



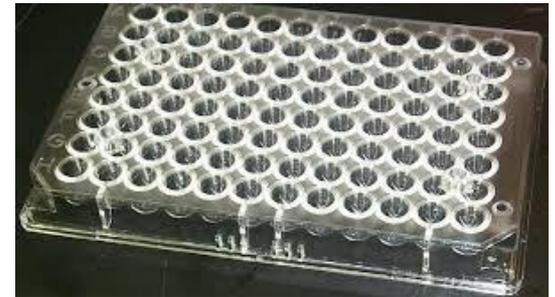
Pitfalls in routine broth microdilution

Jenny Åhman

NordicAST Workshop 2018

Broth microdilution

- Quantitative method to measure the *in vitro* antimicrobial activity against bacteria
- Broth microdilution
 - Microtitration plate (96 wells)
 - Two-fold dilutions of the antimicrobial agent
- **MIC - Minimum Inhibitory Concentration**
 - Expressed in mg/L or $\mu\text{g/mL}$.



ISO Standard 20776-1

Reference method for susceptibility testing of rapidly growing aerobic bacteria

Media	Mueller Hinton broth
Inoculum	5×10^5 CFU/mL
Incubation	Sealed panels Ambient air $35 \pm 1^\circ\text{C}$ 16-20 hours

- For every tested isolate:
Perform viable colony counts from positive control well to confirm 5×10^5 CFU/mL and check for purity.

Fastidious organisms

ISO Standard 20776-1

- Mueller-Hinton broth with 2.5-5 % lysed horse blood for testing of *Streptococcus* species.

EUCAST recommendation

- Mueller Hinton broth with 5% lysed horse blood and 20 mg/L β-NAD.

Streptococcus pneumoniae

EUCAST Clinical Breakpoint Tables v. 8.0, valid from 2018-01-01

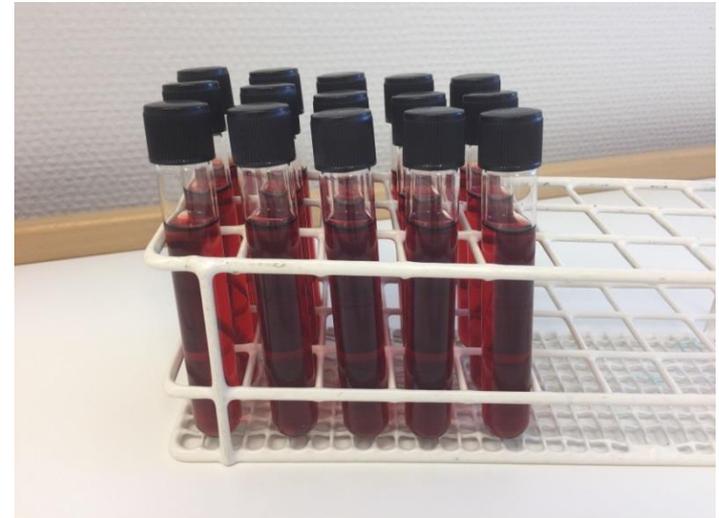
MIC determination (broth microdilution according to ISO standard 20776-1)
Medium: Mueller-Hinton broth + 5% lysed horse blood and 20 mg/L β-NAD (MH-F broth)
Inoculum: 5x10⁵ CFU/mL
Incubation: Sealed panels, air, 35±1°C, 18±2h
Reading: Unless otherwise stated, read MICs at the lowest concentration of the agent that completely inhibits visible growth.
Quality control: *Streptococcus pneumoniae* ATCC 49619. For agents not covered by this strain, see EUCAST QC Tables

Disk diffusion (EUCAST standardised disk diffusion method)
Medium: Mueller-Hinton agar + 5% defibrinated horse blood and 20 mg/L β-NAD (MH-F)
Inoculum: McFarland 0.5 from blood agar or McFarland 1.0 from chocolate agar
Incubation: 5% CO₂, 35±1°C, 18±2h
Reading: Unless otherwise stated, read zone edges as the point showing no growth viewed from the front of the plate with the lid removed and with reflected light.
Quality control: *Streptococcus pneumoniae* ATCC 49619. For agents not covered by this strain, see EUCAST QC Tables.

Penicillins ¹	MIC breakpoint (mg/L)		Disk content (µg)	Zone diameter breakpoint (mm)		Notes
	S ≤	R >		S ≥	R <	
Benzylpenicillin (indications other than meningitis) ²	0.06 ¹	2 ¹		Note ^A	Note ^A	¹ Breakpoints for penicillins other than benzylpenicillin relate only to non-meningitis isolates. Isolates fully susceptible to benzylpenicillin (MIC ≤0.06 mg/L and/or susceptible by oxacillin disk screen, see note C) can be reported susceptible to beta-lactam agents for which clinical breakpoints are listed (including those with "Note"). ² For breakpoints and dosing in pneumonia, see table of dosages. ³ For isolates categorised as intermediate to ampicillin avoid oral treatment with ampicillin, amoxicillin or amoxicillin-
Benzylpenicillin (meningitis)	0.06 ¹	0.06 ¹		Note ^A	Note ^A	
Ampicillin	0.5 ^{1,3}	2 ^{1,3}		Note ^{A,B}	Note ^{A,B}	
Ampicillin-sulbactam	Note ^{1,4}	Note ^{1,4}		Note ^{A,B}	Note ^{A,B}	

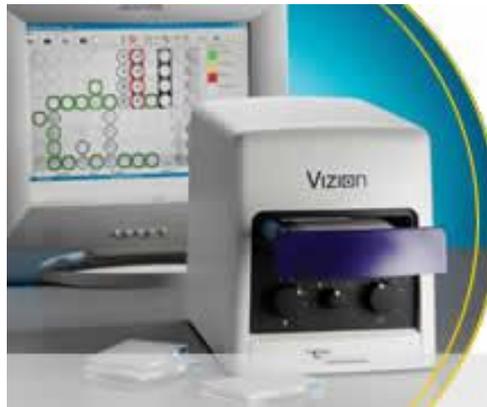
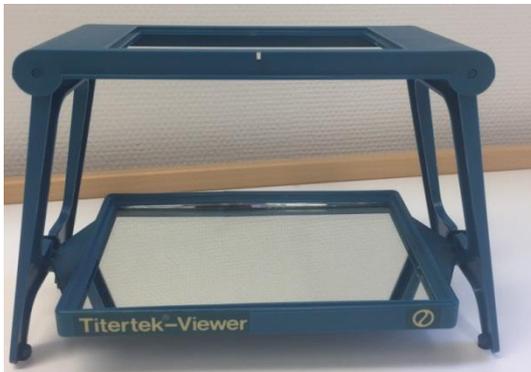
MH-F broth

- Mueller Hinton broth supplemented with 5% lysed horse blood and 20 mg/L β -NAD
- Validated for:
 - *Streptococcus* spp.
 - *Haemophilus influenzae*
 - *Moraxella catarrhalis*
 - *Listeria monocytogenes*
 - *Campylobacter jejuni* and *coli*
 - *Pasteurella multocida*
 - *Corynebacterium* spp.
 - *Aerococcus sanguinicola* and *urinae*
 - *Kingella kingae* etc.



Reading of BMD MICs

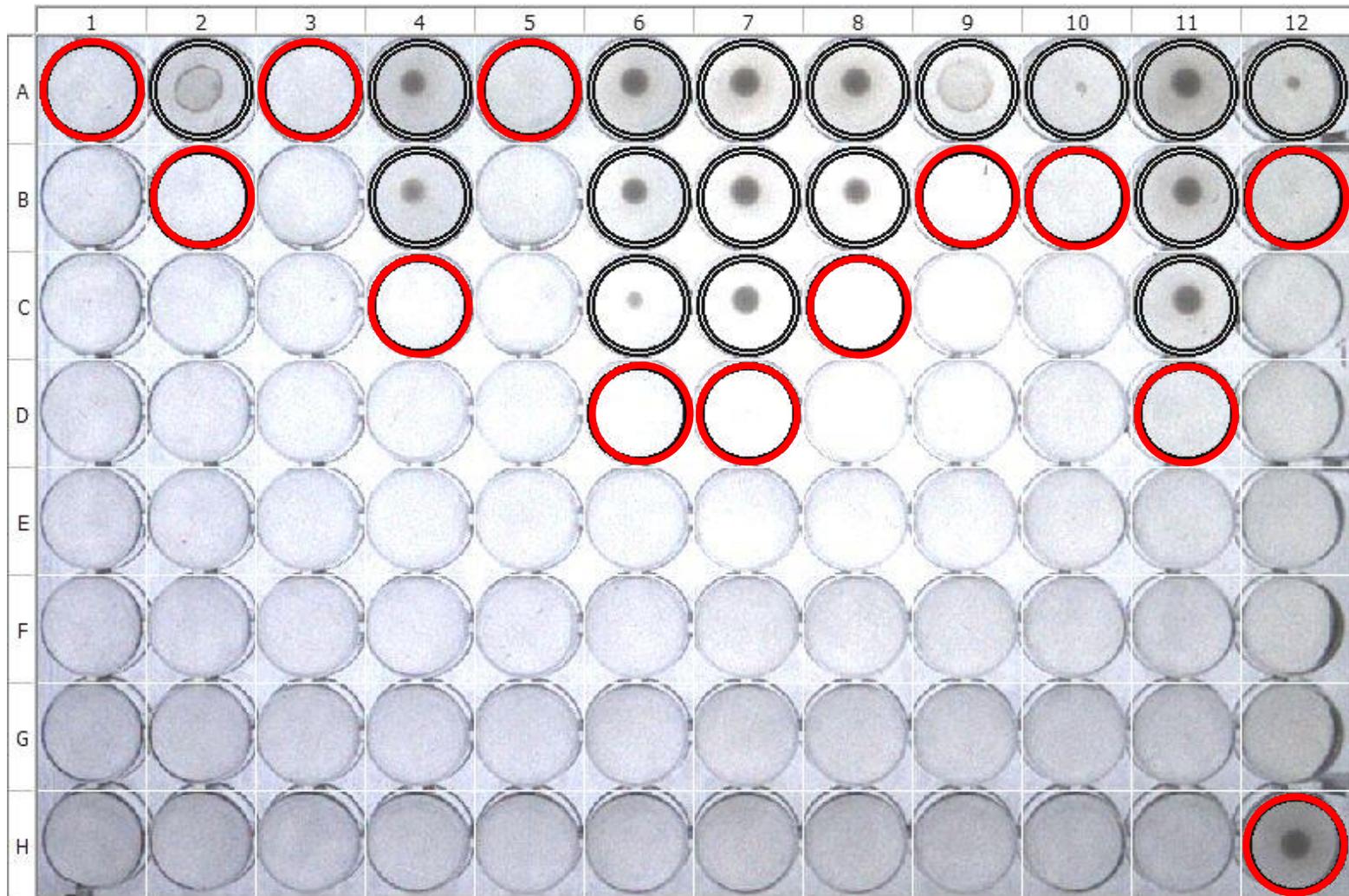
- Read MICs only for pure cultures and when there is sufficient growth in the positive control.
- Manual reading is the reference
- Automated readers or use of camera systems must be calibrated to manual reading.



Reading instructions

- Read the MIC as the lowest concentration of agent that completely inhibits growth as detected by the unaided eye.

Reading of MICs *E. coli*



Positive control OK!

MIC (mg/L)	FEP	PTZ	DOR	AMI	ERT	GEN	CIP	TOB	CRO	CAZ	LEV	TIG
	≤0.06	2	≤0.03	4	≤0.015	2	0.12	1	0.06	0.25	0.25	0.25

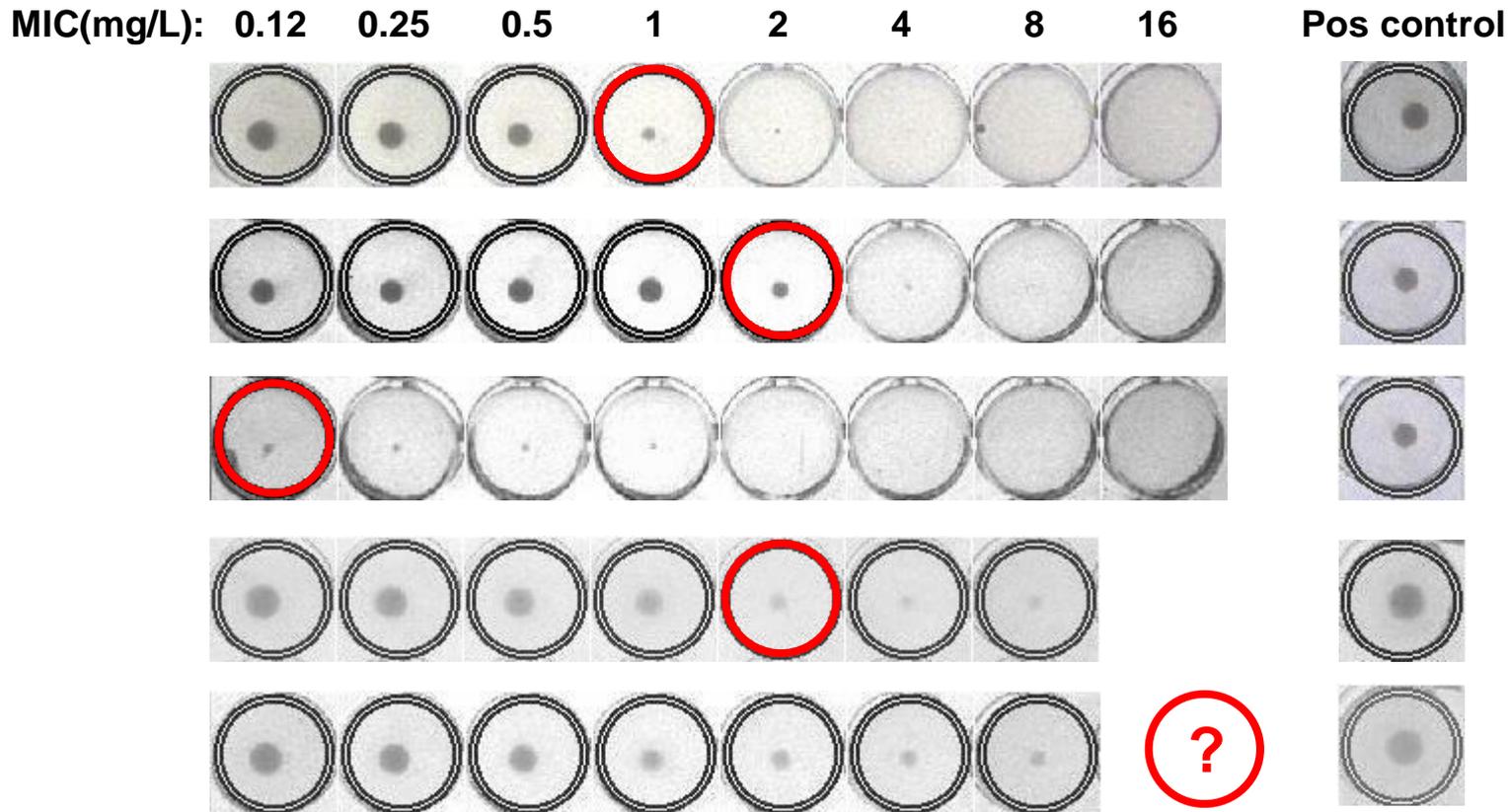
Reading instructions

- Read the MIC as the lowest concentration of agent that completely inhibits growth as detected by the unaided eye.
- Trimethoprim and trimethoprim-sulfamethoxazole:
Read at 80% inhibition!
- Grampositive cocci and bacteriostatic drugs:
Disregard pinpoint growth!

Specific reading instructions

For trimethoprim and trimethoprim-sulfamethoxazole:

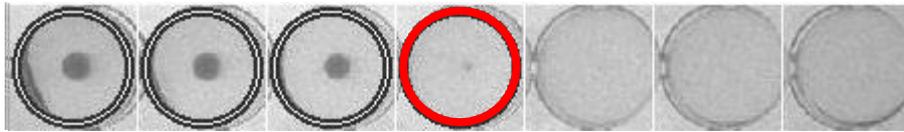
Read at 80% inhibition as compared with the growth control well.



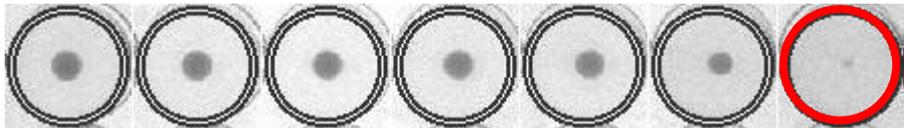
Specific reading instructions

Grampositive cocci and bacteriostatic drugs:
Disregard pinpoint growth.

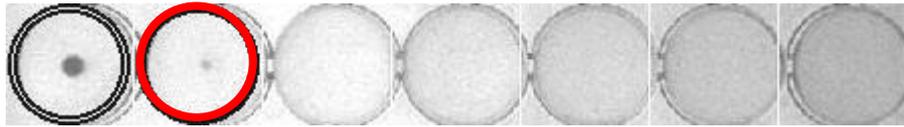
Doxycycline



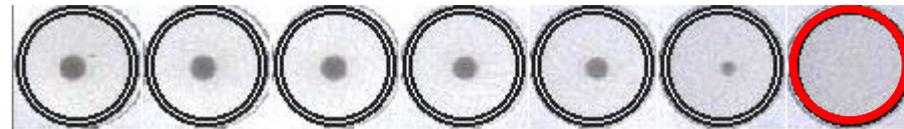
Fusidic acid



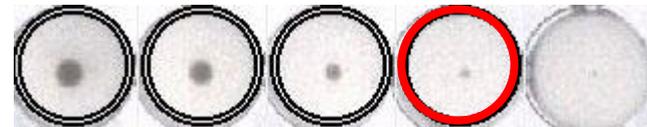
Tetracycline



Doxycycline



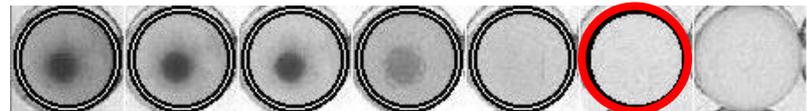
Linezolid



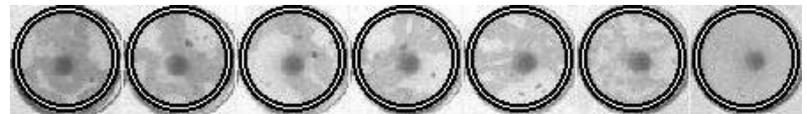
Difficult reading situations

– Haze

- Often seen for *Pseudomonas* and *Acinetobacter*



– Scattered growth



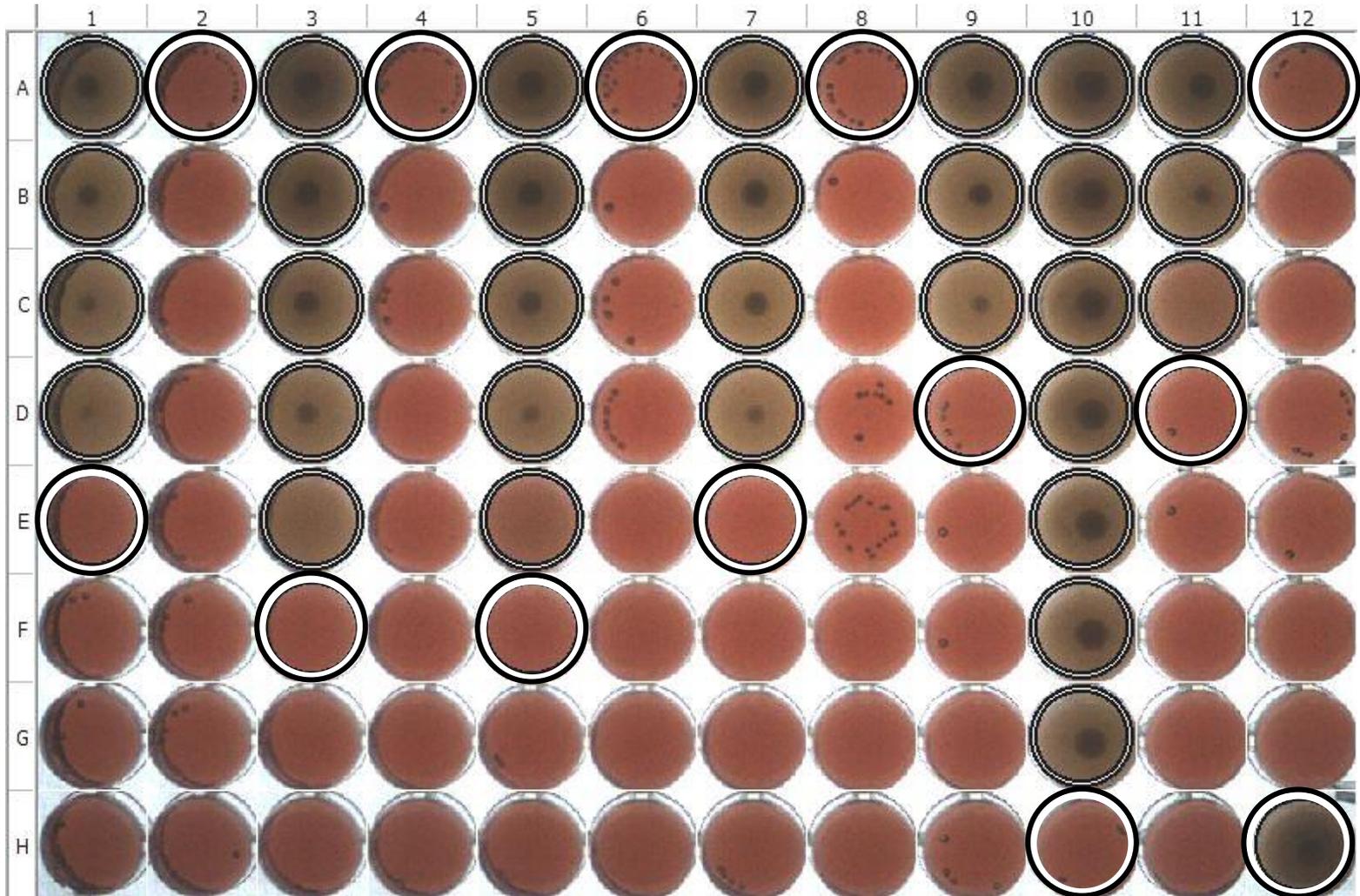
– Very tiny pellets

- Often seen for bacteriostatic drugs

– Haemolysis

- Often seen for *Streptococcus* spp.

Reading of MICs *S. pneumoniae*



Difficult reading situations

– MH-F broth not clear

- Improve lysing and/or centrifugation of horse blood

– Condensation



– Skipped wells

- Repeat the test or report the highest MIC

Quality control

- Check that MICs are within QC ranges (EUCAST website) for relevant QC strains:
 - New lot Mueller Hinton / MH-F broth
 - New lot Microtiter plate
- Frequency to be determined locally
- Use several QC strains to cover all agents
 - Commercial panels often have few concentrations for each agent
- Include resistant QC strains to test β -lactam β -lactamase inhibitor combinations.
- Variation: Within \pm one doubling dilution is acceptable.

Quality control strains

Organism	Number	Characteristics
<i>E. coli</i>	ATCC 25922	Susceptible, wild-type
<i>P. aeruginosa</i>	ATCC 27853	Susceptible, wild-type
<i>S. aureus</i>	ATCC 29213	Weak β -lactamase producer
<i>E. faecalis</i>	ATCC 29212	Susceptible, wild-type
<i>S. pneumoniae</i>	ATCC 49619	Reduced susceptibility to benzylpenicillin
<i>H. influenzae</i>	ATCC 49766	Susceptible, wild-type
Control of the inhibitor component of β-lactam-β-lactamase inhibitor combinations (amoxicillin-clavulanic acid, piperacillin-tazobactam)		
<i>E. coli</i>	ATCC 35218	TEM-1 β -lactamase producer
<i>K. pneumoniae</i>	ATCC 700603	ESBL producer (SHV-18)

Examples of quality control

Pseudomonas panel

QC strain	AMI	AZT	FEP	CAZ	CIP	COL	GEN	IMI	LEV	MER	PTZ	TOB	TSU
P. aeruginosa ATCC 27853	2	8	2	2	0.5	0.5	1	2	1	0.5	4	0.5	16
P. aeruginosa ATCC 27853	4	4	2	2	0.5	1	1	4	1	1	4	0.5	16
Target	2	4	1-2	2	0.5	1-2	1	2	1-2	0.5	2-4	0.5	16
Range	1-4	2-8	0.5-4	1-4	0.25-1	0.5-4	0.5-2	1-4	0.5-4	0.25-1	1-8	0.25-1	8-32

QC strain	AMI	AZT	FEP	CAZ	CIP	COL	GEN	IMI	LEV	MER	PTZ	TOB	TSU
E.coli NCTC 13846	2	≤1	≤1	≤0.5	>8	4	0.5	≤0.25	>8	≤0.12	8	1	>16
E.coli NCTC 13846	2	≤1	≤1	≤0.5	>8	4	0.5	≤0.25	>8	≤0.12	8	0.5	>16
Target	-	-	-	-	-	4	-	-	-	-	-	-	-
Range	-	-	-	-	-	-	-	-	-	-	-	-	-

QC strain	AMI	AZT	FEP	CAZ	CIP	COL	GEN	IMI	LEV	MER	PTZ	TOB	TSU
K. pneumoniae ATCC 700603	1	32	2	32	0.5	0.5	8	0.5	1	≤0.12	16	8	1
K. pneumoniae ATCC 700603	<0.5	32	2	32	0.5	0.5	8	1	1	0.25	16	8	1
Target	-	-	-	-	-	-	-	-	-	-	16	-	-
Range	-	-	-	-	-	-	-	-	-	-	8-32	-	-

On target
Upper limit
Lower limit
Out of range

Examples of quality control

Haemophilus panel

QC strain	AMC c2	AMO	AMP	CTX	CRO	CXM	CIP	IMI	LEV	MER	MOX	TET	TSU
H. influenzae ATCC 49766	≤0.25	0.5	≤0.25	≤0.015	≤0.015	0.5	≤0.015	0.5	≤0.015	≤0.03	≤0.015	≤0.25	≤0.06
H. influenzae ATCC 49766	≤0.25	≤0.25	≤0.25	≤0.015	≤0.015	0.5	≤0.015	0.5	≤0.015	≤0.03	≤0.015	≤0.25	≤0.06
Target	0.25	0.25	0.125	0.008	0.004	0.5	0.008	0.5	0.016	0.06	0.016	0.5	0.03
Range	0.125-0.5	0.125-0.5	0.06-0.25	0.004-0.016	0.002-0.008	0.25-1	0.004-0.016	0.25-1	0.008-0.03	0.03-0.12	0.008-0.03	0.25-1	0.016-0.06

QC strain	AMC c2	AMO	AMP	CTX	CRO	CXM	CIP	IMI	LEV	MER	MOX	TET	TSU
S. pneumoniae ATCC 49619	≤0.25	≤0.25	≤0.25	0.06	0.06	≤0.25	1	≤0.25	1	0.06	0.12	≤0.25	0.5
S. pneumoniae ATCC 49619	≤0.25	≤0.25	≤0.25	0.06	0.06	≤0.25	0.5	≤0.25	0.5	0.06	0.12	≤0.25	0.5
Target	-	0.06	0.12	0.06	0.06	0.5	-	0.06	1	0.06-0.12	0.12	0.12-0.25	0.25-0.5
Range	-	0.03-0.12	0.06-0.25	0.03-0.12	0.03-0.12	0.25-1	-	0.03-0.12	0.5-2	0.03-0.25	0.06-0.25	0.06-0.5	0.12-1

QC strain	AMC c2	AMO	AMP	CTX	CRO	CXM	CIP	IMI	LEV	MER	MOX	TET	TSU
E. coli ATCC 25922	4	4	2	0.12	0.12	8	≤0.015	≤0.25	0.03	≤0.03	0.03	1	≤0.06
E. coli ATCC 25922	4	8	4	0.06	0.06	8	≤0.015	≤0.25	0.03	≤0.03	0.03	1	≤0.06
Target	4	4	4	0.06	0.06	4	0.008	0.12	0.015-0.03	0.015-0.03	0.015-0.03	1	≤0.5
Range	2-8	2-8	2-8	0.03-0.12	0.03-0.12	2-8	0.004-0.015	0.06-0.25	0.008-0.06	0.008-0.06	0.008-0.06	0.5-2	

On target
Upper limit
Lower limit
Out of range

Introducing broth microdilution

- Purpose of testing
 - Which isolates and antimicrobial agents?
- Chose a manufacturer of panels and broth
 - Other materials?
- Introduce to a small group at the laboratory
- Practice the testing procedure and reading of results
- Start with quality control strains and then clinical isolates

BMD products

Multiple agents

- **Sentititre** Thermo Fisher Scientific



- **MICRONAUT-S** Merlin



Single agents

- **MICRONAUT-MIC Strip** Merlin



- **UMIC** Biocentric



- **ComASP** (SensiTest) Liofilchem



MIC panels

Commercial panels	Custom made panels
Fixed agents and concentrations. Often short ranges	Agents and concentrations adapted to local preferences
Concentrations can be adapted to EUCAST or CLSI breakpoints	Concentrations of choice
Concentrations of inhibitor can differ for amoxicillin-clavulanic acid (fixed conc. vs. ratio)	Concentrations of choice
Difficult to QC	Can be easier to QC
For IVD use	Might be RUO
Can be ordered in small quantities	Usually require large orders

Validation of BMD

Quality Control strains

- Evaluate results for relevant QC stains against EUCAST QC ranges and targets. Results should be within QC ranges and close to target values.

Clinical isolates

- Test clinical isolates with known MICs, for example UKNEQAS isolates.
- Compare MIC distributions with reference distributions on the EUCAST website

MIC distributions

www.eucast.org

Antimicrobial wild type distributions of microorganisms

Search
 Method: MIC Disk diffusion
 Antimicrobial: [Antimicrobial...] Species: [Staphylococcus aureus] Disk c...

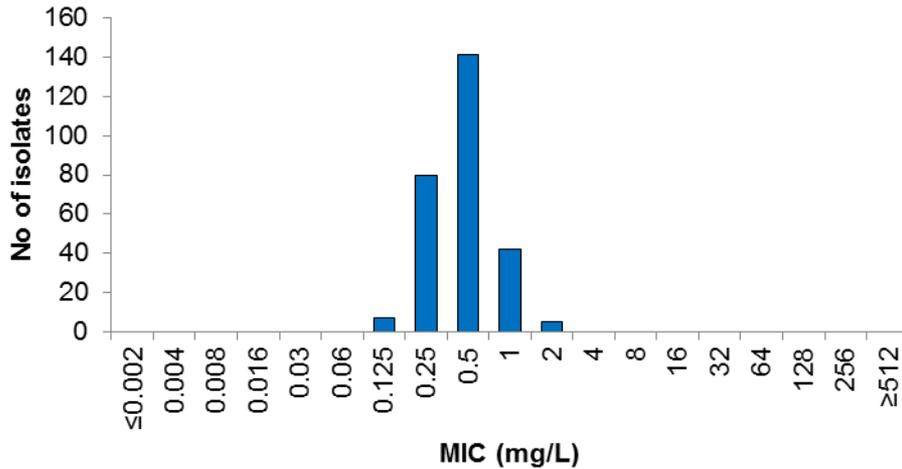
Species: **Staphylococcus aureus** (Method: **MIC**)

MIC distributions include collated data from multiple sources, geographical areas and time periods and can never be used to infer rates of resistance

	0.002	0.004	0.008	0.016	0.032	0.064	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	ECOFF	Distributions	Observations
Amikacin	0	0	2	0	1	0	0	37	101	349	1873	2666	943	322	83	64	3	2	0	8.0	19	6446
Ampicillin	0	0	0	1	4	11	298	248	305	407	182	155	130	87	80	0	2	1	0	ND	6	1911

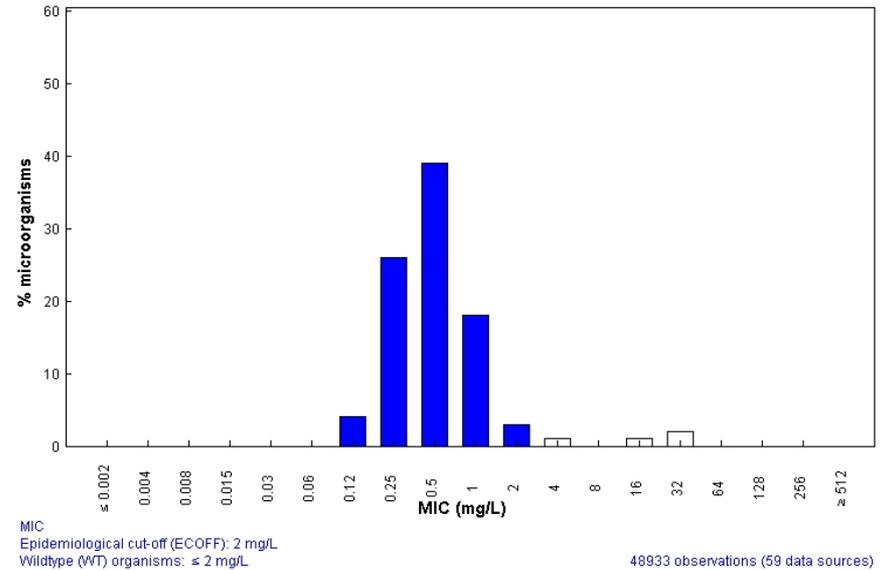
Gentamicin / Staphylococcus aureus
 International MIC Distribution - Reference Database 2018-04-27

S. aureus vs. gentamicin



Routine MIC distribution for clinical isolates

MIC distributions include collated data from multiple sources, geographical areas and time periods and can never be used to infer rates of resistance



Reference MIC distribution EUCAST website

EUCAST Development Laboratory

- Broth microdilution testing for aerobic bacteria can be ordered from the EUCAST Development Laboratory. See information on <http://www.nordicast.org/metoder>



BMD panels

- Available BMD panels:
 - Enterobacterales 26 agents
 - *Pseudomonas* and *Acinetobacter* spp. 13 agents
 - *Staphylococcus* and *Enterococcus* spp. 32 agents
 - *Streptococcus* spp 24 agents
 - *Haemophilus* spp. 13 agents
 - Extended Gram-negative 17 agents
 - Including:
 - Colistin
 - Piperacillin-tazobactam
 - Ceftazidime-avibactam
 - Ceftolozane-tazobactam

Thank you!

