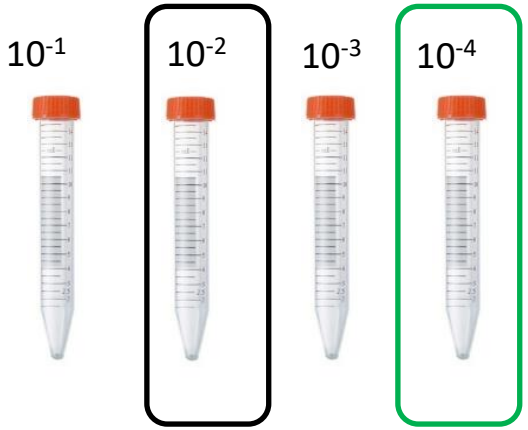
- eucast: Methodology for AST in Mycobacteria

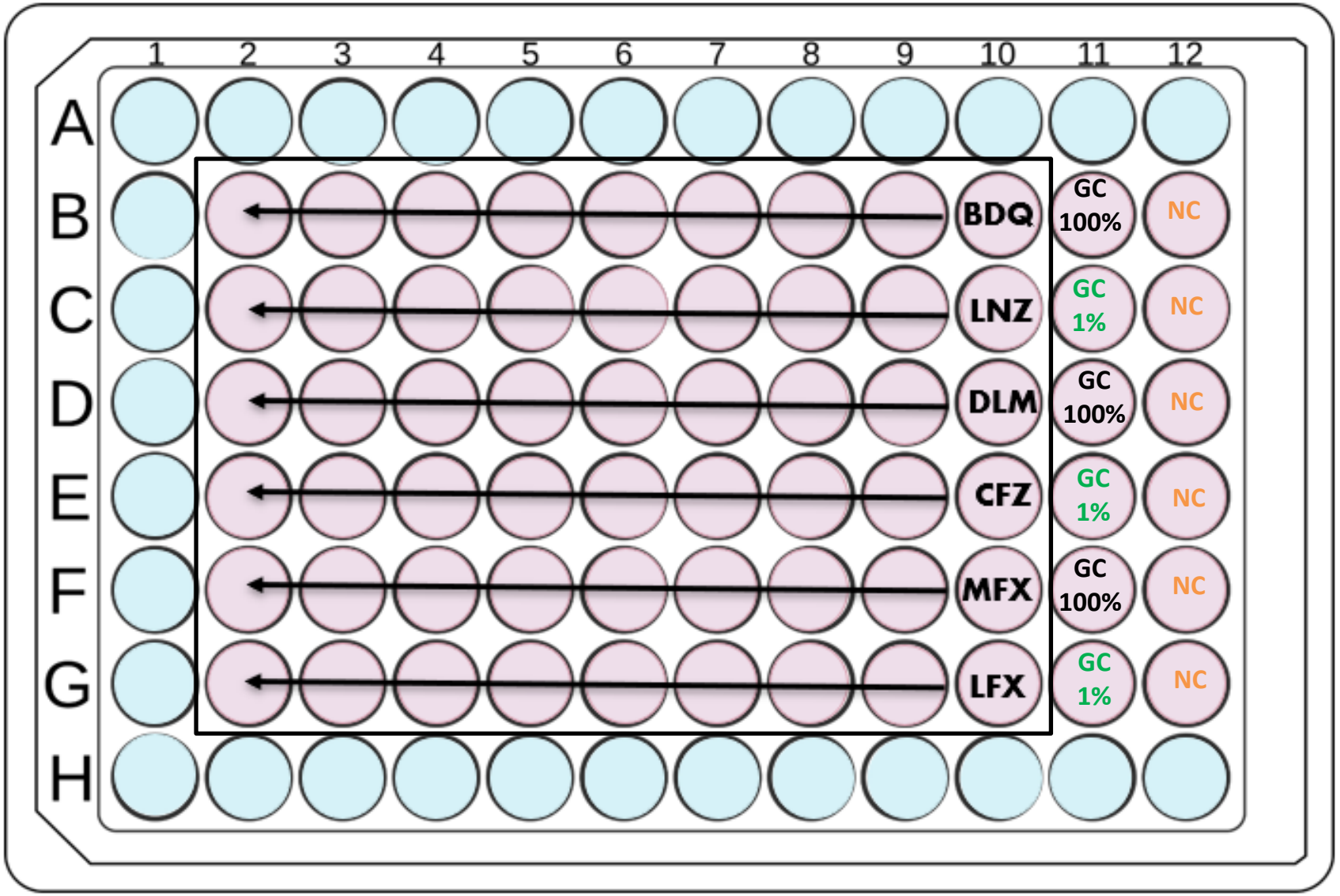
→ MIC determination of anti-TB agents Version 8.2. of January 29th, 2025

Inoculum preparation and inoculation

Bron: https://www.fishersci.ca







Example of plate design

	1	2	3	4	5	6	7	8	9	10	11	12
A	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20
B	negative control	GC 100%	AA1 (10-2) C8	AA1 (10-2) C7	AA1 (10-2) C6	AA1 (10-2) C5	AA1 (10-2) C4	AA1 (10-2) C3	AA1 (10-2) C2	AA1 (10-2) C1	GC 1%	200ul dH20
C	negative control	GC 100%	AA2 (10-2) C8	AA2 (10-2) C7	AA2 (10-2) C6	AA2 (10-2) C5	AA2 (10-2) C4	AA2 (10-2) C3	AA2 (10-2) C2	AA2 (10-2) C1	GC 1%	200ul dH20
D	negative control	GC 100%	AA3 (10-2) C8	AA3 (10-2) C7	AA3 (10-2) C6	AA3 (10-2) C5	AA3 (10-2) C4	AA3 (10-2) C3	AA3 (10-2) C2	AA3 (10-2) C1	GC 1%	200ul dH20
E	negative control	GC 1%	AA4 (10-2) C8	AA4 (10-2) C7	AA4 (10-2) C6	AA4 (10-2) C5	AA4 (10-2) C4	AA4 (10-2) C3	AA4 (10-2) C2	AA4 (10-2) C1	GC 100%	200ul dH20
F	negative control	GC 1%	AA5 (10-2) C8	AA5 (10-2) C7	AA5 (10-2) C6	AA5 (10-2) C5	AA5 (10-2) C4	AA5 (10-2) C3	AA5 (10-2) C2	AA5 (10-2) C1	GC 100%	200ul dH20
G	negative control	GC 1%	AA6 (10-2) C8	AA6 (10-2) C7	AA6 (10-2) C6	AA6 (10-2) C5	AA6 (10-2) C4	AA6 (10-2) C3	AA6 (10-2) C2	AA6 (10-2) C1	GC 100%	200ul dH20
H	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20

Normalised for drug-solvent

AA1-AA6	Antituberculous agent 1-6 (May be expanded to 2 rows/agent depending on target MIC range)
GC	Growth control

Two-fold serial dilutions of drugs

GC100%	Same inoculum as in the drug containing wells
GC1%	Hundredfold diluted inoculum compared to drug containing wells

Should both show growth to have a valid result

Negative Ctrl	200µl 7H9-OADC
dH20	sterile distilled water

To avoid drying out of plates

Good practice and quality assurance

WHO recommended critical concentrations (2024)

Table 2.2. CCs and clinical breakpoints for medicines recommended for the treatment of TB

Drug groups	Drug	LJ	7H10	7H11	MGIT[a]
First-line agents *(25)*[29]	Isoniazid	0.2	0.2	0.2	0.1
Isoniazid and rifamycins	Rifampicin[b]	40	0.5	1.0	0.5
	Rifabutin[c]	–	–	–	–
	Rifapentine[d]	–	–	–	–
First-line agents *(26)*[30]	Ethambutol[e]	2.0	5.0	7.5	5.0
Ethambutol and pyrazinamide	Pyrazinamide	–	–	–	100.0
Agents for the treatment of rifampicin-resistant and multidrug-resistant TB *(27)*[31]					
Group A	Fluoroquinolones:[f]				
	Levofloxacin (CC)[g]	2.0	1.0	–	1.0
	Moxifloxacin (CC)[g]	1.0	0.5	0.5	0.25
	Moxifloxacin (CB)[h]	–	2.0	–	1.0
	Bedaquiline[i]	–	–	0.25	1.0
	Linezolid	–	1.0	1.0	1.0
Group B	Clofazimine[j]	–	–	–	1.0
	Cycloserine/ terizidone[j]	–	–	–	–
Group C	Ethambutol	2.0	5.0	7.5	5.0
	Delamanid[i]	–	–	0.016	0.06
	Pyrazinamide	–	–	–	100.0
	Imipenem-cilastatin/ meropenem	–	–	–	–
	Amikacin	30.0	2.0	–	1.0
	Streptomycin	4.0	2.0	2.0	1.0
	Ethionamide	40.0	5.0	10.0	5.0
	Prothionamide	40.0	–	–	2.5
	p-aminosalicylic acid	–	–	–	–

CB: clinical breakpoint; CC: critical concentration; DST: drug-susceptibility testing; LJ: Löwenstein–Jensen; MGIT: mycobacterial growth indicator tube; RFB: rifabutin; RIF: rifampicin; RPT: rifapentine; TB: tuberculosis; WHO: World Health Organization.

Note: All concentrations are in mg/L and apply to the proportion method, with 1% as the critical proportion. Unless otherwise stated, they are CCs rather than CBs. Red font indicates updated CC for RIF in 2021.

[a] MGIT is proposed as the reference method for performing DST for second-line anti-TB medicines.

Drug concentrations and preparations

- In case of commercial kits → follow manufacturer's instructions (e.g. MGIT)
- In case of in-house preparation:
  - Stock solutions, usually
    - 10.000 µg/ml, considering potency (see later)
    - Stored in aliquots at -20°C or -80°C
    - Solvent specific for drug (classes)
    - Not to be refrozen after thawing
  - Working solutions
    - Dilutions to achieve lower concentrations
    - Not to be stored after use
  - Assure minimal volumes to be added to the medium
    - To avoid diluting the growth medium too much

Potency of a drug

- Antimicrobial agents are assayed for standard units of activity or potency
  - Defined based on in vitro and in vivo experiments
  - May differ from the actual weight of the powder
  - May differ by production lot

- Standardize stock and working solutions of a drug based on the lot used
  - Potency value provided by manufacturer
  - Apply the formula to measure the actual weight

$$\text{Weight (mg)} = \frac{\text{Volume (ml)} \times \text{Concentration (µg/ml)}}{\text{Potency (µg/mg)}}$$

Example: For an agent with a potency of 904 µg/mg requiring 415 µg/ml stock solution in 50 ml of diluent:

50 x 415/ 904 = 22.9 mg of the agent

Quality assurance(QA): Why?

- To ensure accurate and reliable testing
- A requirement for analysis under ISO 15189 accreditation

- To detect, evaluate and correct errors due to test system failure, environmental conditions or operator performance, before patient results are reported
  - Product may be mishandled during shipment
  - Laboratory performance/ability to accurately perform test

QA: How?

- A comprehensive and systematic QA programme should be implemented, to enable laboratories to achieve and maintain high levels of accuracy and proficiency in testing
  - Through Supranational Reference Laboratory Network (https://sites.google.com/view/srln/home)

- Key QA activities include:
  - Training and competence assessment
  - Instrument verification
  - Equipment maintenance
  - Method validation
  - Quality control (QC) of reagents by lot testing
  - External quality assessment (EQA) by proficiency panel testing
  - Quality indicators monitoring
  - Continuous quality improvement.

Quality indicators for phenotypic DST

Indicator[a]	Description	Target
Number and proportion of isolates with monoresistance and multidrug resistance to all combinations of drugs tested (e.g. isoniazid monoresistance, rifampicin monoresistance, MDR)	Number of isolates resistant to single or multiple drug combination/total number of isolates tested Stratify by each drug tested	Dependent on population tested and drug resistance prevalence and patterns
Number and proportion of isolates inoculated for DST that were discarded due to contamination	Number of isolates discarded due to contamination/total number of isolates inoculated for DST	<3%
Number and proportion of isolates inoculated for DST that were uninterpretable due to lack of growth of control (drug-free) tubes/plates	Number of isolates discarded due to lack of growth on drug-free media/ total number of isolates inoculated for DST	<3%
Laboratory turnaround time	Time between inoculation of DST and result reporting (mean, range and 90th percentile)	Solid media: 3–4 weeks Liquid media: 2–3 weeks
	Total DST turnaround time including time for primary culture to produce inoculum	Solid media: 8–16 weeks Liquid media: 4–6 weeks

DST: drug-susceptibility testing; MDR: multidrug resistance.

[a] See *MGIT procedure manual* (https://www.finddx.org/wp-content/uploads/2016/02/mgit_manual_nov2006.pdf) *(2)*, *GLI mycobacteriology laboratory manual* (https://stoptb.org/wg/qli/qat.asp) *(3)*.and *Technical manual for drug susceptibility testing of medicines used in the treatment of tuberculosis* (https://apps.who.int/iris/handle/10665/275469) *(5)*.

Quality control (QC) of DST

- QC of DST is critical to ensure its accuracy & properly functioning
  - On all new lots or new shipment of drugs
  - Weekly with each batch of testing
  - To be done for the method used for routine testing
  - Testing reference strains
    - Pan-susceptible H37Rv
    - Specific drug-resistant strains optional (recommended at least for implementation validation)

    - QC organism suspension can be prepared from MGIT tube or solid media
    - Organism suspension must be homogeneous, without clumps

  - Accurate pipettes must be used, not disposable transfer pipettes

WHO recommended quality control strains (2024)

Annex Table 2. Recommended quality control strains (37)

Drug grouping	Drug	ATCC	BCCM/ITM	Lineage	Resistance mechanism[b]
	Susceptible H37Rv strain	27294[a]	500735	4	none
First-line	Rifampicin-R	35838[a]		4	*rpoB* S450L[c,d]
	Isoniazid low-level-R	BAA-812[a]		not known	*inhA* C-15T[e]
	Isoniazid high-level-R	35822[a]		4	complete *katG* deletion[c,d,f]
	Ethambutol-R	35837[a]		4	not known
	Pyrazinamide-R	35828[a]		4	*pncA* G132S[c]
Group A	Fluoroquinolone high-level-R		500831[g]	2	*gyrA* D94G[h]
	Bedaquiline-R		500807	4	*atpE* A63P
	Linezolid-R		501291[g]	4	*rplC* C154R
Group B	Clofazimine-R		500861[g]	1	*Rv0678* Y92Stop[i]
	Cycloserine-R		501136[g]	4	*alr* D320N[j]
Group C	Delamanid-R/		501095	2	*ddn* Q58Stop[k]
	Carbapenems-R	No DST method exists			
	Amikacin-R		501330[g]	4	*rrs* A1401G
	Streptomycin-R	35820[g]		4	*rpsL* K43R[d]
	Ethionamide/prothionamide-R	BAA-812[g]		not known	*inhA* C-15T[e]
	Para-aminosalicylic acid-R	DST not recommended			
Other	Pretomanid-R		501095	2	*ddn* Q58Stop[k]

Trouble shooting

Trouble shooting MGIT

Event	Probable cause	Troubleshooting
X400 for MGIT DST: GC or drug containing tubes turn positive in less than 4 days	Inoculum too heavy or contamination	Ensure that the correct MacFarland bacterial suspension is made. Ensure the use of only pure cultures for DST with the correct aseptic techniques.

Trouble shooting LJ

Event	Probable cause	Troubleshooting
Contaminated slants in LJ DST	Inoculum contained mixed growth of MTBC and contaminants. Materials used were not sterile. Poor aseptic technique that introduces contaminates during the DST manipulation	Ensure the use of only pure cultures for DST with the correct aseptic techniques. Ensure that only sterile materials are used for DST manipulations. Verify that aseptic practices are always maintained.

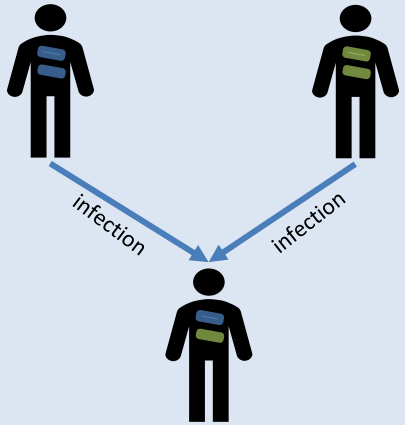
Summary of common errors and their impact

Examples of typical confounding factors / sources of error of DST	Drug activity	Resulting shift of measured MIC	Typical outcome of DST in MGIT
Calculation errors			
False weight or volume caluclated (e.g. due to a shift of the dot position: 10 mg instead of 1.0 mg)	↘ or ↗	↗ or ↘	FR or FS
Wrong or forgotten potency /activity factor	↘ (or ↗)	↗ (or ↘)	FR (or FS)
Measuring factors / errors			
Balance or pipettes not or wrongly calibrated	↘ or ↗	↗ or ↘	FR or FS
Transfer of false volumes (e.g. 25 instead of 50 ml)	↘ or ↗	↗ or ↘	FR or FS
Chemical factors / errors			
Incomplete dissolution (e.g. due to wrong diluent, wrong temperature)	↘	↗	FR
Precipitation (e.g. due to wrong diluent, wrong temperature)	↘	↗	FR
Absorption to surfaces (e.g. of plastic tubes or pipettes)	↘	↗	FR
Loss of drug activity (e.g. by wetting, irradiation, decay)	↘	↗	FR
Storage errors			
Fluctuating or too high storage temperature	↘	↗	FR
False stem solution management (multiple thawing / freezing cycles)	↘	↗	FR
Exposure of drug powder to light / humidity	↘	↗	FR

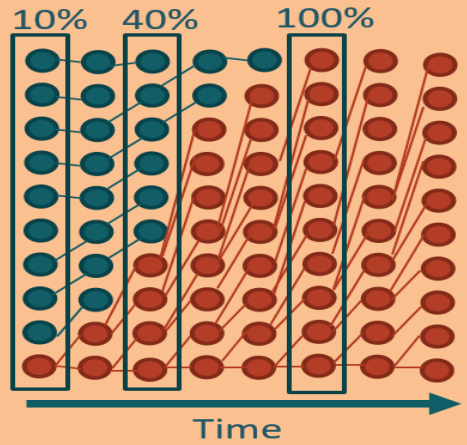
Hetero-resistance

Heteroresistance = simultaneous presence of R (mut) and S (WT) bacilli

Mixed infection: S & R strains

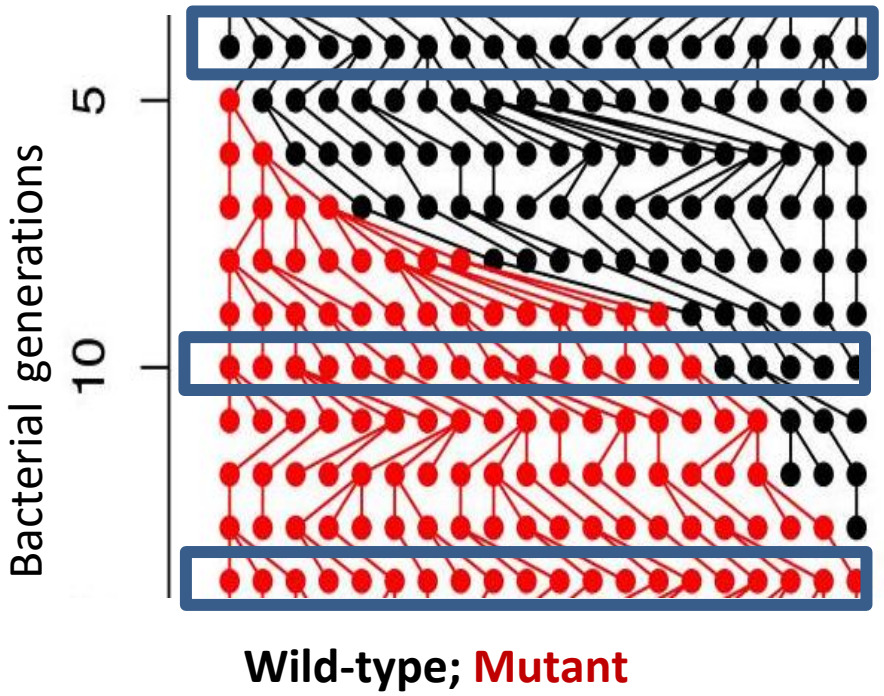


Within **patient ongoing evolution**
→ S & R populations from same strain



Both can lead to bacillary variation between sputum specimens

Limit of detection for heteroresistance varies by technique

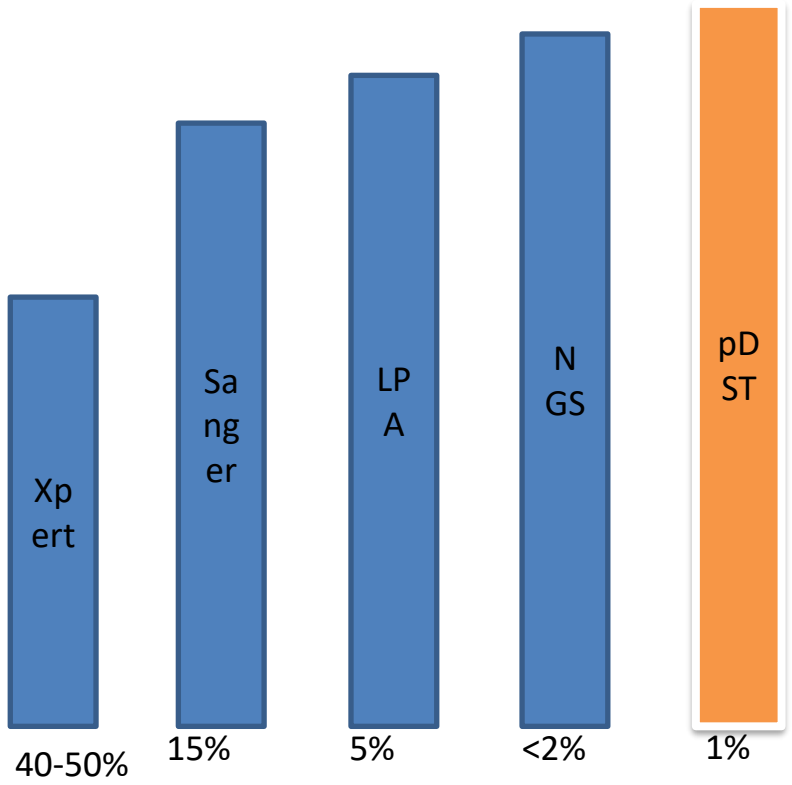


Pure wildtype population

Heteroresistance "variable mutation"
75% mutant- 25% wildtype

Pure mutant population-
"fixed mutation"

Proportion of mutants at which a test will identify resistance



Cross-resistance

Cross-resistance

Defined as

Organisms being resistant to a drug without being exposed to that drug

→ resistance to multiple anti-TB agents caused by a single genetic change

- Common among drugs of the same class, e.g. fluoroquinolones

  → Same underlying mode of action: Gyrase enzyme (*gyr*A and *gyr*B gene mutations)

- Also among drugs from different classes

  - Bedaquiline & clofazimine

    → Same drug resistance mechanism through efflux pump (regulator)

  - Isoniazide & ethionamide

    → Same drug resistance mechanism through mutations in the promotor region of the *inhA* gene