



Other common challenges in clinical laboratories

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Case 1

- 75 year old patient
- diabetic, colorectal cancer-> surgery
- post surgical complications: intra abdominal abcess and sepsis
- Treatment with piperacillin-tazobactam initiated
- Blood cultures are positive: *E. coli*
- Pip/tazo = 18-19mm (depending on the reader !)

	MIC breakpoints (mg/L)			Disk content (µg)	Zone diameter breakpoints (mm)		
	S ≤	R >	ATU		S ≥	R <	ATU
Piperacillin-tazobactam	8 ⁵	8 ⁵	16	30-6	20	20	19

Mentimeter

What do you do?

Report R

Perform a MIC with gradient test

Perform a MIC with BMD

Mentimeter

A gradient test for PTZ was performed and the MIC is 4 (= S). Choose relevant answer(s)?

MIC result is always more reliable than DD

Report Suceptible

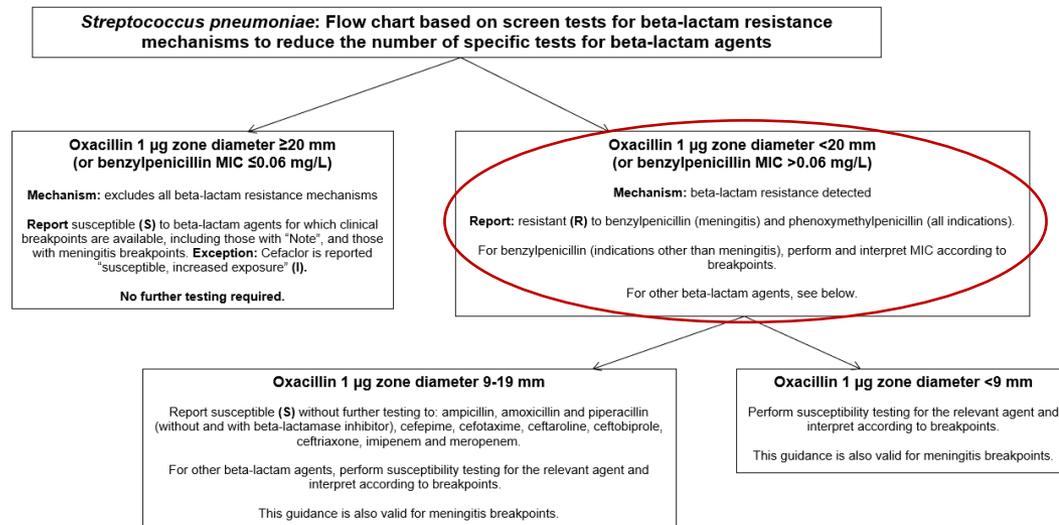
Report Resistant

Do not report S/R but comment on possible treatment failure

Perform BMD

Case 2

- 58 year old, usually healthy
- hospitalized with high fever, chills and fatigue
- CRP: 250, thorax radiography: pneumonia with lobar consolidation, pneumococcal Ag from urine positive
- Treatment with benzylpenicillin
- Blood cultures are positive: *Streptococcus pneumoniae*
- Oxacillin = 15 mm



Mentimeter

What method should you use to determine the MIC for benzylpenicillin?

Agar dilution

Gradient test

BMD

All methods perform equally

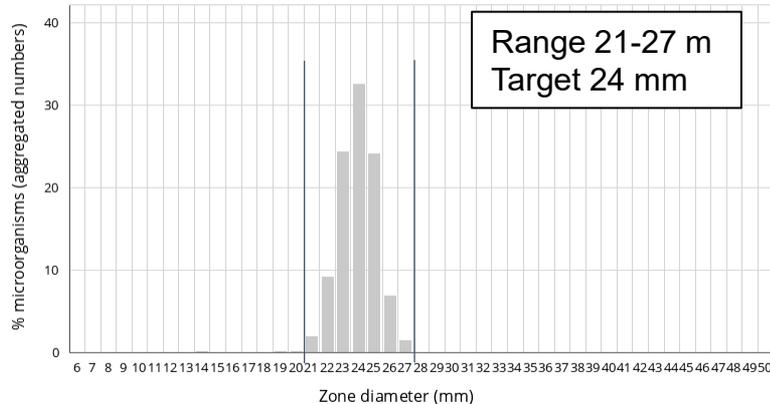
Discrepancies between disk diffusion and MIC

- Is QC ok?
 - Mean values from repeated tests on target ± 1 mm (mode MIC on target)?
 - BL-BLI combinations checked with both a susceptible and a resistant strain?

Piperacillin-tazobactam / *Escherichia coli* ATCC 25922
International zone diameter distribution - Reference database 2024-05-20
EUCAST disk diffusion method

Based on aggregated distributions

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



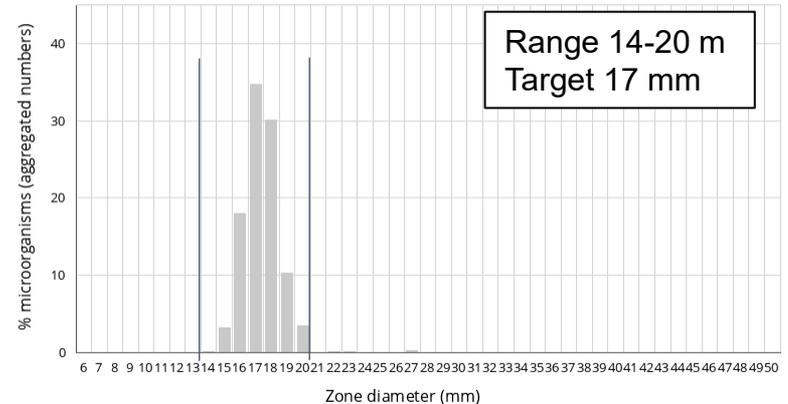
Disk content: 36
Epidemiological cut-off (ECOFF): -
Wildtype (WT) organisms: -

Confidence interval: -
2706 observations (32 data sources)

Piperacillin-tazobactam / *Klebsiella pneumoniae* ATCC 700603
International zone diameter distribution - Reference database 2024-05-20
EUCAST disk diffusion method

Based on aggregated distributions

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

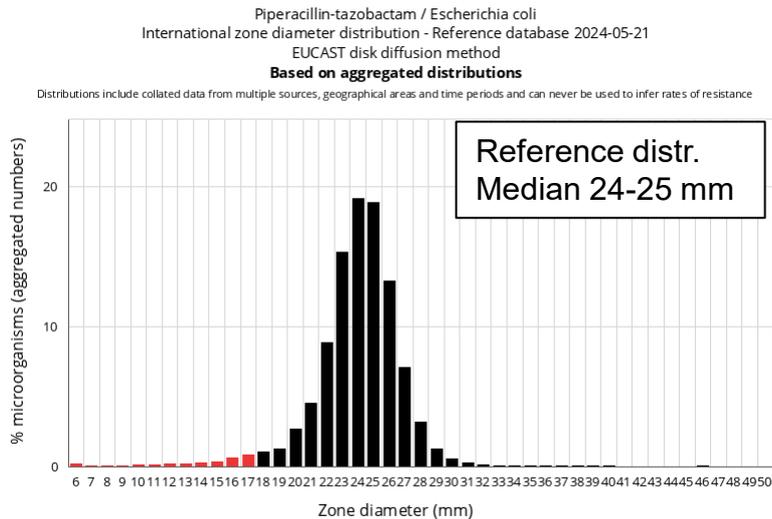


Disk content: 36
Epidemiological cut-off (ECOFF): -
Wildtype (WT) organisms: -

Confidence interval: -
1243 observations (21 data sources)

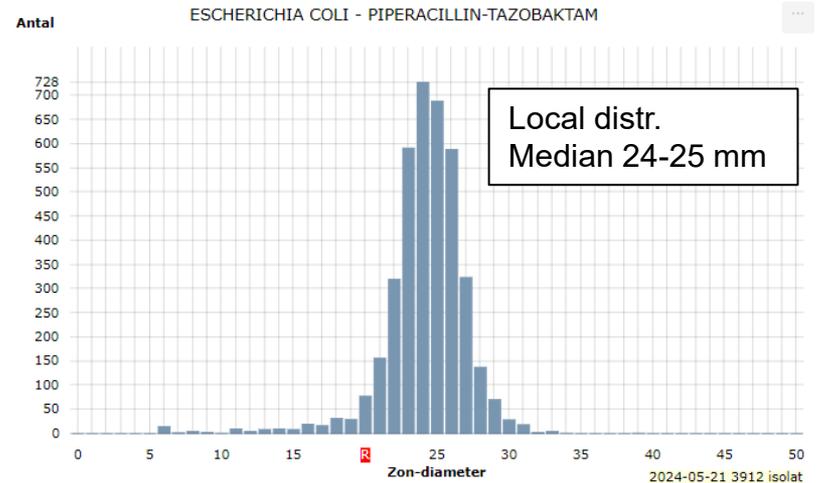
Discrepancies between disk diffusion and MIC

- Is QC ok?
 - Mean values from repeated tests on target ± 1 mm (mode MIC on target)?
 - BL-BLI combinations checked with both a susceptible and a resistant strain?
 - Median for clinical isolates = median in reference distribution ± 1 mm?



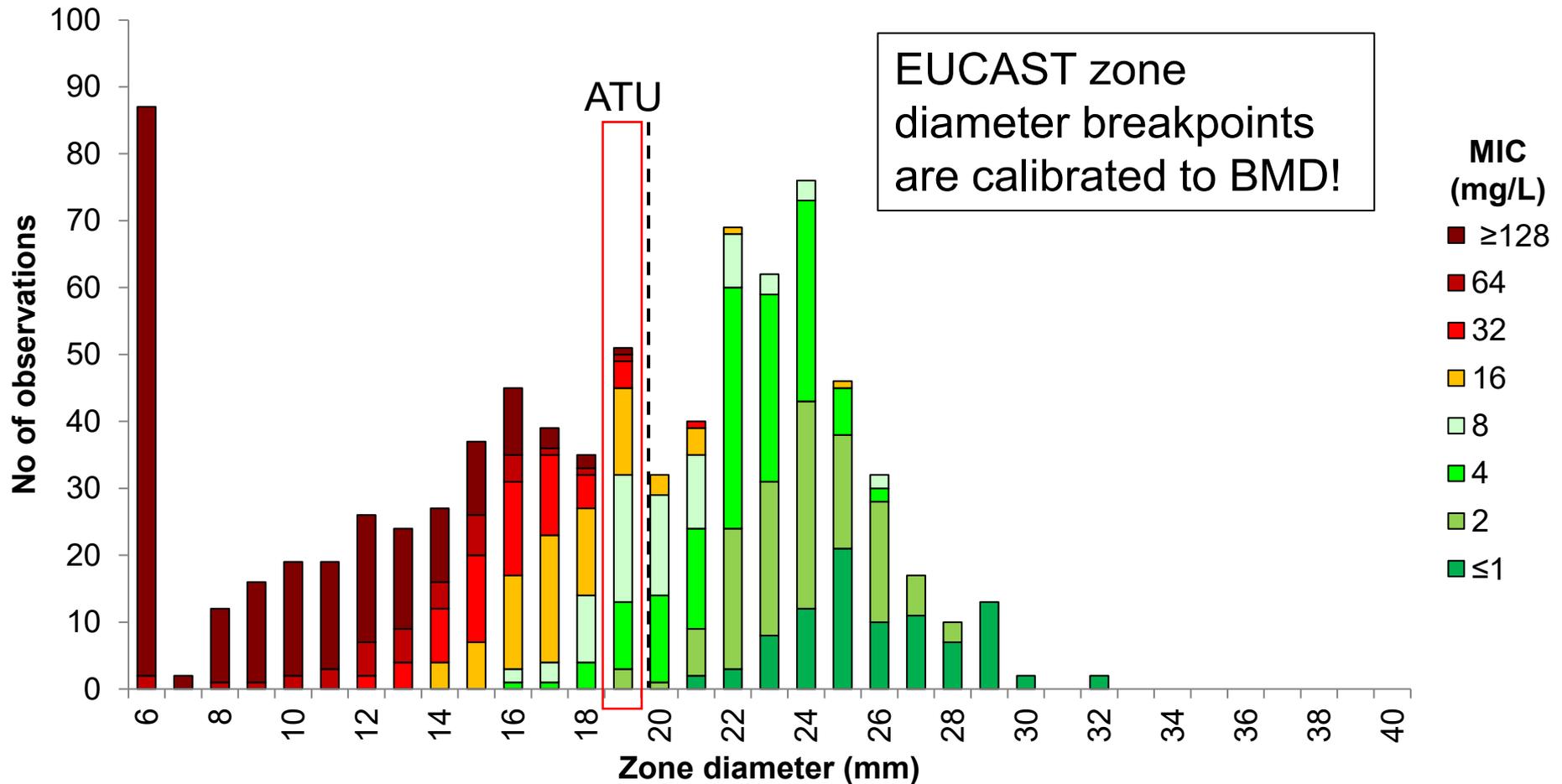
Disk content: 36
Epidemiological cut-off (ECOFF): 18 mm
Wildtype (WT) organisms: ≥ 18 mm

Confidence interval: 18 - 19
57404 observations (12 data sources)



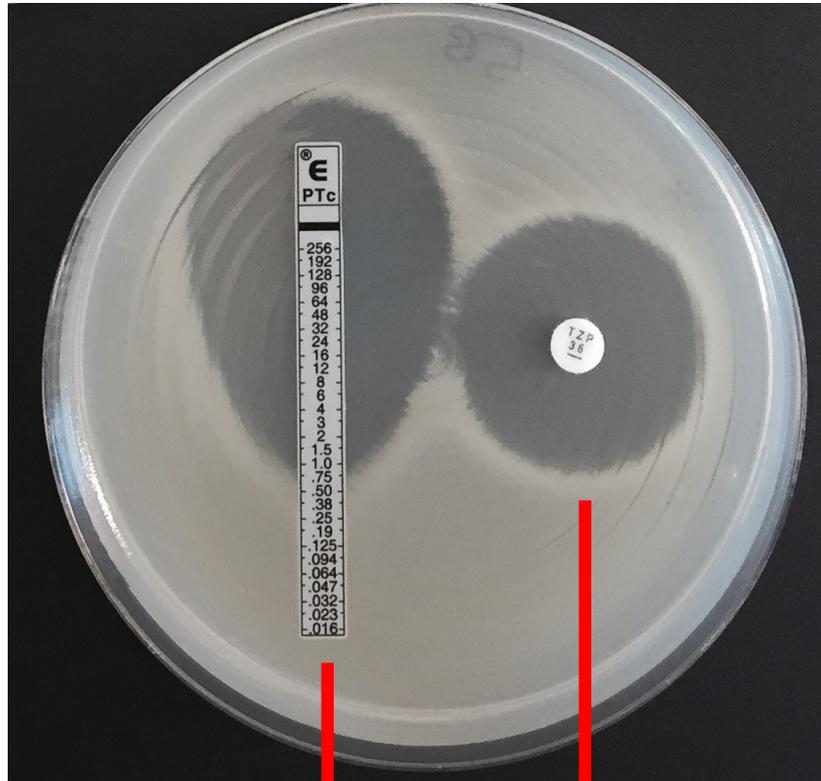
2024-05-21 3912 isolat

Piperacillin-tazobactam 30-6 μ g vs. MIC *Enterobacteriales*, 531 isolates (840 correlates)



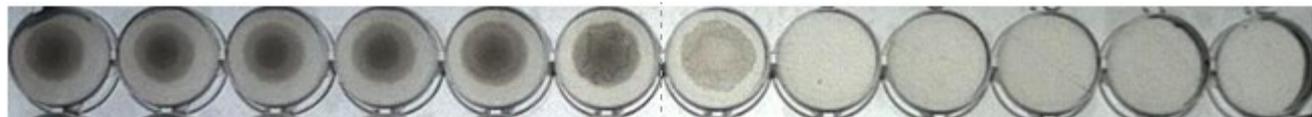
Breakpoints	
MIC	S \leq 8, R>8 mg/L
Zone diameter	S \geq 20, R<20 mm

Calibration of AST methods/products



Piperacillin and tazobactam diffuse into the agar and form concentration gradients for piperacillin and tazobactam respectively.

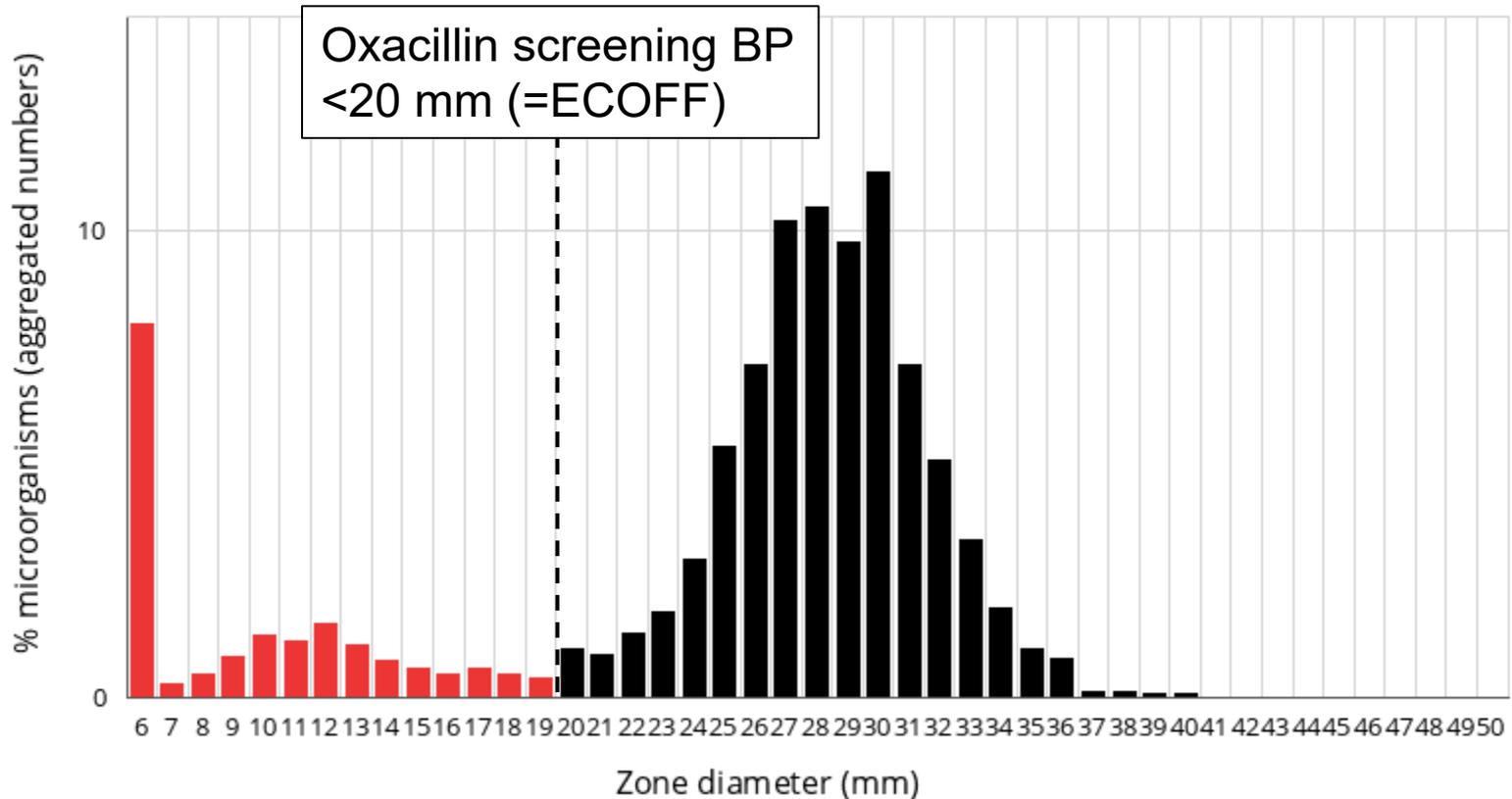
BMD = gold standard



Fixed 4 mg/L tazobactam

Oxacillin / *Streptococcus pneumoniae*
International zone diameter distribution - Reference database 2024-05-17
EUCAST disk diffusion method
Based on aggregated distributions

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



The oxacillin 1 µg screening test is a sensitive method to detect beta-lactam resistance mechanisms in *S. pneumoniae*.

Screen-positive isolates are resistant to one or several beta-lactam agents.

Results benzylpenicillin gradient tests

Etest

		PCG BMD									
		0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	
PCG Etest	0.008										
	0.016	7	1								
	0.03	1	2	2	3						
	0.06		1	10	9						
	0.125				2	2					
	0.25					11	6				
	0.5					1	4	4	4		1
	1						2	8	14		2
	2								6	8	
	4										3

Etest

EA = 91%

Bias = -41%

MTS

		PCG BMD									
		0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	
PCG MTS	0.008	3	1								
	0.016	5	1								
	0.03		1	4	5						
	0.06		1	8	9						
	0.125					8	5				
	0.25					6	2	1			
	0.5						3	7	9		
	1						2	4	15		4
	2									8	
	4										2

MTS

EA = 78%

Bias = -69%

ISO 20776-2

EA = $\geq 90\%$

Bias = $\leq \pm 30\%$

EUCAST warning on benzylpenicillin gradient tests

- Etest and MTS systematically underestimate benzylpenicillin MICs in *S. pneumoniae*
 - Trust the oxacillin 1 µg screening test
 - Isolates with gradient test MIC values of 0.5-2 mg/L should be checked with broth microdilution.
- A panel of 10 *S. pneumoniae* with varying beta-lactam susceptibility is available from CCUG for validation of MIC determination of benzylpenicillin in *S. pneumoniae*. Reference MICs are available on the EUCAST website https://www.eucast.org/ast_of_bacteria/strains_with_defined_susceptibility/.

https://www.eucast.org/ast_of_bacteria/warnings/

Case 3

- 2 year old child
- fever and complains of left ear pain for 2 days
- otoscopy: acute otitis media, tympanic membrane perforation with purulent otorrhea
- pus sample taken and send to bacteriology
- *Haemophilus influenzae* identified in pure culture (++++)
- AST performed and... it is difficult to read with haze in the inhibition zones and fuzzy zone edges



Mentimeter

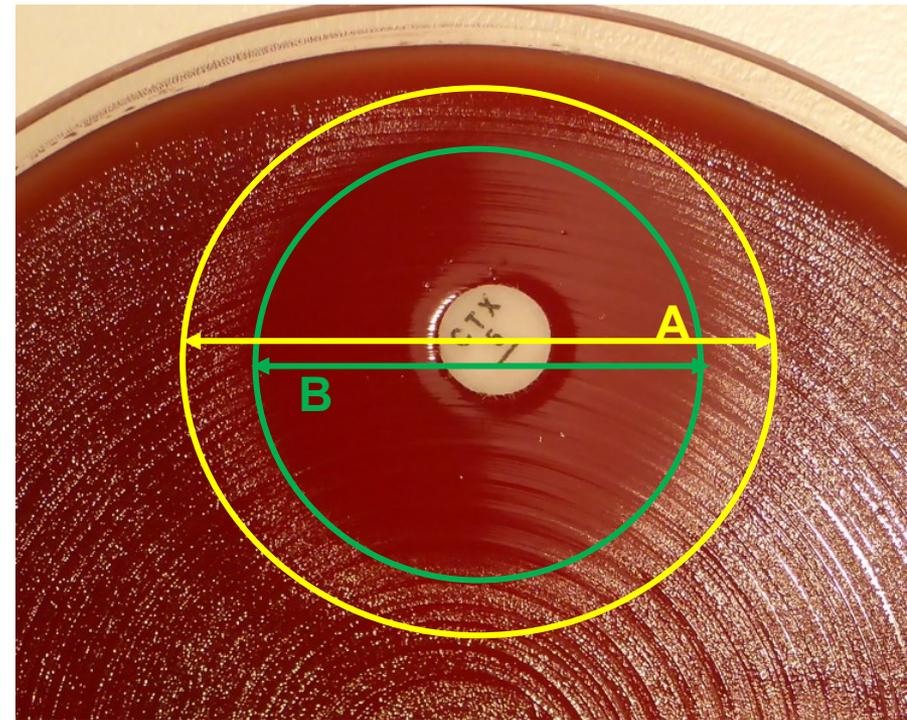
In case of *Haemophilus influenzae*, how do you read cefotaxime inhibition zone?

A: read the most obvious inhibition zone

B: look closely and take all the growth into account

C: hold the plate in a 45-degree angle and estimate where the zone edge is

D: repeat the test



Mentimeter

In case of *Haemophilus influenzae* with this AST, choose the correct answer(s):

Repeat the AST

Check the QC of PCG disc

Confirm cefotaxime result with MIC gradient test

If PCG is S, I do not need to read the other β -lactam disks

If PCG is S, I do not need to perform a β -lactamase test

- Penicillin screen S
- β -lactamase negative
- Ampicillin S
- Amox-clav. acid I
- Cefotaxime R

Mentimeter

In case of Haemophilus influenzae with this AST, choose the correct answer(s):

Repeat the AST

Check the QC of AMP disk

Check the QC of AMC disk

Confirm AMC result with MIC gradient test
Report R to AMP and AMC

Report S to AMP and suppress AMC

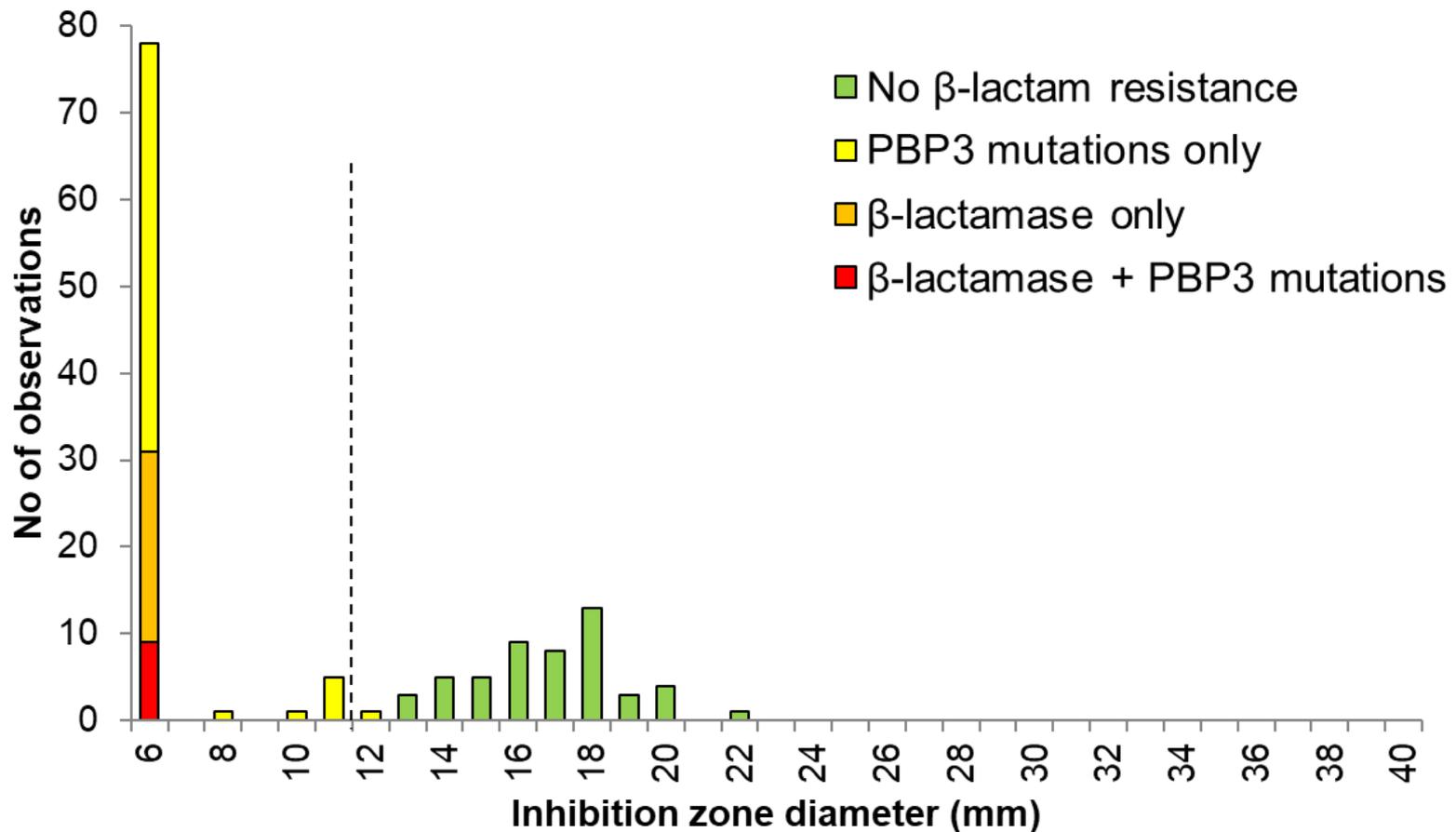
- Penicillin screen 
- β -lactamase negative
- Ampicillin 
- Amox-clav. acid 
- Cefotaxime 

AST of *H. influenzae*

- Problems with AST of *H. influenzae* can be related to
 - Resistance mechanisms
 - Breakpoints splitting NWT populations
 - Testing issues
 - Too humid MH-F plates
 - Reading of zones

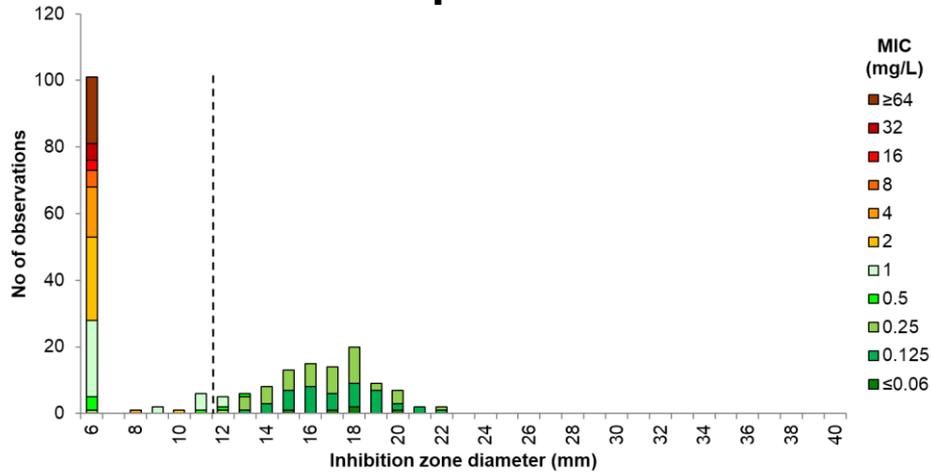
Benzylpenicillin 1 unit screening test

Benzylpenicillin 1 unit vs. β -lactam resistance mechanism

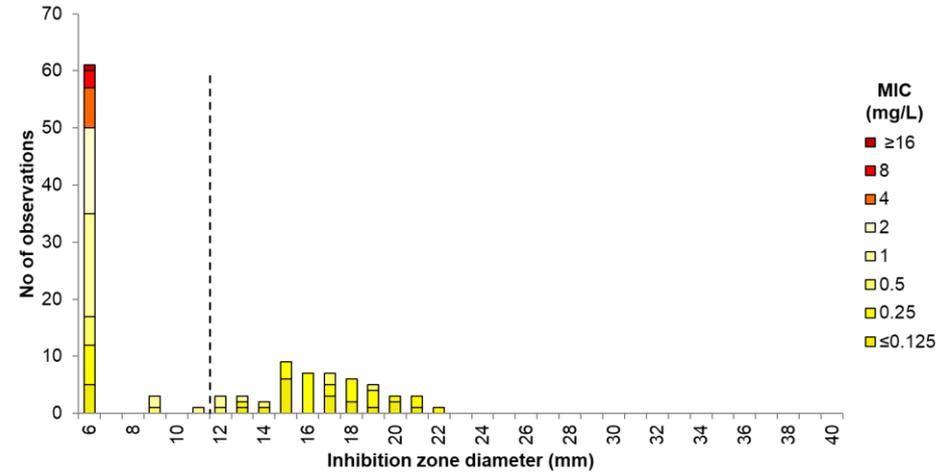


Benzylpenicillin 1 unit vs. MICs for other beta-lactams

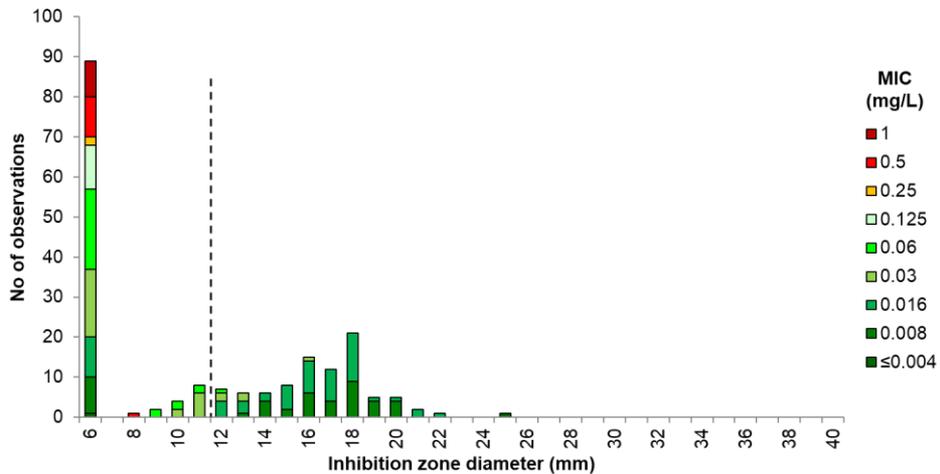
Ampicillin



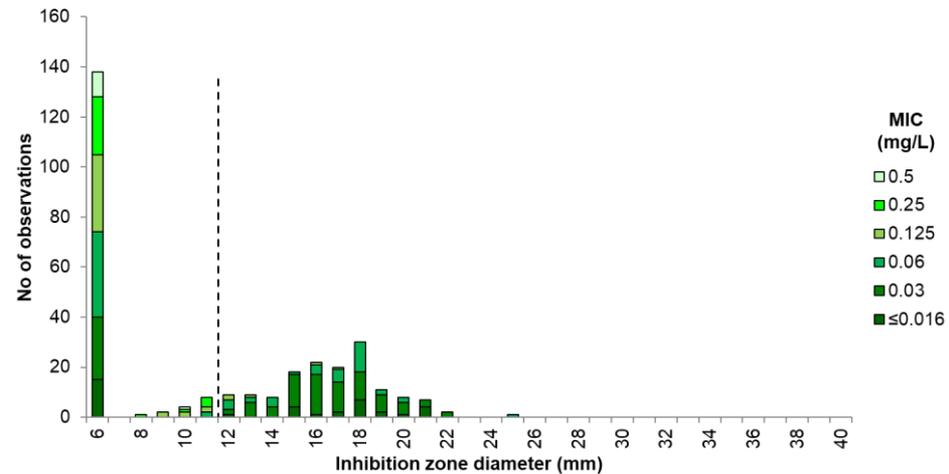
Amoxicillin-clavulanic acid



Cefotaxime



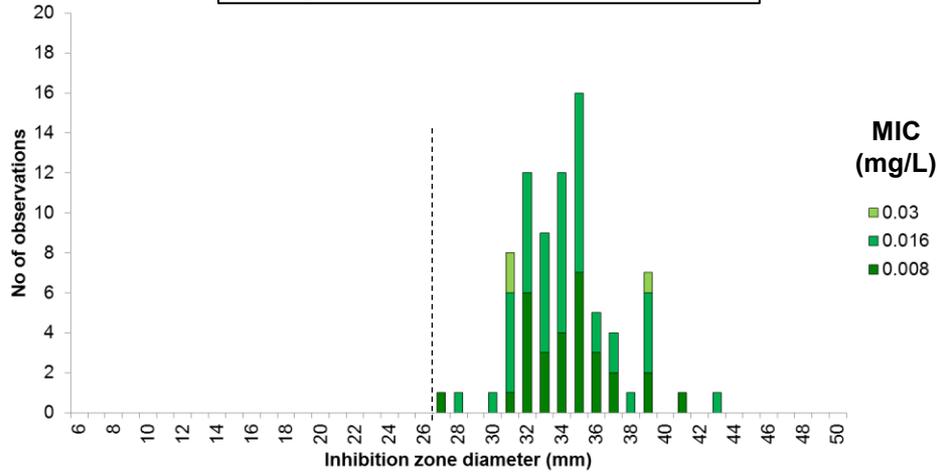
Meropenem



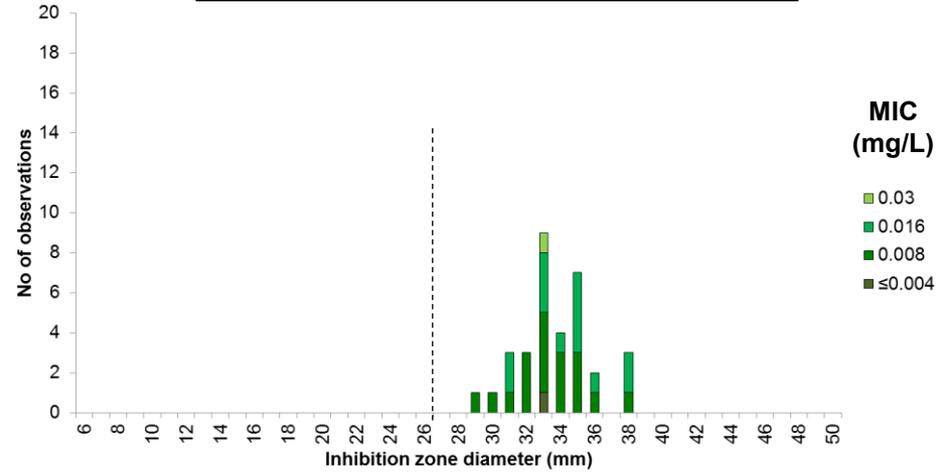
H. influenzae

Cefotaxime 5 µg disk diffusion per beta-lactam resistance mechanism

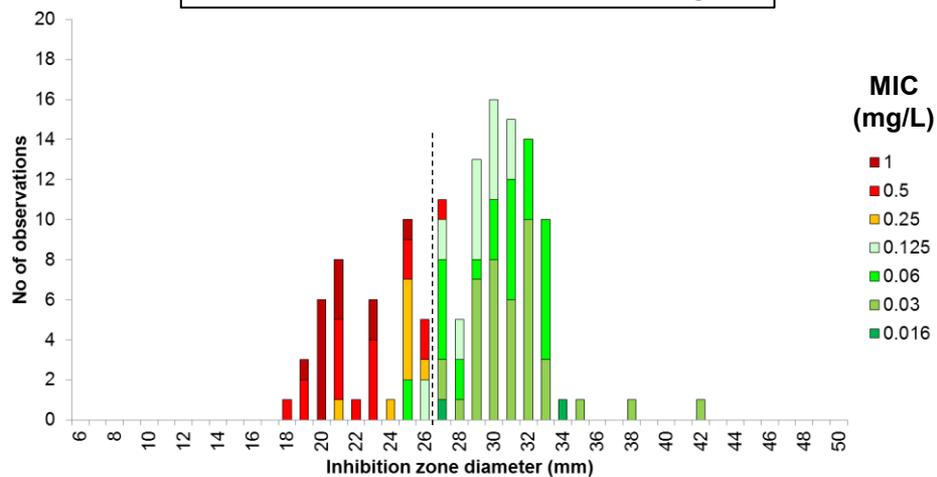
No β-lactam resistance



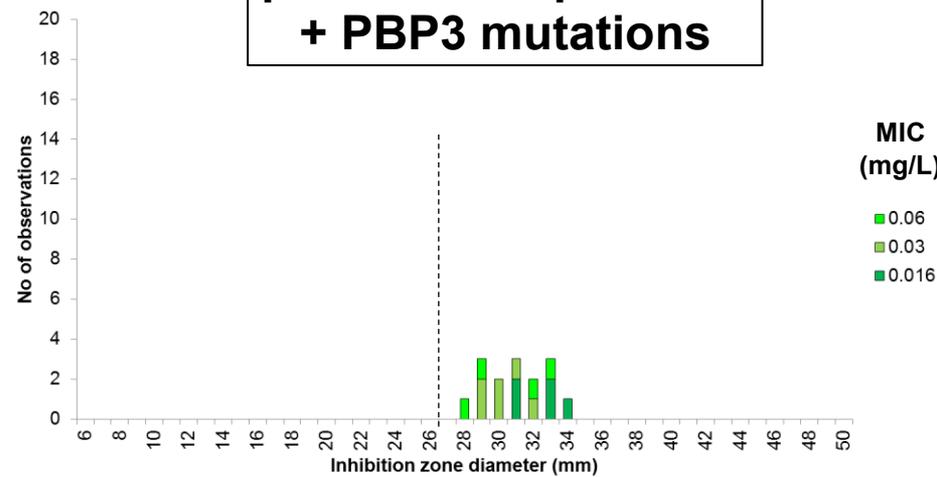
β-lactamase positive only



PBP3 mutations only



β-lactamase positive + PBP3 mutations

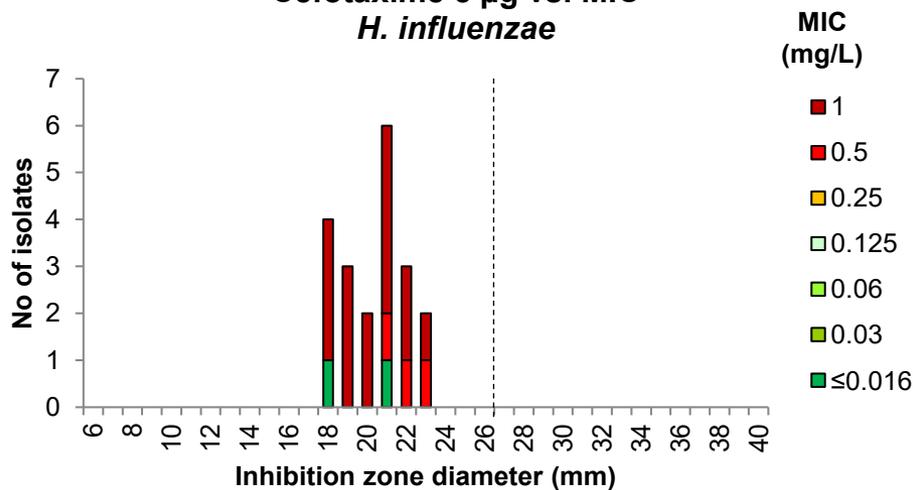


Confirmation of CTX R *H. influenzae* with BMD

Data from Clinical Microbiology Karlskrona and Växjö, 2023

Routine DD test result

Cefotaxime 5 µg vs. MIC
H. influenzae



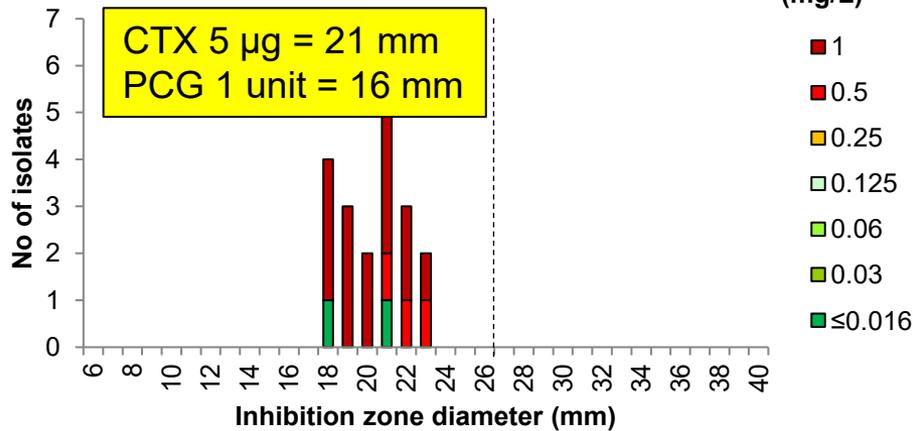
Two isolates with CTX 5 µg zones
<27 mm had BMD MICs ≤0.016 mg/L!

BMD of CTX R *H. influenzae* with BMD

Data from Clinical Microbiology Karlskrona and Växjö, 2023

Routine DD test result

Cefotaxime 5 µg vs. MIC
H. influenzae



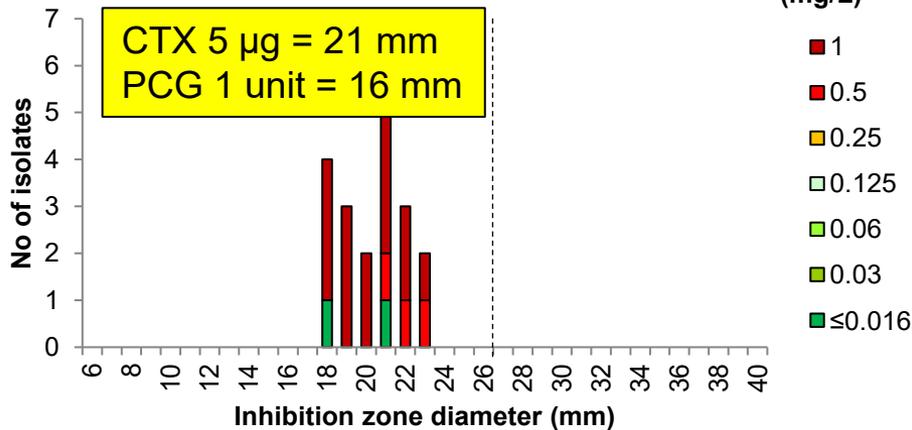
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BMD of CTX R *H. influenzae* with BMD

Data from Clinical Microbiology Karlskrona and Växjö, 2023

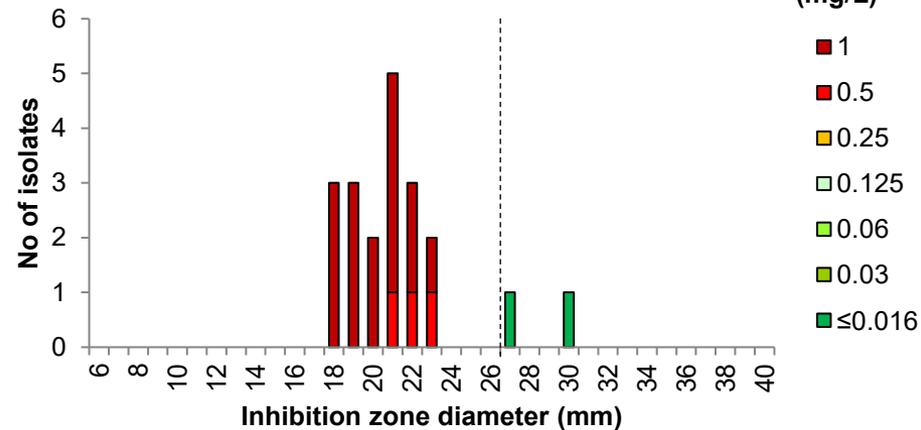
Routine DD test result

Cefotaxime 5 µg vs. MIC
H. influenzae



After retesting

Cefotaxime 5 µg vs. MIC
H. influenzae



Two isolates with CTX 5 µg zones
<27 mm had BMD MICs ≤0.016 mg/L!

MH-F agar plates

Excess moisture and fuzzy zone edges



Excess moisture may cause fuzzy zone edges and/or haze within zones. If necessary, dry plates before use.

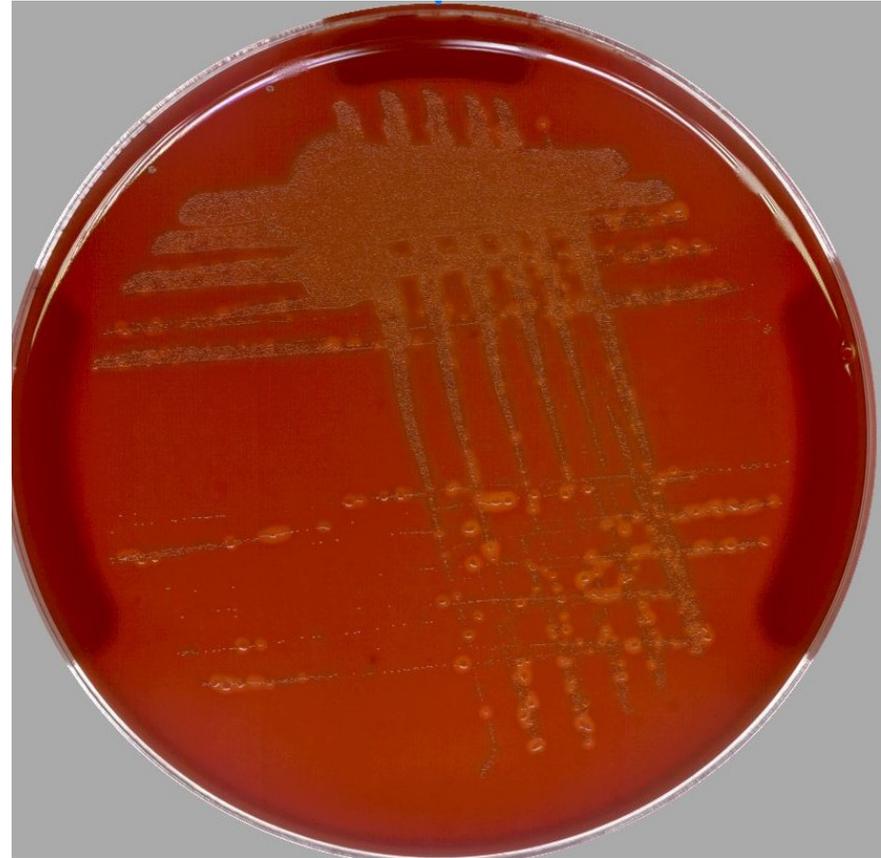
Reading zone diameters for *H. influenzae* and beta-lactam agents

- Read the outer edge of zones where an otherwise clear inhibition zone contains an area of growth around the disk



Case 4

- 14 year old with sore throat and fever
- GAS-Ag from throat is positive
- The patient has a lot of allergies and specifically against β -lactams with history of anaphylactic shock
- Clinician orders throat culture for AST
- Easter is coming... impossible to do directly AST (not enough isolated CFU)
- Technician made a subculture on 1 isolated colony, grew it for 6 hours and did the AST



Mentimeter

In case of GAS with this AST, choose the correct answer(s)

Penicillin R is an unexpected phenotype

It doesn't matter, patient is allergic to penicillin

Check for purity

Check identification

Repeat AST from an overnight culture

- Penicillin 
- Erythromycin 
- Clindamycin 

Mentimeter

Klebsiella isolates with large colonies will often give a "thickly inoculated" AST plate. Choose the correct answer(s)?

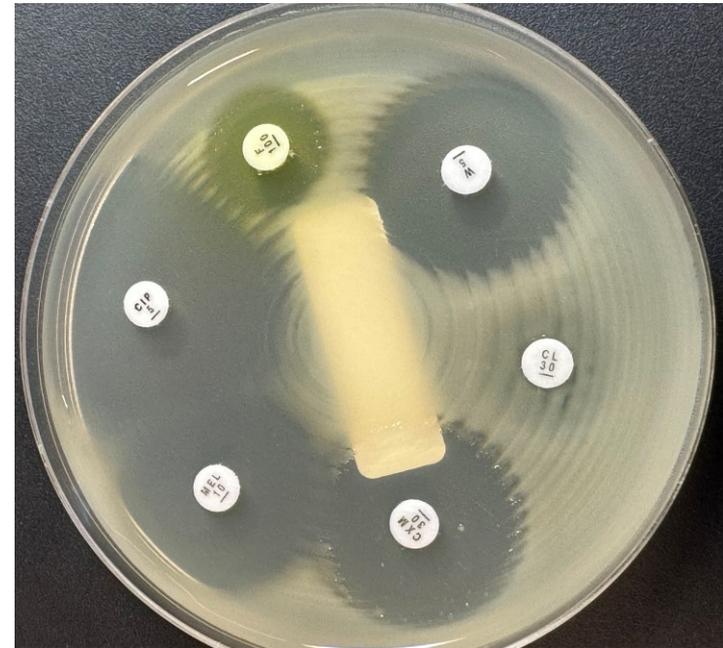
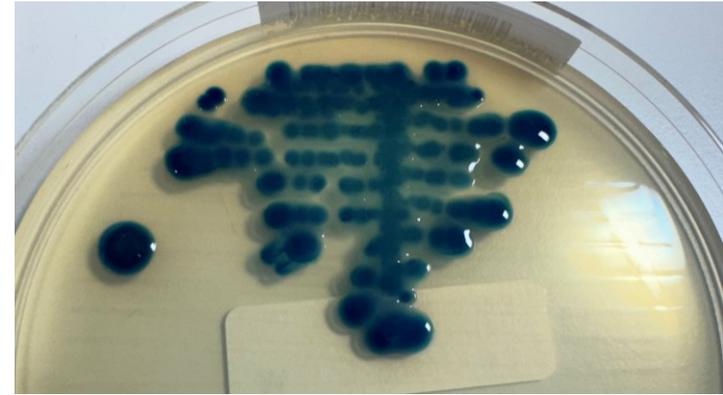
If large inhibition zones, read as it is

If inhibition zones close to BP, repeat AST with 0,3 McF inoculum

Remove excess fluid from the swab before streaking

Incubate less than 16 h

In case of RAST from BC bottle, repeat from an overnight growth if zones are close to BP



Mentimeter

What should you do when there are colonies within the inhibition zone?

Take all the colonies into account

Take only the large colonies into account

Ignore the isolated colonies

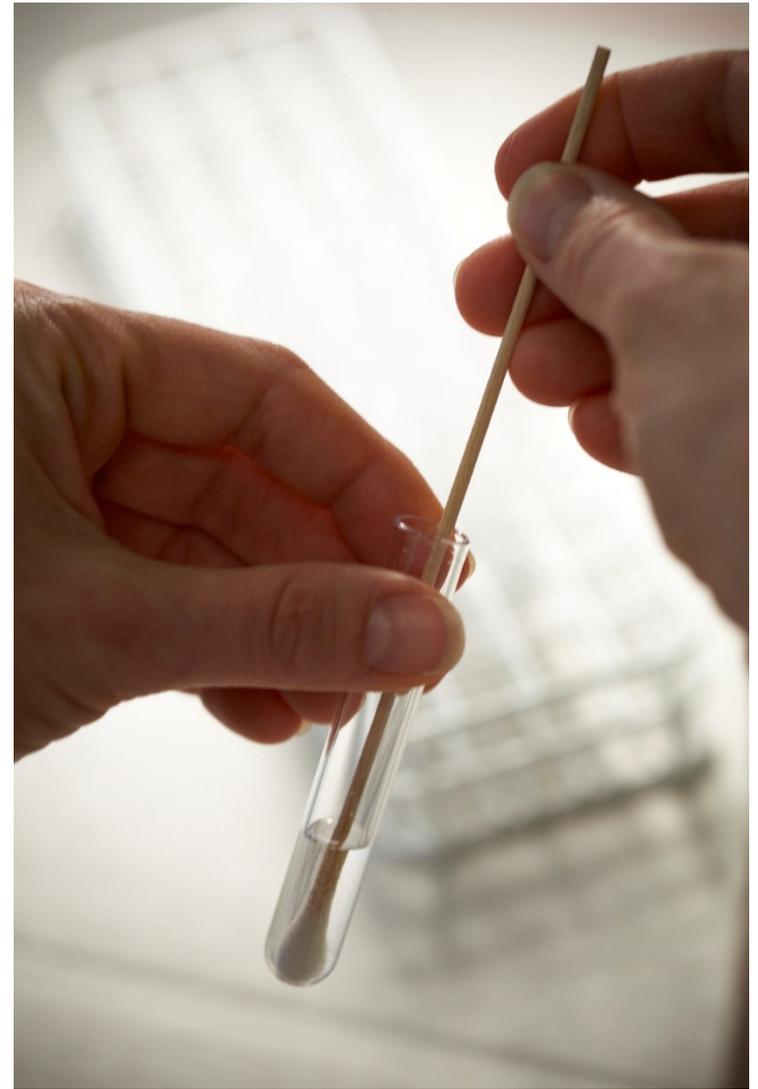
Check for purity

Retest from the isolated colonies



Inoculum

- The method requires an inoculum suspension with a turbidity equivalent to a 0.5 McFarland standard.
- Exceptions:
 - *Streptococcus pneumoniae* (McF 0.5 from blood agar plate, McF 1.0 from chocolate agar plate)
 - Anaerobic bacteria (McF 1.0)



Select well-isolated colonies from an overnight culture on non-selective medium



Overnight culture = Bacterial culture incubated for 16-24 h

Inoculum

- If possible, use several morphologically similar colonies to avoid selecting an atypical variant.



Do not repeat disk diffusion from colonies growing within the inhibition zone!

Inoculum

- If possible, use several morphologically similar colonies to avoid selecting an atypical variant.



Reading of zones

- Contamination?
- If cultures are pure, colonies within zones should be taken into account when measuring the diameter.

Inoculation of plates

- For **Gram-negative bacteria**, remove excess fluid by pressing and turning the swab against the inside of the tube **to avoid over-inoculation**.
- For Gram-positive bacteria, do not press or turn the swab against the inside of the tube and take particular care to ensure that there are no gaps between streaks.



Inoculum from positive blood culture bottles

- Use the EUCAST RAST method
 - Breakpoints for 4, 6, 8 and 16-20 h incubation
- Other local method
 - Laboratories are responsible for validating all deviations from the standard methodology
 - Too thick inoculum and/or zone diameters close to the breakpoints: Repeat with standard disk diffusion from an overnight culture!

Age of inoculum

- Overnight culture = 16-24 h incubation
 - The growth phase of the bacteria affects the results
 - Using bacterial cultures incubated less than 16-24 h results in deviating zone diameters

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Mean zone diameters from 6 tests (mm)

<i>S. pneumoniae</i> ATCC 49619	OXA 1	PCG 1	NOR 10	ERY 15	KLI 2	TET 30	RIF 5	TSU 25
4 h culture	10.4	17.0	17.8	24.4	20.6	26.8	24.6	18.8
6 h culture	10.8	17.2	17.6	24.0	20.6	26.6	24.2	19.0
Overnight culture	10.8	17.4	18.4	25.2	21.4	28.8	26.0	19.8
Difference	0.4	0.4	0.8	1.2	0.8	2.2	1.8	1.0

<i>H. influenzae</i> ATCC 49766	PCG 1	CEC 30	CTX 5	MER 10	NAL 30	TET 30	TSU 25
4 h culture	16.7	23.2	29.8	25.8	25.5	27.8	28.2
6 h culture	16.5	22.3	29.2	23.5	25.3	27.8	27.7
Overnight culture	17.3	24.8	32.0	27.5	26.8	29.2	29.0
Difference	0.8	2.5	2.8	4.0	1.5	1.4	0.5

Largest mean zone diameter

>1 mm difference between largest and smallest value