The EUCAST approach for drug susceptibility testing of mycobacteria: past, present, future

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Chair of the Antimycobacterial susceptibility testing subcommittee of EUCAST

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European Society of Clinical Microbiology and Infectious Diseases

- Organized by ESCMID, ECDC and national antimicrobial committees (NAC)
- Since 1997, restructured in 2002
- Defining ECOFF and breakpoints for all antimicrobials against bacteria and fungi
- Request by EMA for all new antimicrobials
- http://www.eucast.org

How EUCAST was involved in mycobacteria susceptibility testing?

EUCAST/EMA meetings

- **Delamanid** or OPC-67683 (*Otsuka*)
  - November 22, 2011
  - April 3, 2012
  - July 2, 2012
  - November 5, 2012
  - February 2013, EMA
- **Bedaquiline** or TMC-207 or R207910 (Janssen, Johnson and Johnson)
  - February 2013
  - April 30th 2013
  - September 23, 2013
  - February 19, 2014

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started 2011

=> Examples of results produced for the EMA submission of new ATTB

Discrepancies between two MIC methods after repeated testing

MTB Resistance Subtype	Sample Collection Date	Agar MIC (mg/L)	REMA MIC (mg/L)
XDR-TB	28-Dec-09	0.03	≤0.008
PRE-XDR-TB	27-May-10	0.03	0.03
PRE-XDR-TB	8-Sep-09	0.06	0.015
MDR-TB	15-Oct-07	0.25	0.015
MDR-TB	6-Oct-09	0.25	0.03
XDR-TB	14-Jun-10	0.25	0.06
MDR-TB	26-May-08	0.5	0.015
PRE-XDR-TB	14-Jan-10	≥1	0.12
MDR-TB	22-Jul-07	≥1	0.25
PRE-XDR-TB	18-Aug-10	≥1	0.25
PRE-XDR-TB	1-Jul-10	≥1	0.25
XDR-TB	21-Feb-11	≥1	0.25
XDR-TB	16-Mar-11	≥1	0.5
XDR-TB	29-Oct-10	≥1	1

How to set breakpoints with so different MIC values?

MTB Resistance Subtype	Sample Collection Date	Agar MIC (mg/L)	REMA MIC (mg/L)
XDR-TB	28-Dec-09	0.03	≤0.008
PRE-XDR-TB	27-May-10	0.03	0.03
PRE-XDR-TB	8-Sep-09	0.06	0.015
MDR-TB	15-Oct-07	0.25	0.015
MDR-TB	6-Oct-09	0.25	0.03
XDR-TB	14-Jun-10	0.25	0.06
MDR-TB	26-May-08	0.5	0.015
PRE-XDR-TB	14-Jan-10	≥1	0.12
MDR-TB	22-Jul-07	≥1	0.25
PRE-XDR-TB	18-Aug-10	≥1	0.25
PRE-XDR-TB	1-Jul-10	≥1	0.25
XDR-TB	21-Feb-11	≥1	0.25
XDR-TB	16-Mar-11	≥1	0.5
XDR-TB	29-Oct-10	≥1	1

Guidelines to be done for new ATTB drugs (European medical agency)

1. MIC determination methods
2. Strains tested
3. Interlaboratory reproducibility
4. Study on non-susceptible or non wild type isolates
5. Selection of in vitro mutants
6. Diagnosis methods for susceptibility testing

EUCAST subcommittee on antimycobacterial susceptibility testing (AMST) created in 2016

- Chair: Emmanuelle Cambau
- Scientific Secretary: Thomas Schön
- ESGMYC members: Miguel Santin and Miguel Viveiros
- ERLTB-net members: Daniella Cirillo and Sven Hoffner/Jim Werngren
- EUCAST members: Gerard Lina and Johan Mouton, Christian Giske and Gunnar Kahlmeter

http://www.eucast.org/mycobacteria/

Cambau E and Rush-Gerdes S, ECDC laboratory Handbook: http://ecdc.europa.eu, Schon T et al. Clinical Microbiology and Infection 2017

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AMST objectives

- Define a reference method for MIC determination on *M. tuberculosis* complex
- setting breakpoints for anti-mycobacterial agents, especially new ones
- Calibration of DST methods with the reference method
- Draft SOP for NTM reference MIC method

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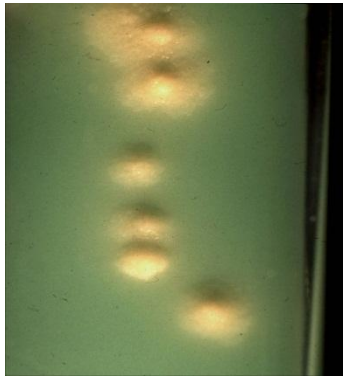
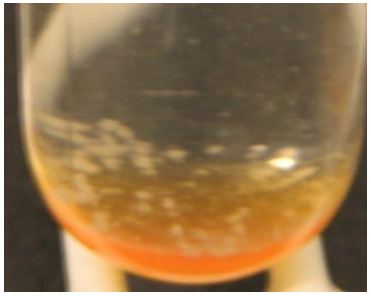
AMST objective 1

- **Define a reference method for MIC determination on *M. tuberculosis* complex**
- setting breakpoints for anti-mycobacterial agents, especially new ones
- Calibration of DST methods with the reference method
- Draft SOP for NTM reference MIC method

Technical issues related to *Mycobacterium tuberculosis*





Highly pathogenic
(BSL3)

Slow growth
(20h MTB vs 20 min E. coli)

in vitro growth
requires rich medium
(no Mueller Hinton
as used for other bacteria)

Poor reproducibility within labs
MIC of bedaquiline for reference strains

WHO systematic review 2018

Table 53. BDQ MICs for pWT isolates on 7H11.

Studies	Lab	Isolate origin	Unique isolates	Total MICs	Type of isolates	Genotypic results	BDQ MIC (mg/L) 0.008	0.015	0.03	0.06	0.12	0.25	0.5	1
4) Kaniga 2016	1	lab strain	1	30	H37Rv ATCC 27294			1	12	11	6			
	2		1	32	H37Rv ATCC 27294		1	3	25	3				
	3		1	30	H37Rv ATCC 27294				23	7				
	4		1	30	H37Rv ATCC 27294					30				
	5		1	30	H37Rv ATCC 27294				3	13	5	9		
	6		1	30	H37Rv ATCC 27294			13	8	9				
	7		1	30	H37Rv ATCC 27294			10	12	8				
	8		1	30	H37Rv ATCC 27294				5	24	1			
4) Kaniga 2016	1-8	sum of dataset 5	1	242	H37Rv ATCC 27294		1	27	88	105	12	9		
5) Diacon 2014a &	10		325	325	baseline isolates from trial 208 & 209	gWT	4	35	77	157	49	3		
2014b, Pym 2016 &	10	clinical	1	1	post-baseline isolates from trial 209				1					
Villellas 2016	10		3	3	post-baseline isolates from trial 209	gWT			1	2				
6) Torrea 2015	10	clinical	77	77	different levels of R		2	3	13	42	16	1		
7) Andries 2005	11		1	2	H37Rv				1	1				
	11	clinical	22	22			3	7	2	8	2			
8) Veziris 2017	12		1	1	H37Rv				1					
	12	clinical	1	1	MDR	gWT							1	
	12		1	1	MDR	gWT parent		1						
9) Zimenkov 2017	13		1	1	H37Rv ATCC 25618				1					
	13	clinical	21	21	mostly XDR	gWT			15	4	2			

The purple line denotes the current EUCAST CB for BDQ testing (0.25 mg/L). Notable limitation: Studies 5 and 6 were conducted in the same laboratory.

Reproducibility is a challenge

TABLE 3 Concordance between CDC and PHL phenotypic DST results for MTBC isolates

Discordance	MDDR phenotypic DST result	Phenotypic DST result from PHL (no. of isolates)				Total no. of isolates
		MDR[a]	RMP-R	INH-R	Susceptible	
No	MDR	63	0	0	0	63
	RMP-R	0	3	0	0	3
	INH-R	0	0	21	0	21
	Susceptible	0	0	0	75	75
Yes	MDR	0	0	2	0	2
	RMP-R	1	0	0	1	2
	INH-R	3	1	0	2	6
	Susceptible	3	1	4	0	8
Total		70	5	27	78	180

[a] For an explanation of abbreviations, see Table 1.

Yakrus MA et al JCM 2014

Impact of inoculum size

Example of bedaquiline

Table 1

Impact of inoculum size on BDQ MIC against *M. tuberculosis* H37Rv strain in 7H9 broth[a] and on 7H11 agar.

Influence de la taille de l'inoculum sur la CMI de la BDQ vis-à-vis de la souche M. tuberculosis *H37Rv mesurée dans un bouillon 7H9 et dans une gélose 7H11.*

	MIC (µg/mL) in 7H9 broth			MIC (µg/mL) on 7H11 agar		
Inoculum size (CFU/mL)	10^5	10^6	10^7	10^5	10^6	10^7
BDQ	0.06	0.25	1	0.03	0.06	0.12
	0.06	0.25	1	–	–	–
INH	0.06	0.06	>32	0.03	0.03	1
	0.06	0.06	>32	–	–	–

MI: minimal inhibitory concentration; BDQ: bedaquiline; INH: isoniazid.

[a] Tests in broth were conducted in duplicate and both sets of results are shown.

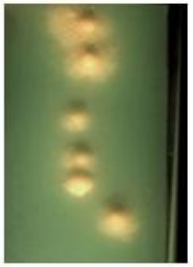
Lounis N, Med Mal Inf 2016, 46: 220

Standardization ++++

- Same protocol for preparing the inoculum
- Same antibiotic concentrations
- Same presentation of results
- Same medium
- Same antibiotic pure powders
- Same protocol for antibiotic stocks
- Same protocol for antibiotic dilutions
- Solvant issues when needed

Inoculum preparation +++ => homogenous and reproducible counting

Culture on LJ
Within 2 weeks
of growth



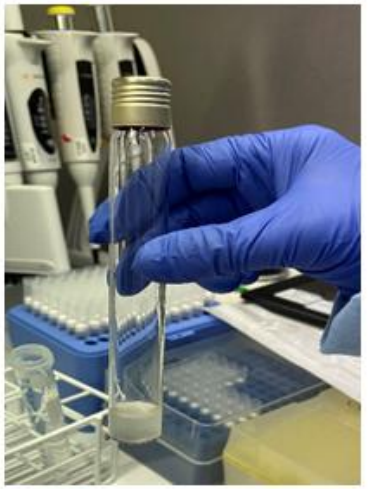
Add
4-5 loops of 1ul
or a full 3 mm loop

Vortex >2 min with
3 mm glass beads

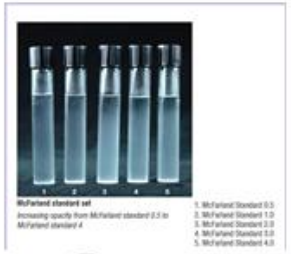
THEN

Add 5 ml sterile water
Allow to settle 30 min

Homogenous
suspension



Adjust with more dH20
if necessary



0.5 Mc Farland
suspension

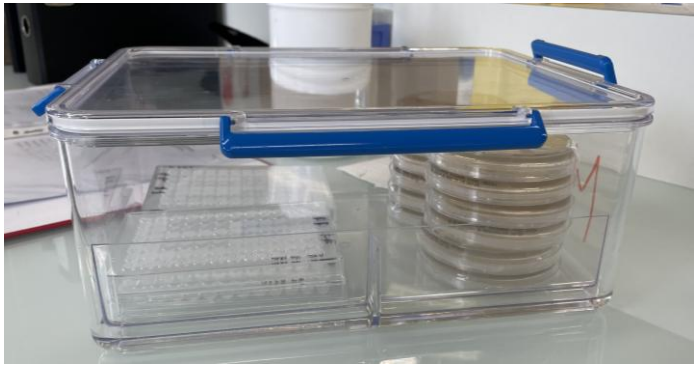
Final inoculum
(10^{-2})

Inoculum target
$1x10^5$ CFU/ml

10^4 CFU per well

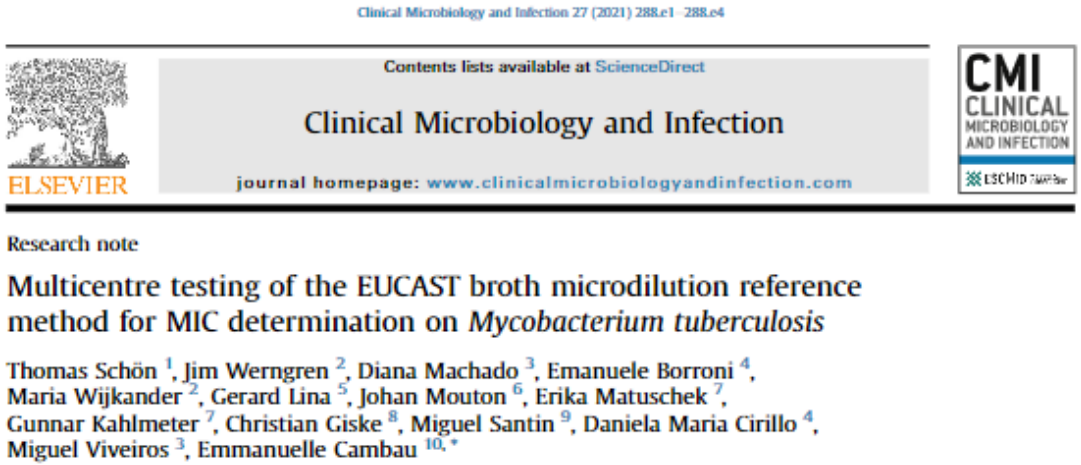
Setting the broth microdilution plate

- Drug dilutions (at least 8 values surrounding the target value)
- 7H9-10% OADC broth
- 100 µl inoculum
- Microtiter plates in polystyrene
- Covered with lid
- Incubated 36 ± 1°C



AMST labs testing and intra- and inter-lab comparisons: broth and agar dilution

- Reproducibility was good with 7H9 microdilution (BMD) and 7H10 agar dilution
- 7H9: intralaboratory agreement for 100% for INH, LEV, AMI. and interlaboratory agreement Day 7, 100% and 88-100% at day 14 (lowest for INH)
- Repeatibility and reproducibility with $10^{-2} > 10^{-1}$
- MIC reading 100% inhibition, 99% inhibition



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Contents lists available at ScienceDirect

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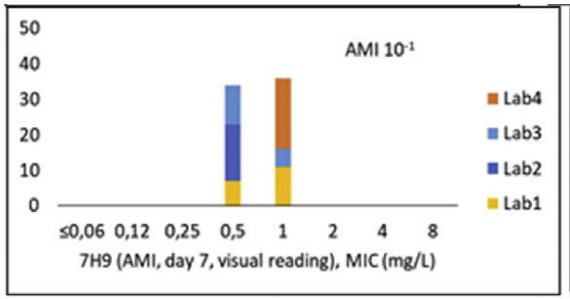
journal homepage: www.clinicalmicrobiologyandinfection.com

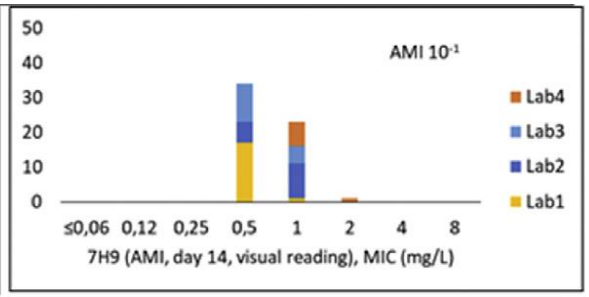
Research note

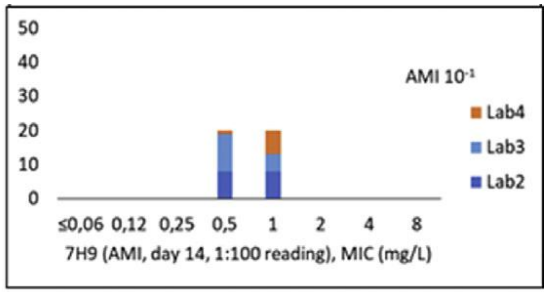
Multicentre testing of the EUCAST broth microdilution reference method for MIC determination on *Mycobacterium tuberculosis*

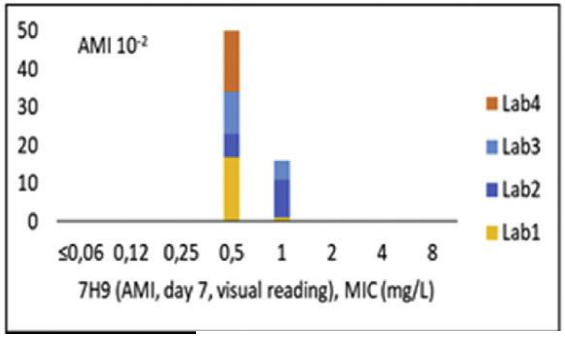
Thomas Schön [1], Jim Werngren [2], Diana Machado [3], Emanuele Borroni [4], Maria Wijkander [2], Gerard Lina [5], Johan Mouton [6], Erika Matuschek [7], Gunnar Kahlmeter [7], Christian Giske [8], Miguel Santin [9], Daniela Maria Cirillo [4], Miguel Viveiros [3], Emmanuelle Cambau [10,*]

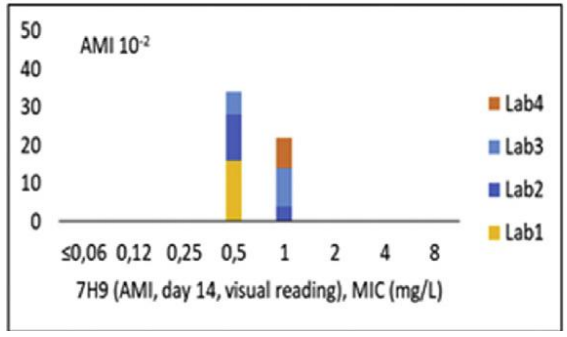
Easy to analyse, e.g. amikacin

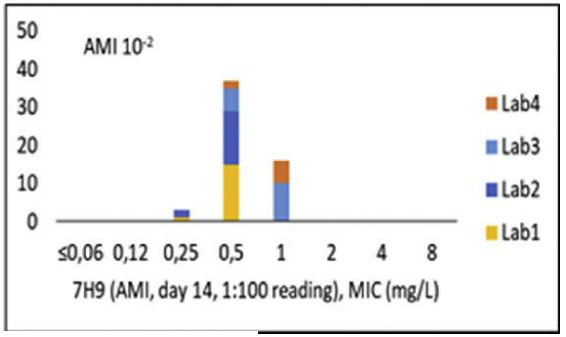


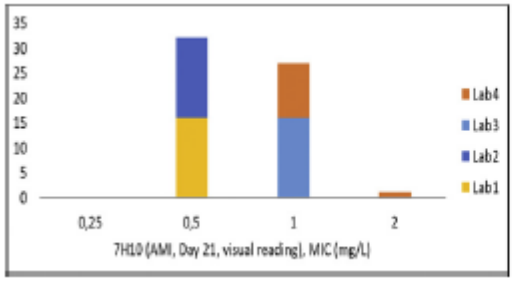


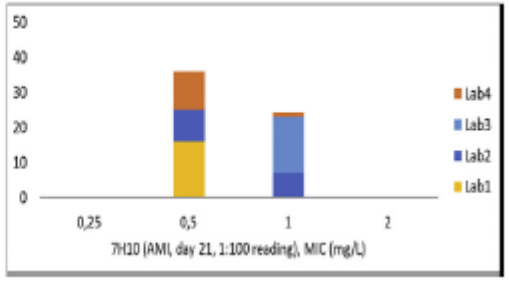






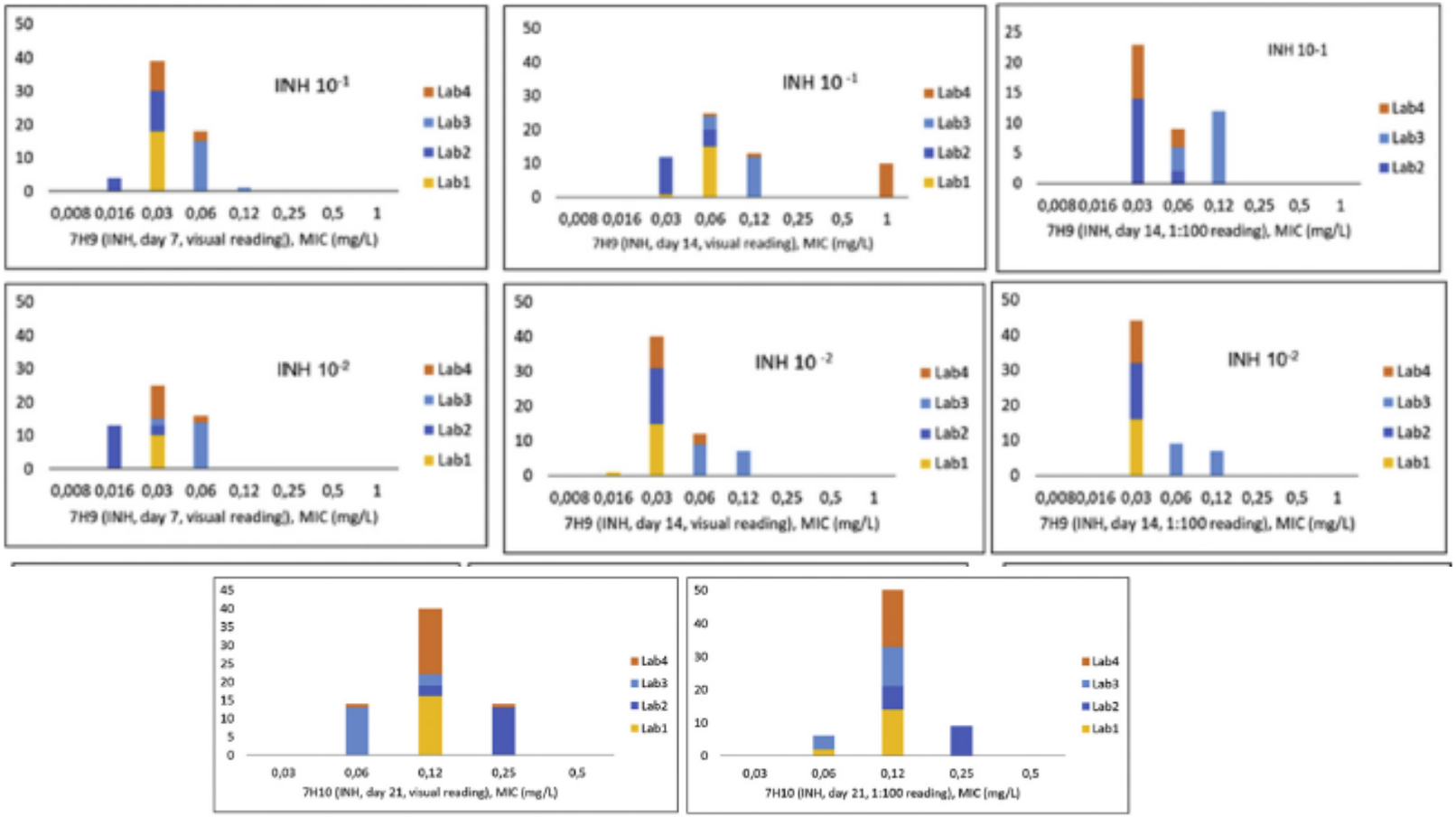






MIC range : 0.5 – 1 mg/L

More difficult: e.g. isoniazid



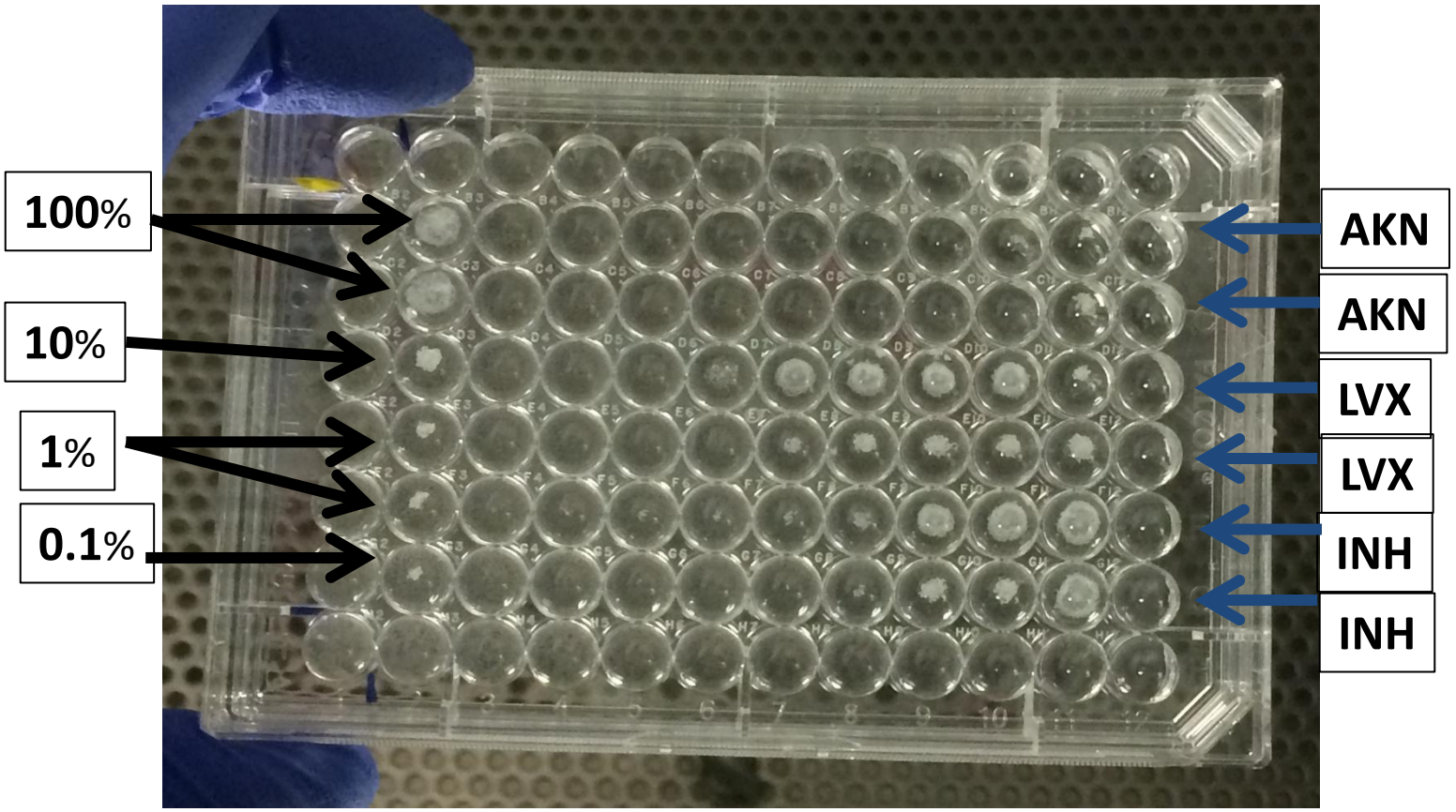
MIC range : 0.016 – 1 mg/L

EUCAST Reference protocol for MIC determination of antituberculous agents

- Broth microdilution method
- Microtiter plate in **polystyrene** with a **lid**
- Medium: **Middlebrook 7H9 + 10% OADC**
- Inoculum: 10^{-2} of 0.5 Mc Farland => **10^4 per well** (100µL)
- **Two growth controls (GC) wells as 1:1 (100%) and 1:100 (1%)**
- **Reading when GC 1% is positive, between 7 to 14 days**.
- MIC is the lowest concentration that inhibits visual growth.

https://www.eucast.org/mycobacteria/methods_in_mycobacteria/
released July 2019

MIC testing : growth controls with different inoculum



In ATB containing wells, growth could be as 100%, 10% , 1% or no growth

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2019

Reference protocol for MIC determination of anti-tuberculous agents against isolates of the *Mycobacterium tuberculosis* complex in Middlebrook 7H9 broth

Version 6.1. 4th of July, 2019.

public consultation and EUCAST endorsement

Appendix 1. Plate outline (7H9 AMST In-house).

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CMI CLINICAL MICROBIOLOGY AND INFECTION ESCMID

Guidelines

Antimicrobial susceptibility testing of *Mycobacterium tuberculosis* complex isolates – the EUCAST broth microdilution reference method for MIC determination

Thomas Schön [1], Jim Werngren [2], Diana Machado [3], Emanuele Borroni [4], Maria Wijkander [2], Gerard Lina [5, 6], Johan Mouton [7], Erika Matuschek [8, 9], Gunnar Kahlmeter [8, 9], Christian Giske [10], Miguel Santin [11], Daniela Maria Cirillo [4], Miguel Viveiros [3], Emmanuelle Cambau [12, 13, *]

3	4	5	6	7	8	9	10	11	12
200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20
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
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
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
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
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
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
200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20

ulous agent 1-6 (May be expanded to 2 rows/agent depending on target MIC range)
ntrol
ulum as in the drug containing wells
id diluted inoculum compared to drug containing wells

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

http://www.eucast.org/mycobacteria/

Schon et al. CMI 2020, CMI 2021

Ongoing annexe for DMSO-dissolved agents

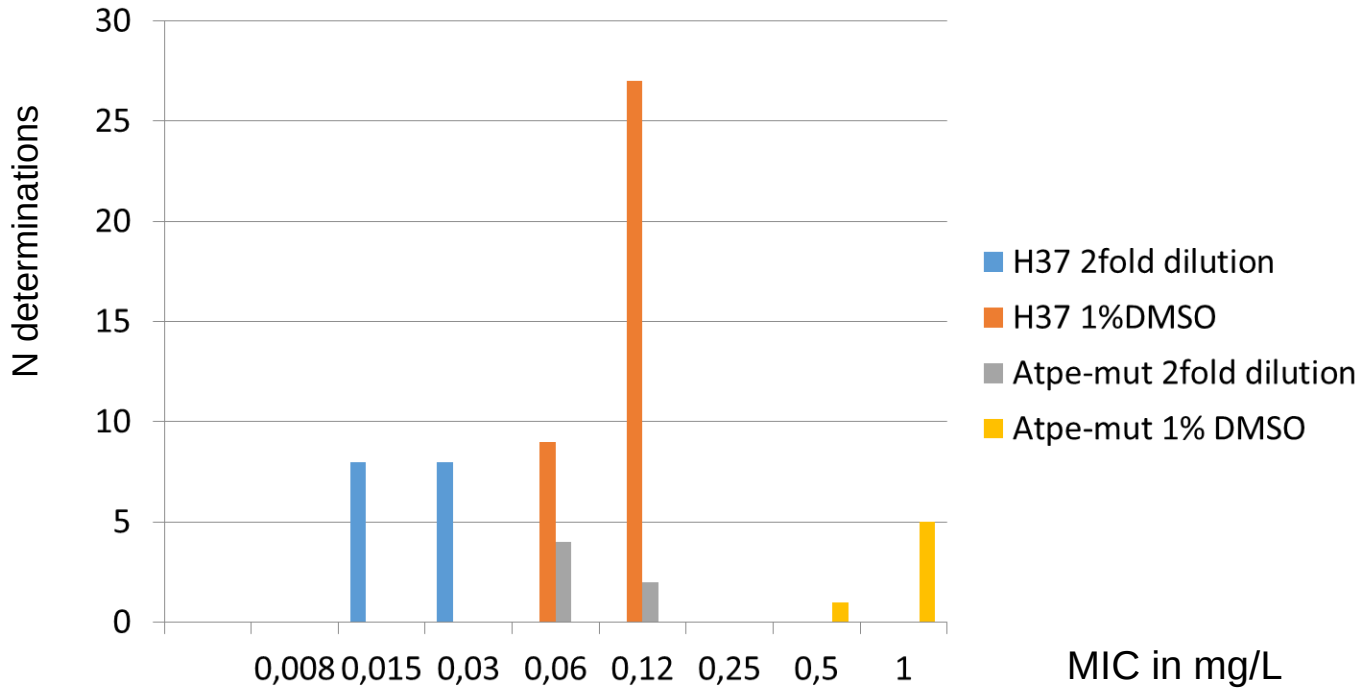
Antituberculous drugs

- Bedaquiline
- Delamanid
- Pretomanid
- Clofazimine
- …

Issues

- Source of the powder
- Stock solution preparation
- Final % DMSO in the wells
- Drug dilutions for reaching the final % DMSO without precipitation
- Polystyrene tubes and tips

Effects of DMSO% on BDQ MIC (10-2 inoculum, Eucast protocol)



Cambau E, personal results

Delamanid MIC testing (preliminary results with the Eucast protocol)

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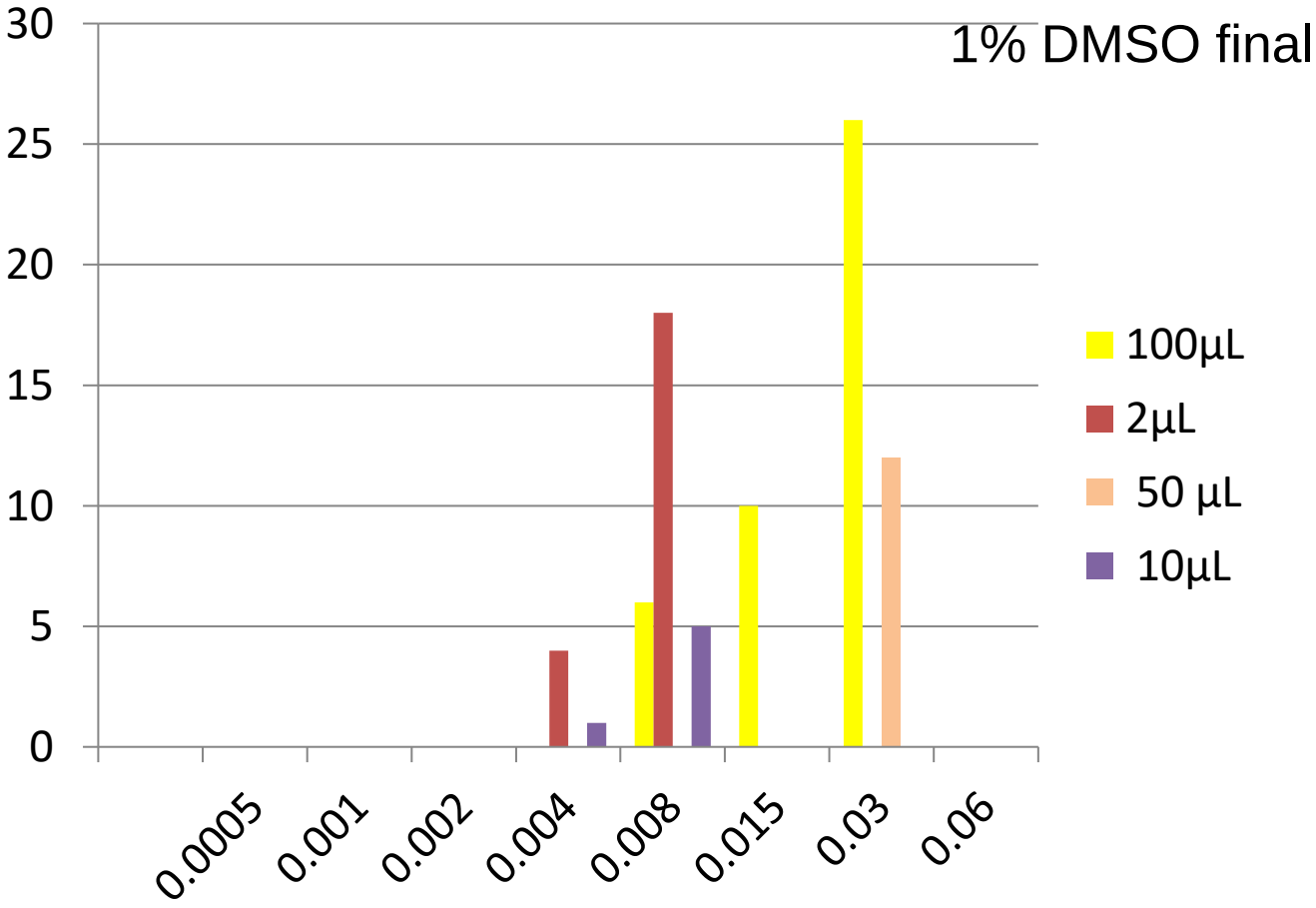

Cambau E, personal results

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AMST objective 2

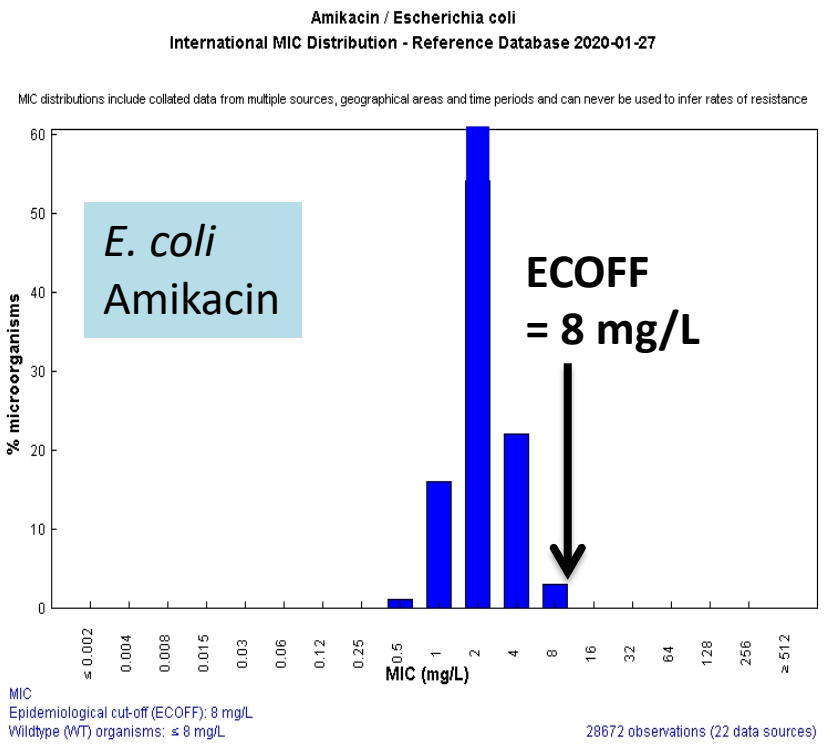
- Define a reference method for MIC determination on *M. tuberculosis* complex
- **Setting breakpoints for anti-mycobacterial agents, especially new ones**
- Calibration of DST methods with the reference method
- Draft SOP for NTM reference MIC method

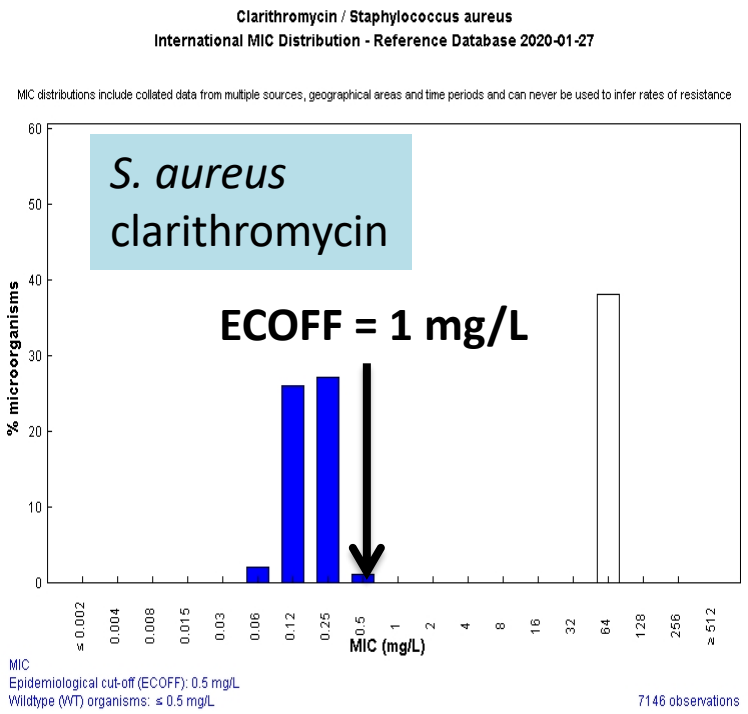
Setting epidemiological cut off (ECOFF) MIC values

ECOFF is the highest MIC observed for wild type strains or strains without acquired resistance mechanisms

At least 15 wt strains and total 100 , and 5 different distributions

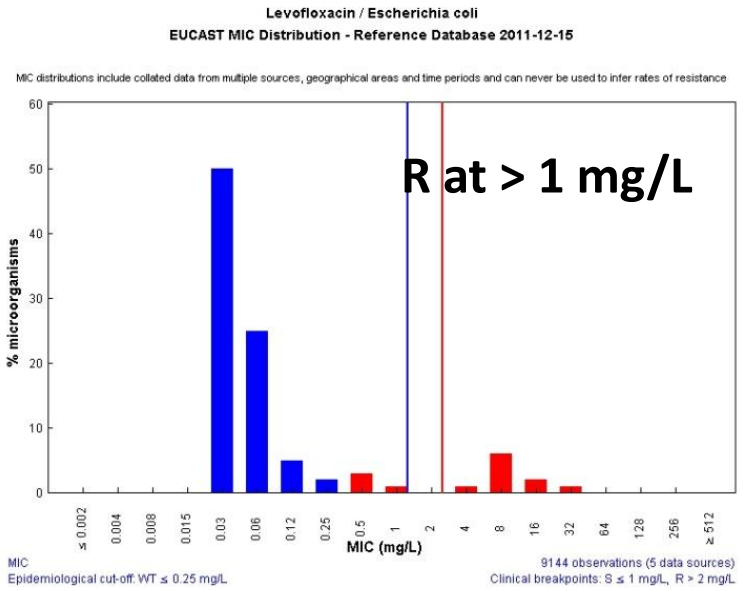
5 dilutions, end and first with 1-5%; if 3-4 dilutions, 5-10% population





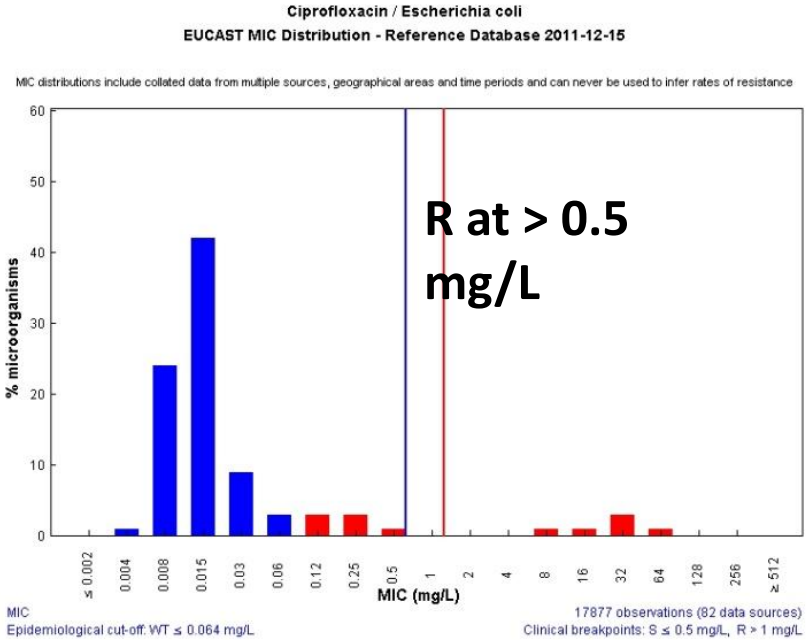
Setting clinical breakpoints

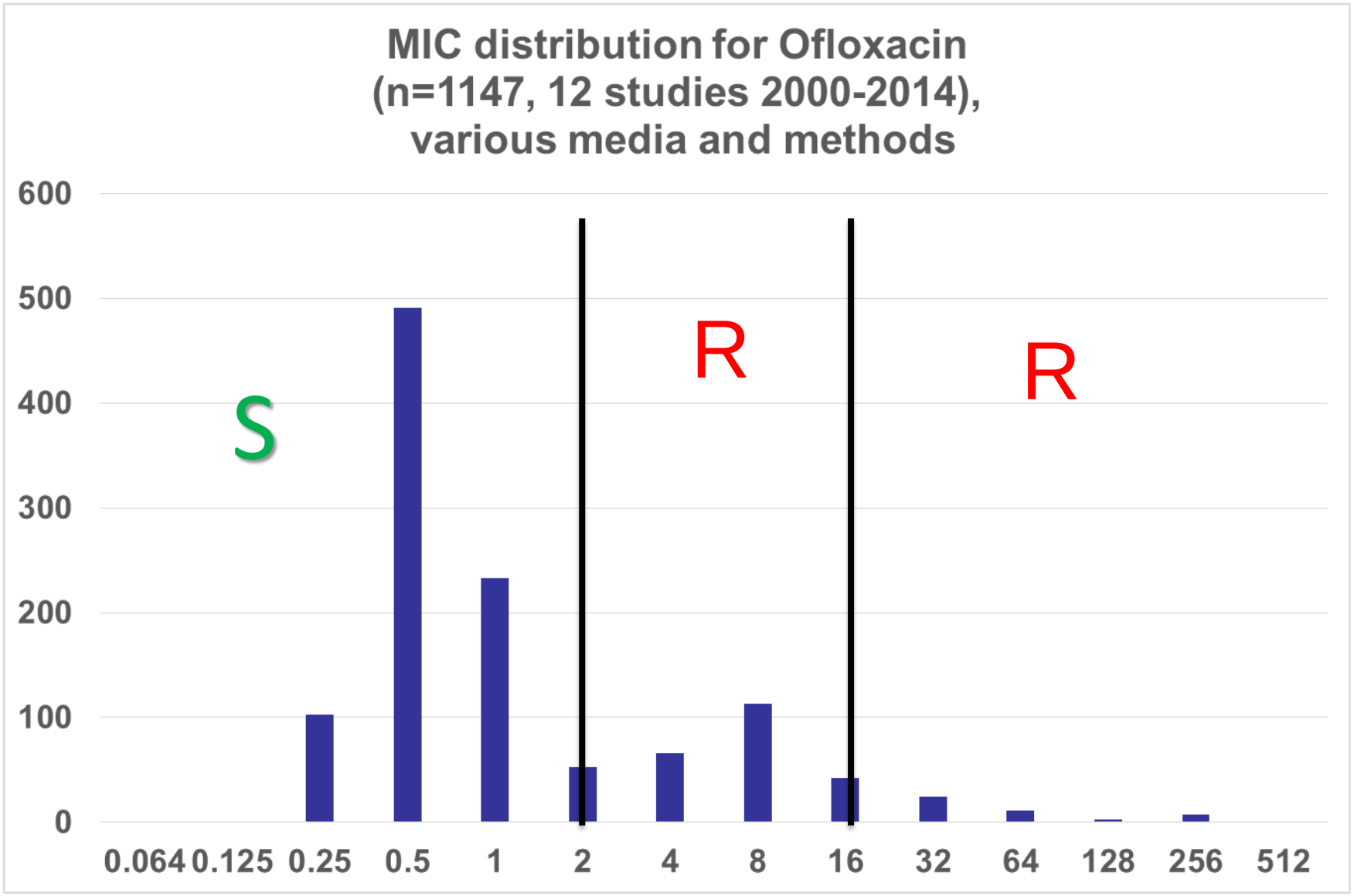
eg MIC distribution for quinolones and *Escherichia coli*



Levofloxacin

Ciprofloxacin

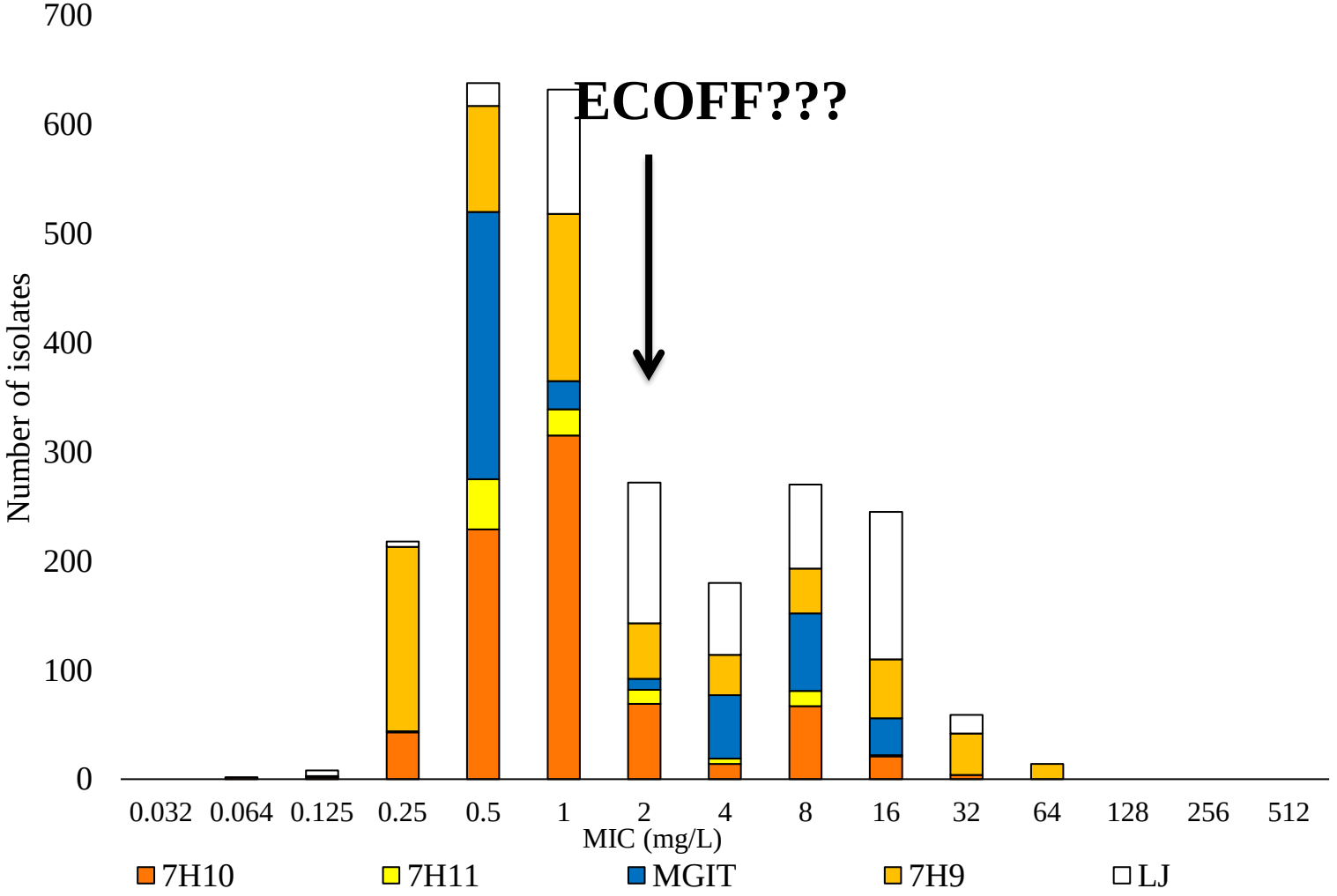




T. Schon, Eucast –Esgmyc workshop 2014,

Ofloxacin MIC according to the medium

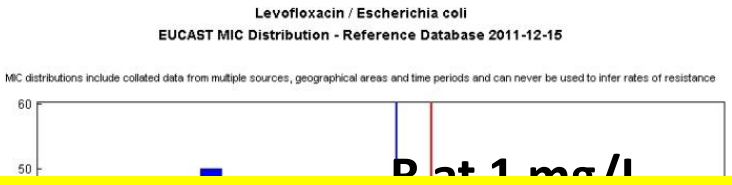
2538 isolates, 22 studies



Schön T et al. 2017

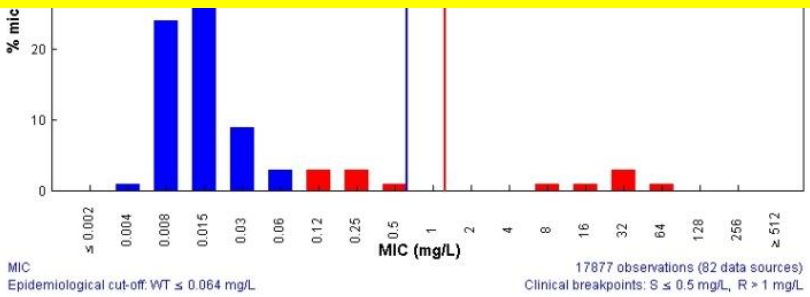
Setting clinical breakpoints

eg MIC distribution for quinolones and *Escherichia coli*



Setting breakpoints requires more than ECOFF

- Pharmacokinetics (normal dosage)
- Pharmacodynamics
- Patient variation
- Treatment outcome data



Lessons taken from the past: AST should separate wild-type strains from resistant ones

- **Resistance** : decrease in sensitivity compared to wild type strains (never came into contact with the drug).
  - ➢ unlikely to show clinical responsiveness to the drug
- **Susceptibility** : level of sensitivity not significantly different from wild type strains
  - ➢ likely to show clinical responsiveness to the drug
- **Critical concentration :** the lowest drug concentration at which wild type strains did not grow

Canetti 1963, Mitchison DA 1968, Heifets L ARRD 1988

Bedaquiline provisory breakpoint

4. Breakpoints Prior to Harmonisation (mg/L) S≤ / R>							
				WHO – MGIT (2018)*	WHO – 7H11 (2018)*	EUCAST** (2014)	CLSI (2018)
General breakpoints	No breakpoints						
Species-related breakpoints							
Mycobacterium tuberculosis				1	0.25	0.25	NA

*Tentative breakpoint based on limited data.

**Provisional clinical breakpoint valid for Middlebrook 7H10 and 7H11 media until MIC distribution data in a reference method is available. EUCAST does not issue breakpoints in commercial methods such as BACTEC 960 MGIT. When definite clinical breakpoints are set in a EUCAST reference method for *M. tuberculosis* it may be

9. Clinical Breakpoints				
PK/PD breakpoints	Not available.			
Species-related breakpoints	**Organism group**	**MIC breakpoints (mg/L)*** S ≤	R >	
	Mycobacterium tuberculosis	0.25	0.25	*Valid for Middlebrook 7H10 and 7H11
Clinical qualifications	Breakpoints apply for use in MDR/XDR-TB patients.			
Dosage	Breakpoints apply to a dosing schedule of 400 mg x1 for 2 weeks and then 200 mg three times weekly.			
Additional comment	*Provisional clinical breakpoint will be revised if needed as soon as MIC distribution data and ECOFFs are set in the EUCAST reference method for *M. tuberculosis*. After that, other commercial and non-commercial methods used should be calibrated against the reference method to apply for the clinical breakpoint. Breakpoints do not apply to non-tuberculous mycobacteria.			

Delamanid provisory breakpoint

4. Breakpoints prior to harmonisation (mg/L) S≤ / R>							
				WHO (2018) MGIT*	WHO (2018) 7H11*	EUCAST (2014)**	CLSI (2018)
General breakpoints	No previous breakpoints						
Species-related breakpoints	No previous breakpoints						
Mycobacterium tuberculosis				0.06	0.016	0.06	NA

*Interim breakpoint based on very limited data.
**Provisional clinical breakpoint based on limited data until MIC distribution data in a EUCAST reference method is available. EUCAST does not issue breakpoints in commercial methods. NA=Not assessed.

9. Clinical breakpoints				
PK/PD breakpoints				
Species-related breakpoints	Organism group	MIC breakpoints (mg/L) S ≤	MIC breakpoints (mg/L) R >	
	Mycobacterium tuberculosis	0.06*	0.06	*Media not specified
Clinical qualifications	Breakpoints apply for use in MXDR-TB patients.			
Dosage	Breakpoints apply to a dosing schedule of 100 mg twice daily.			
Additional comment	This is a provisional clinical breakpoint which is subject to revision as soon as more MIC, PK/PD and clinical outcome data are available in the EUCAST reference MIC method for *M. tuberculosis*. *Based on the available data, it is recommended that the ECOFF at 0.016 mg/L for Middlebrook 7H11 should be used for 7H10/7H11 until the next revision, in order to avoid classifying potentially low-level resistant isolates as susceptible. The few isolates with resistance mutations described so far in 7H11/7H10 show MICs of at least 0.5-1 mg/L (Gler 2012, Stinson 2016 and Schena 2016) but that does not exclude the possibility of low-level resistance slightly above the tentative ECOFF (0.016 mg/L) but below the current media-unrelated tentative clinical breakpoint (0.06 mg/L).			

Difficulty in choosing the best AMST technique to be used for MTBC

Cheap

Do-able in series

Accurate

Simple

Easy to implement

Safe

Non commercial based

Rapid results

Affordable in low-income countries

Robust

Commercially available

Reproducible

Techniques used for antimycobacterial susceptibility testing (AST)

Table 1

Phenotypic methods and techniques used for drug susceptibility testing in *Mycobacterium tuberculosis* [14]

Methods	Detection of *M. tuberculosis* growth	
	Direct evidence	Indirect evidence
Critical concentration	Resistance ratio	Nitrate reductase assay
	Absolute concentration	Resazurin test
	Microscopy observed direct susceptibility testing	Alamar blue test
	Microtitre plates (e.g. Versatrek)	
Critical proportion and critical concentrations	WHO and ECDC recommended protocol on Löwenstein–Jensen	Automated BACTEC MGIT960
	CLSI recommended protocol on 7H10 or 7H11	

T. Schön et al. / Clinical Microbiology and Infection 23 (2017) 154–160

Techniques detailed in Cambau E and Rush-Gerdes S, ECDC laboratory Handbook: http://ecdc.europa.eu, will be revised 2022

Calibration is needed between reference protocol and techniques used for DST

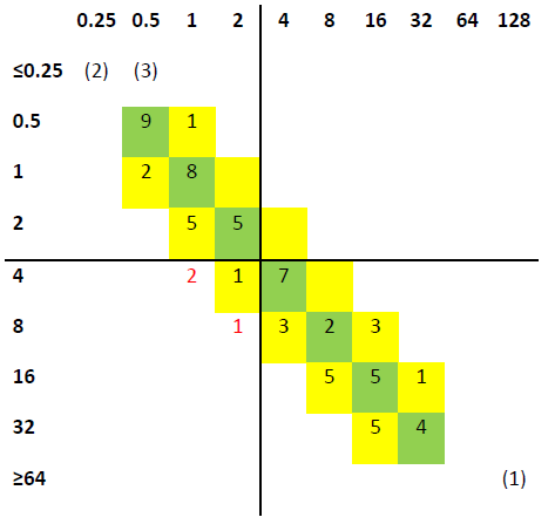
EUCAST EUROPEAN COMMITTEE ON ANTIMICROBIAL SUSCEPTIBILITY TESTING

European Society of Clinical Microbiology and Infectious Diseases

SOP for calibrating surrogate MIC methods for *M. tuberculosis* against the EUCAST reference MIC method

Version 1.0, 18th of July 2019

Based on EUCAST SOP 9.1, CLSI (M23-A5, M52), and ISO-20776-2:2007 by EUCAST-AMST in collaboration with the EUCAST development laboratory (Gunnar Kahlmeter and Erika Matuschek).



Conclusions

- EUCAST-AMST started to work by defining a standardized reference protocol
- Enabling to compare MIC values from different laboratories on different MTBC strains
- Enabling to calculate ECOFF on wild type isolates
- Enabling to calibrate for methods used in clinical microbiology labs for susceptibility testing

Many thanks to AMST colleagues