



Broth microdilution

Methodology and technical aspects

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NordicAST Workshop 2017

Broth microdilution

- Dilution methods
 - Broth (macro)dilution $>200 \mu\text{l}$ per tube
 - Broth microdilution $\leq 200 \mu\text{l}$ per well
- Broth microdilution
 - 96-well microtitration plate
 - Two-fold dilutions of the antimicrobial agent
- Advantages with BMD:
 - Test multiple antibiotics at once
 - Small amount of reagents and space required
 - Commercial plates available with dehydrated antibiotics



Broth microdilution

- Quantitative method to measure the *in vitro* antimicrobial activity against bacteria
- **MIC - Minimum Inhibitory Concentration**
 - The lowest concentration of antimicrobial agent that completely inhibits growth of the organism as detected by the unaided eye.
 - Expressed in mg/L or $\mu\text{g/mL}$.
- Several factors influencing the MIC value:
 - Inoculum size, type of growth medium, incubation time and temperature, test procedure etc.

ISO Standard 20776-1

- Reference method for antimicrobial susceptibility testing of rapidly growing aerobic bacteria.
- Use the colony suspension method
 - Several morphologically similar colonies from an overnight culture on non-selective agar suspended in saline to McF 0.5, then diluted in **Mueller Hinton broth** to a final conc of **5×10^5 CFU/mL**.
- Inoculate each microdilution well within 30 min of inoculum preparation.
- Apply a tight lid or adhesive seal and incubate in **34-37°C** in ambient air for **16-20 hours**.

ISO Standard 20776-1

- Read the MIC as the lowest conc. of the agent that completely inhibits visible growth.
- Do not read results if no growth in positive control well
- Perform viable colony counts from positive control well to confirm 5×10^5 CFU/mL and to check for purity.

MH-F broth

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

Preparation of MH-F broth

- Preparation of 50% lysed horse blood:
 - Dilute mechanically defibrinated horse blood with an equal amount of sterile deionized water.
 - Freeze the blood at -20°C overnight and thaw. Repeat the cycle until the cells are completely lysed (at least three cycle).
 - Clarify by centrifugation and discard the pellet. Repeat if necessary to improve clarity.
- Preparation of β -NAD stock solution:
 - Dissolve β -NAD (purity $\geq 98\%$) in sterile deionized water to a concentration of 20 mg/mL.
 - Sterilize through a 0.2 μm membrane filter.
- Store stock solution of lysed horse blood and β -NAD at -20°C .

Preparation of MH-F broth

- Preparation of MH-F broth:
 - Prepare and autoclave MH broth according to the manufacturer's instructions.
 - Cool medium to 42-45°C, add 50% lysed horse blood and β -NAD.
- Store MH-F broth at 4-8°C.



Reading of BMD MICs

- Check for purity and check the positive control well.
- Read the MIC as the lowest conc. of the agent that completely inhibits visible growth.
- Difficulties:
 - Skipped wells
 - Haemolysis
 - Haze
 - Scattered growth
 - Very tiny pellets
 - Condensation
 - MH-F broth not clear

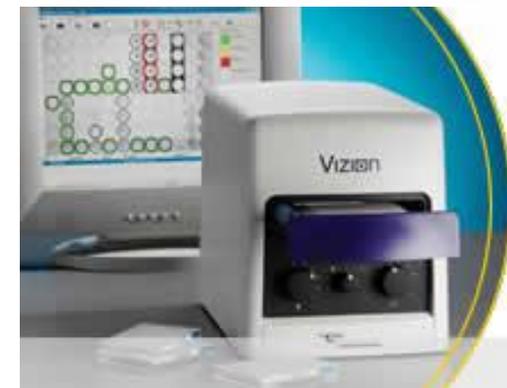
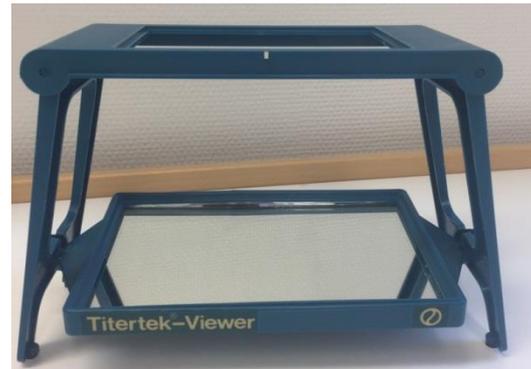
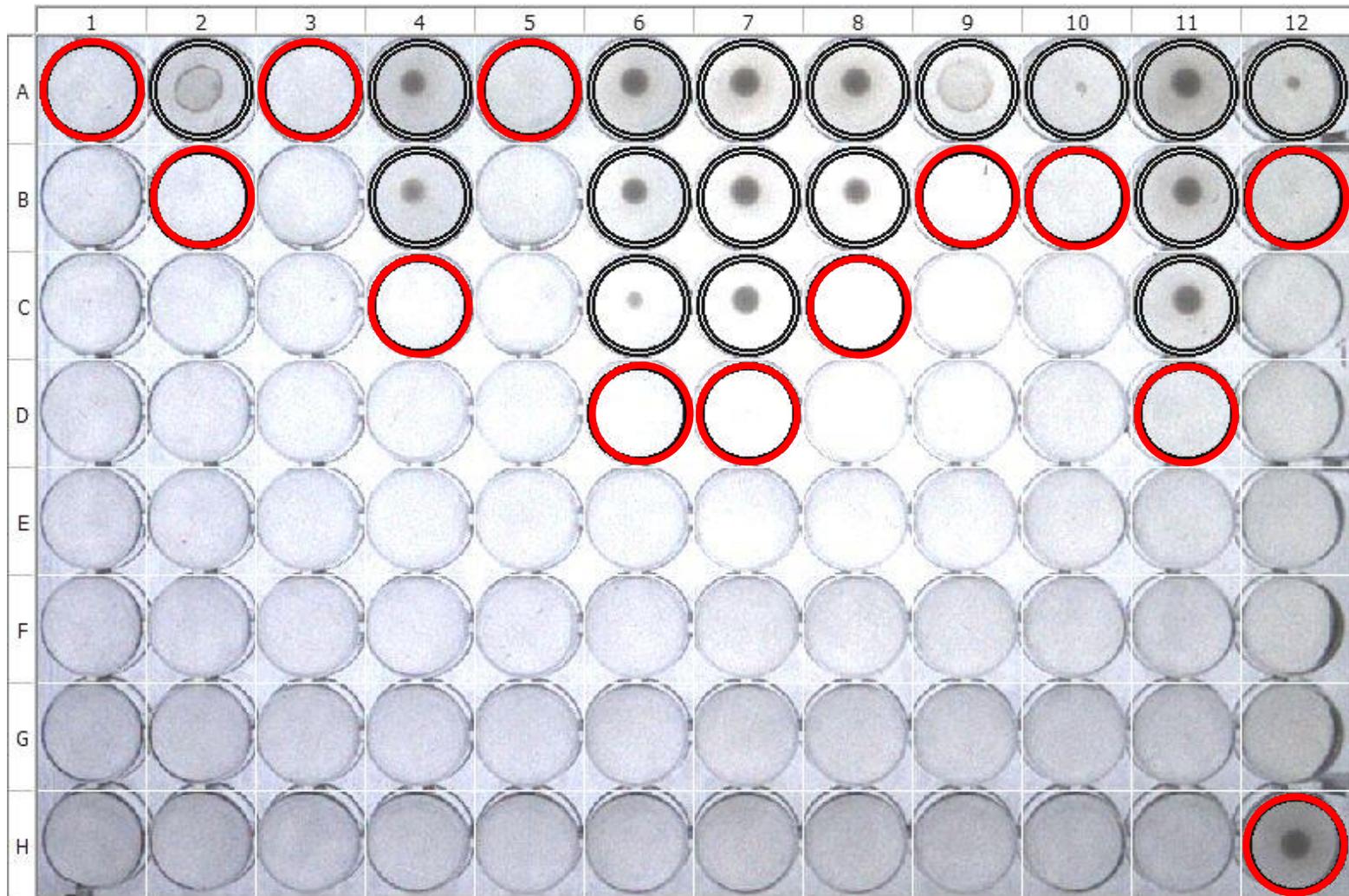


Plate format

	1	2	3	4	5	6	7	8	9	10	11	12
A	FEP 0.06	P/T4 1/4	DOR 0.03	AMI 1	ETP 0.015	GEN 0.25	CIP 0.015	TOB 0.25	AXO 0.03	TAZ 0.12	LEVO 0.03	TGC 0.12
B	FEP 0.12	P/T4 2/4	DOR 0.06	AMI 2	ETP 0.03	GEN 0.5	CIP 0.03	TOB 0.5	AXO 0.06	TAZ 0.25	LEVO 0.06	TGC 0.25
C	FEP 0.25	P/T4 4/4	DOR 0.12	AMI 4	ETP 0.06	GEN 1	CIP 0.06	TOB 1	AXO 0.12	TAZ 0.5	LEVO 0.12	TGC 0.5
D	FEP 0.5	P/T4 8/4	DOR 0.25	AMI 8	ETP 0.12	GEN 2	CIP 0.12	TOB 2	AXO 0.25	TAZ 1	LEVO 0.25	TGC 1
E	FEP 1	P/T4 16/4	DOR 0.5	AMI 16	ETP 0.25	GEN 4	CIP 0.25	TOB 4	AXO 0.5	TAZ 2	LEVO 0.5	TGC 2
F	FEP 2	P/T4 32/4	DOR 1	AMI 32	ETP 0.5	GEN 8	CIP 0.5	TOB 8	AXO 1	TAZ 4	LEVO 1	TGC 4
G	FEP 4	P/T4 64/4	DOR 2	AMI 64	ETP 1	GEN 16	CIP 1	TOB 16	AXO 2	TAZ 8	LEVO 2	TGC 8
H	FEP 8	FEP 16	DOR 4	DOR 8	ETP 2	ETP 4	CIP 2	CIP 4	AXO 4	TAZ 16	LEVO 4	POS CON

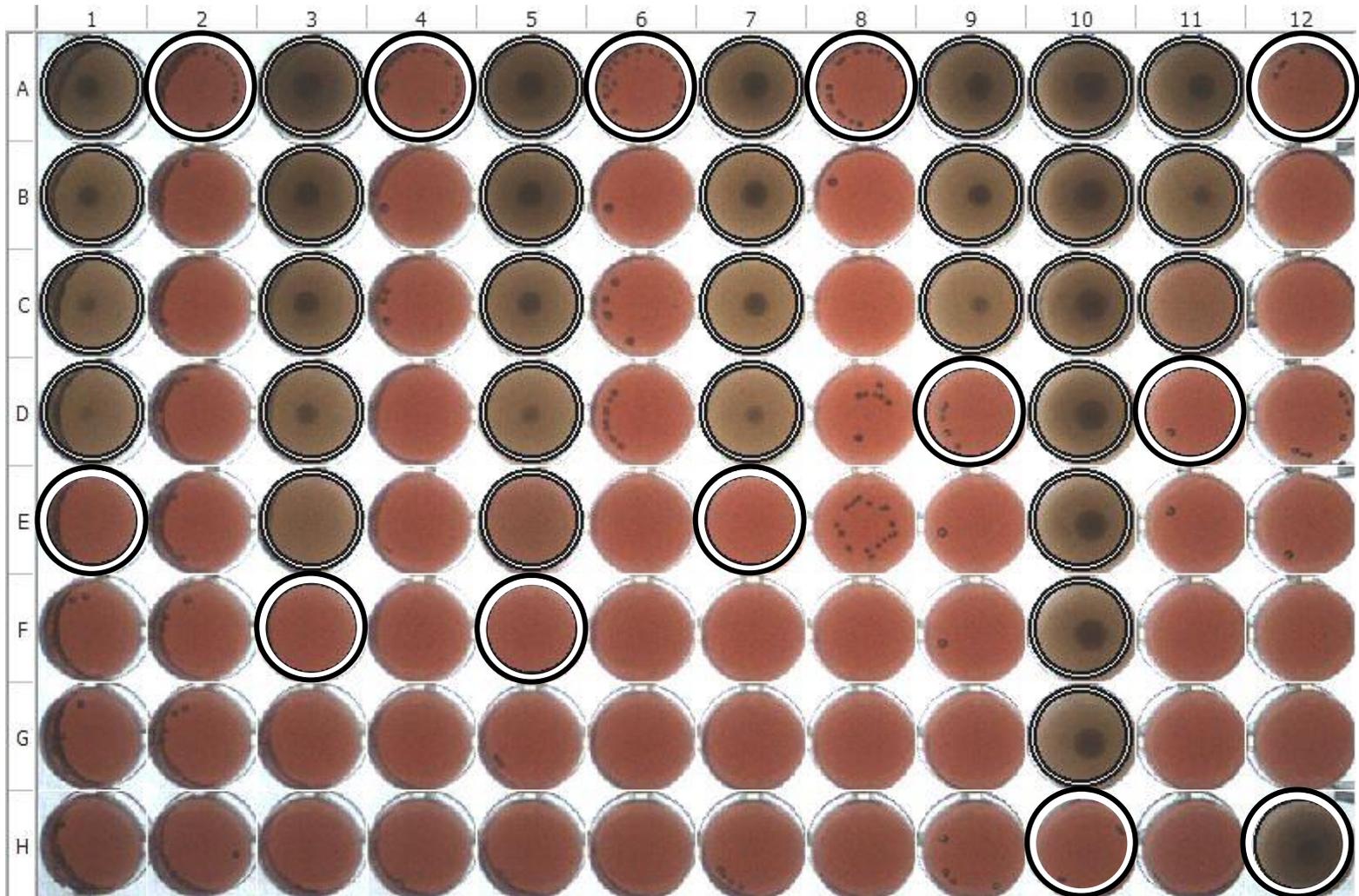
Reading of MICs *E. coli*



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

Positive control OK!

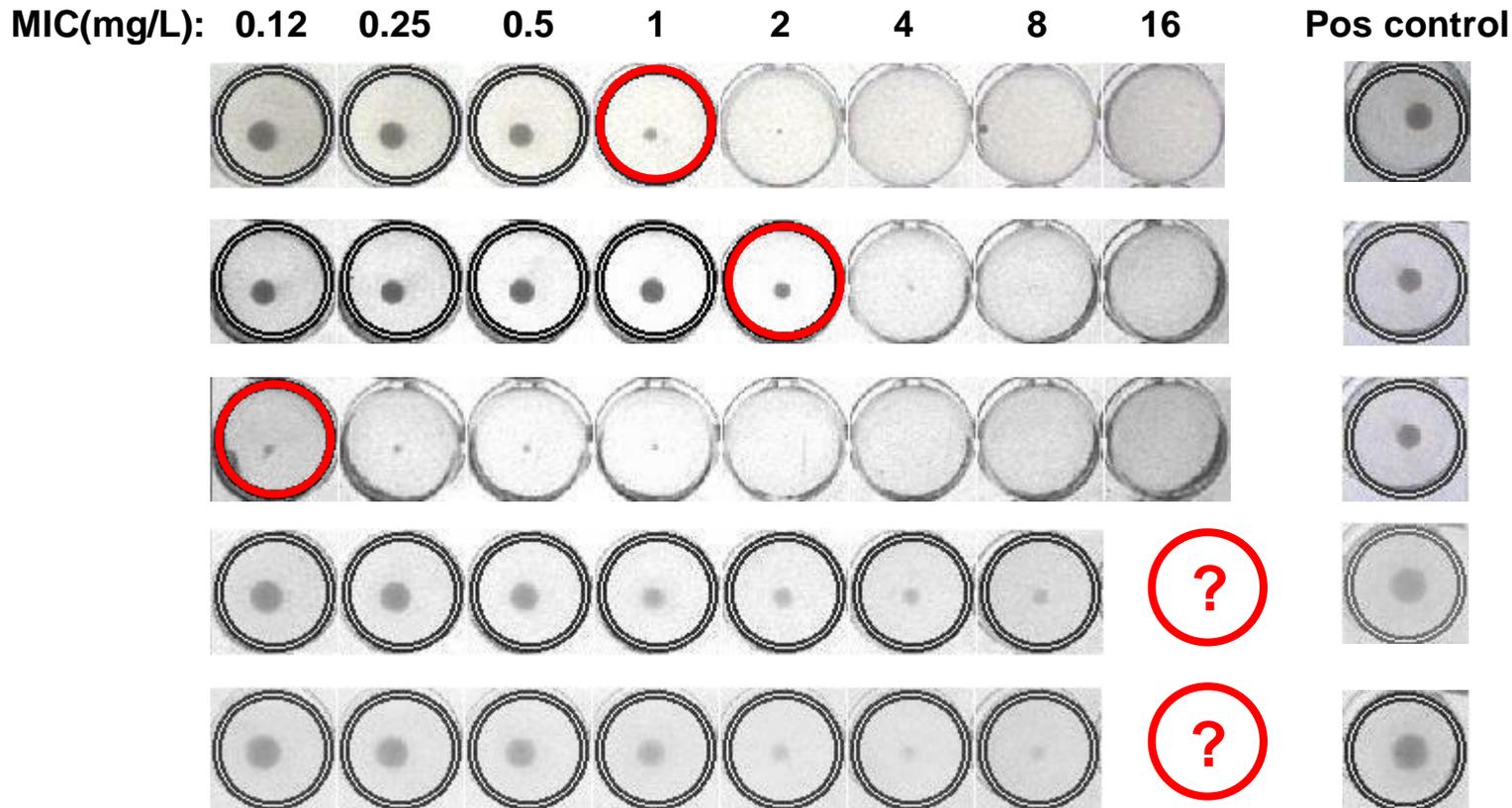
Reading of MICs *S. pneumoniae*



Specific reading instructions

For trimethoprim and trimethoprim-sulfamethoxazole:

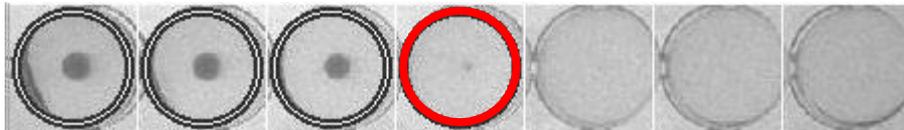
Read at 80% inhibition as compared with the growth control well.



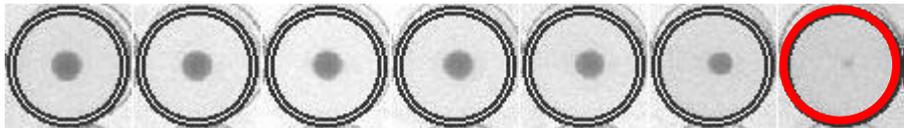
Specific reading instructions

Grampositive cocci and bacteriostatic drugs:
Disregard pinpoint growth.

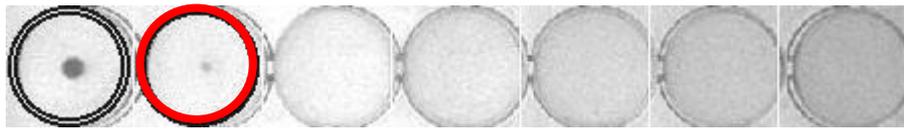
Doxycycline



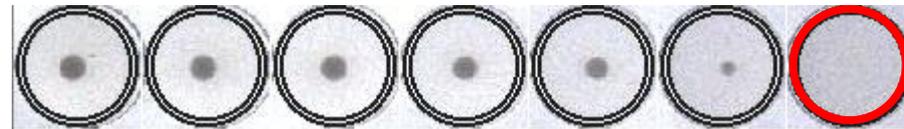
Fusidic acid



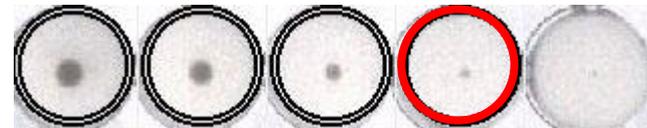
Tetracycline



Doxycycline



Linezolid



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
- 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)

Potential sources of error

- Microtitre plate:
 - Antimicrobial agents
(loss of potency, labile agent)
 - Storage
 - Contamination
 - Expiry date
- Test conditions:
 - Inoculum preparation
 - Incubation
(temperature, atmosphere, time)
 - Inoculation
 - Reading
- MH/MH-F broth:
 - Not prepared according to instructions
 - pH
 - Storage
 - Contamination
 - Expiry date
- Control organism:
 - Mutation
 - Contamination
 - Age of culture

EUCAST Development Laboratory

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



www.nordicast.org

Methods

[EUCASTs lappdiffusionsmetod](#)

[Förklarande metoddokument](#)

[Metodutveckling](#)

Metodbeskrivningar

NordicAST rekommenderar att resistensbestämning utförs enligt EUCASTs standardiserade metoder.

Utförlig metodbeskrivning finns på [EUCASTs hemsida](#).

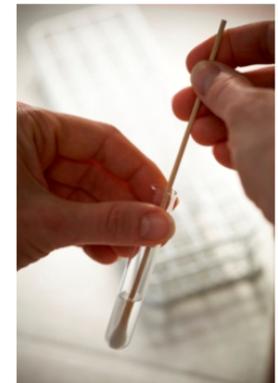
Här finns metodbeskrivningar översatta till svenska samt förklarande metoddokument på skandinaviska språk.

MIC-värden med referensmetodik från EUCAST Development Laboratory (EDL)

Vid beställning av MIC-värden med buljongspädningsmetod använd följande [remiss](#)
[Tillgängliga MIC-paneler och priser](#)

Anmälan av metodologiska problem inom resistensbestämning

För frågor inom resistensbestämning och anmälan av metodologiska problem, kontakta [EUCASTs referenslaboratorium](#).



Nyheter metoder

[MIC-värden med buljongspädning -uppdaterad information och remiss](#)

Erika Matuschek | 2017-03-20

[New method document, Screening for carbapenemase-producing](#)

[Enterobacteriaceae \(CPE\), published](#)

Mikala Wang | 2017-01-31

[Lappdiffusionsmanual och tillhörande dokument uppdaterade](#)

Erika Matuschek | 2017-01-10

[English version of the Haemophilus](#)

BMD panels

- Available BMD panels:

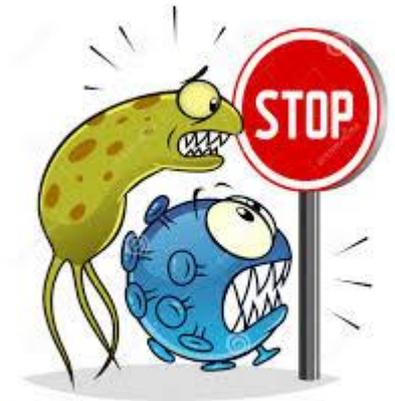
- Enterobacteriaceae 26 agents
- Pseudomonas and Acinetobacter 13 agents
- Staphylococcus and Enterococcus spp. 32 agents
- Streptococcus spp 24 agents
- Haemophilus spp. 13 agents

- New panel:

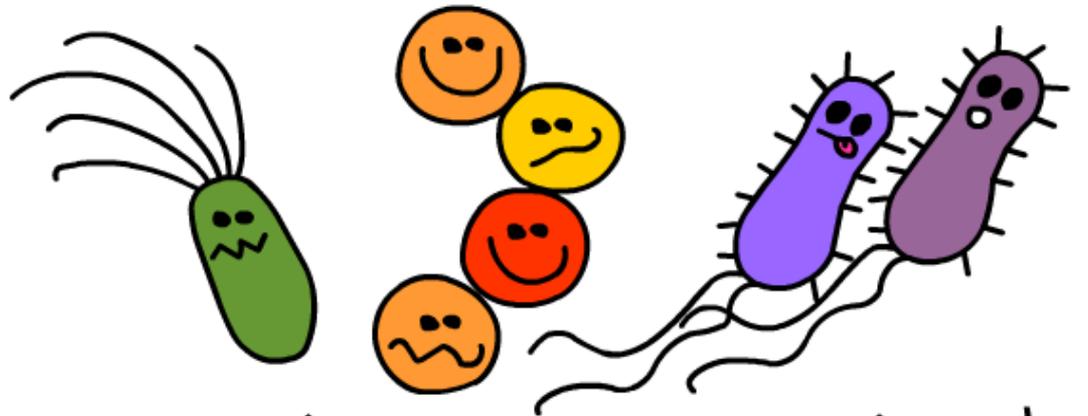
- Extended Gram-negative 17 agents

Including:

- Colistin
- Piperacillin-tazobactam
- Ceftazidime-avibactam
- Ceftolozane-tazobactam



Thank you!



bacteria are my friends