

Background for resistance mechanism testing in every-day routine: a case-based session

Anna Hill, Dennis Back Holmgaard & Barbara Holzknecht



2024 workshop, Malmö
Meet-the-Experts 28.5.2024

Agenda

S. aureus

Enterobacterales

Pseudomonas spp.

H. influenzae

~~*Enterococcus* spp.~~

1. case: *Staphylococcus aureus*

Bone tissue isolate (ostitis)

Which agents would you test for?

Staphylococcus aureus

Disk diffusion testing, Dept. Clinical Microbiology Herlev, DK:

Standard panel

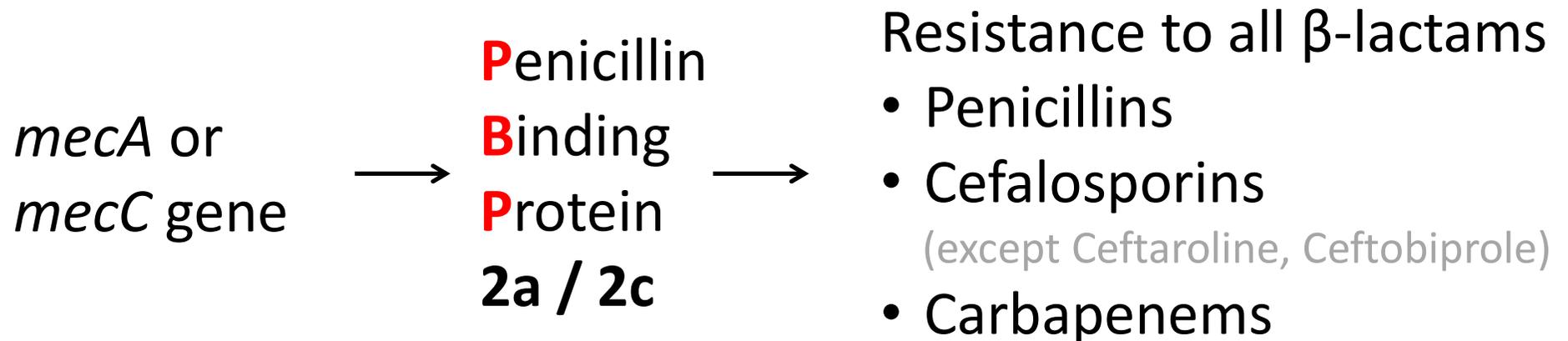
- Penicillin
- Cefoxitin
- Erythromycin
- Clindamycin
- Fusidinic acid
- Moxifloxacin

“extended panel”

- Tetracycline
- Rifampicin
- Linezolid
- Mupirocin
- Trimethoprim/Sulfamethizol
- Gentamicin

If you should choose one single disk, which one would you choose?

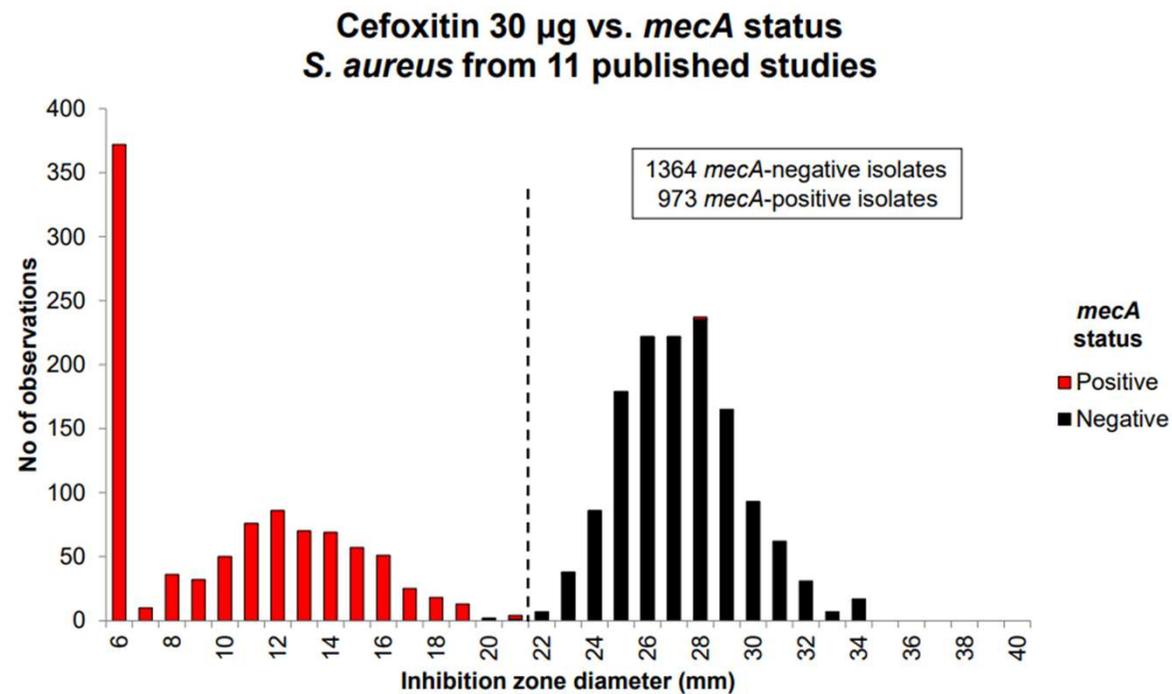
Methicillin-resistant *S. aureus* (MRSA): background



Nordic countries:

- Low prevalence (invasive isolates 2022: 1,1 – 2,8%)
- Reportable disease

MRSA: susceptibility testing



Case: *S. aureus* otitis isolate. Cefoxitin zone 12 mm.

Would you perform a confirmatory test?

<i>Staphylococcus</i> spp.				EUCAST kliniske brytningspunkter v. 14.0, NordicAST v. 14.0, 2024-01-19				
Expert Rules and Expected Phenotypes								
For abbreviations and explanations of breakpoints, see the Notes sheet								
Cephalosporins	MIC breakpoints (mg/L)			Disk content (µg)	Zone diameter breakpoints (mm)			Kommentarer
	S ≤	R >	ATU		S ≥	R <	ATU	
								<p>Cefoksitin benyttes for å utelukke meticillinresistens. Cefoksitinfølsomme isolater kan besvares S for samtlige cefalosporiner som har kliniske brytningspunkter eller Note unntatt cefotaksim og ceftriakson som rapporteres "I-Følsom, økt eksponering". For midler som gis peroralt, må man sørge for at doseringen gir tilstrekkelig eksponering ved infeksjonsstedet.</p> <p>Cefoksitinresistens hos <i>S. aureus</i> konfirmeres med <i>mecA</i>-/<i>mecC</i>-undersøkelse. Cefoksitinresistente isolater besvares R for cefalosporiner, unntatt ceftarolin og ceftobiprol som rapporteres i henhold til resistensbestemmelse. Alle <i>S. aureus</i> isolater som har <i>mecA</i> eller <i>mecC</i> betraktes som MRSA.</p> <p>For <i>S. pseudintermedius</i>, <i>S. intermedius</i>, <i>S. schleiferi</i> og <i>S. coagulans</i> benyttes oksacilin i stedet for cefoksitin til screening for meticillinresistens, se Note under cefoksitin.</p>
Cefoxitin (screen only) , <i>S. aureus</i> and coagulase-negative staphylococci except <i>S. epidermidis</i> and <i>S. lugdunensis</i>	Note	Note		30	22	22		<p>Cefoksitin MIC kan benyttes for påvisning av meticillinresistens hos <i>S. aureus</i> (>4 mg/L) og <i>S. saprophyticus</i> (>8 mg/L). For andre stafylokokker, benytt kun lappediffusjon. Om koagulasenegative stafylokokker ikke identifiseres til artsnivå, benytt sonebrytningspunkter S ≥ 25 / R < 22 mm. Isolater med sonediameter 22–24 mm bør rapporteres R eller undersøkes for <i>mecA</i>/<i>mecC</i>.</p>

Which test?

PCR (*mecA*, *mecC*)

Latex agglutination (PBP2a)



Picture:

http://www.oxid.com/dk/BLUE/prod_detail/prod_detail.asp?pr=DR0900&c=dk&lang=en&org=&img=DR0900A&sec=

Cefoxitin screening negative *S. aureus* isolate (inhibition zone 25 mm).
Would you test for other beta-lactams?

S. aureus: Benzylpenicillin susceptibility

Mechanism ***blaZ*** gene \longrightarrow penicillinase
beta-lactamase

Testing

Penicillin S

Inhibition zone ≥ 26 mm
AND "beach"



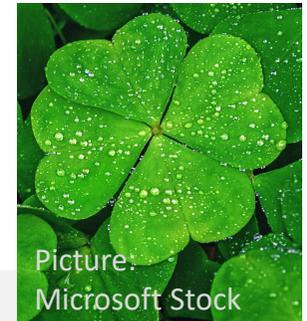
Penicillin R

Inhibition zone < 26 mm
OR "cliff"



Confirmatory test?

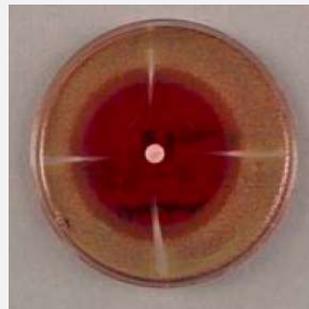
Clover leaf test



Principle

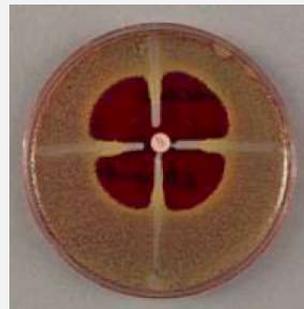
seeding with Penicillin-susceptible *S. aureus* (PSSA), cross: test strain
Penicillinase producing test strain (Penicillin R): cleavage of Penicillin → PSSA can grow

negative



Penicillin S

positive



Penicillin R

Pictures: Hanne Wiese

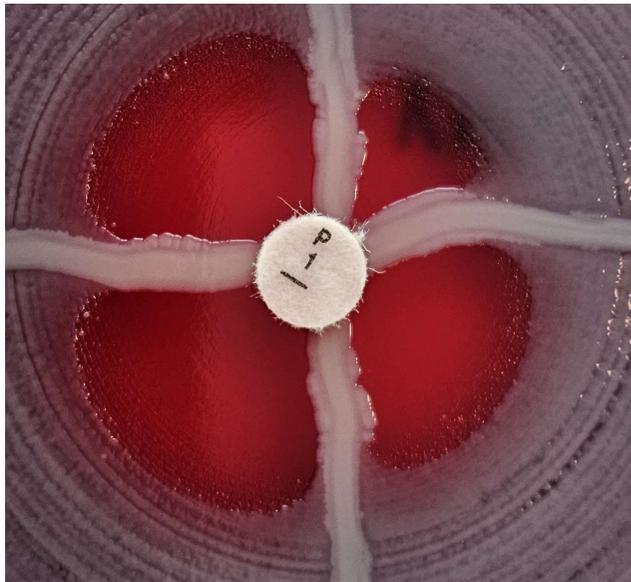
Ørstavik,
Acta Path Microbiol Scand,
1971. 79:855-856.

Performance

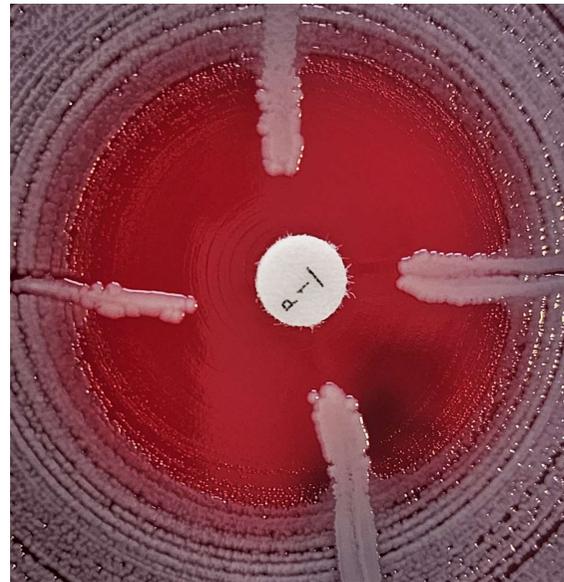
100 % sensitivity vs. 98% sensitivity for disk diffusion (zone diameter + edge)

Skov et al. J Antimicrob Chemother 2021; 76: 1487–1494

Clover leaf test: inoculum matters!



High inoculum
(testisolate streak from
colony)



Same isolate, low inoculum
(testisolate streak from
0,5 McFarland suspension)

Your *S. aureus* otitis patient is beta-lactam allergic.

The isolate is erythromycin R (8 mm), but has a large clindamycin inhibition zone (24 mm).

Can you report clindamycin S?

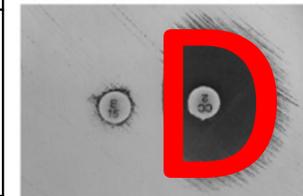
S. aureus: Macrolides/Lincosamides

for testing: Erythromycin

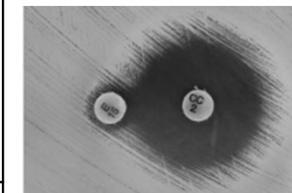
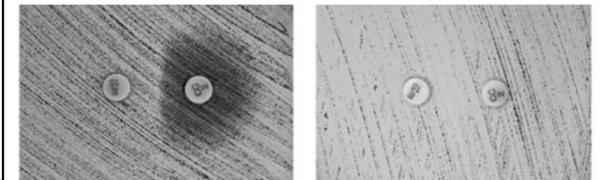
Clindamycin

12-20 mm apart (edge to edge)

Gene	Phenotype	Resistance mechanism	Macrolides	Clindamycin
<i>erm</i> (A,C)	MLS _B -inducible	Ribosomal methylation	R	(s)
<i>erm</i> (A,C)	MLS _B -constitutive	Ribosomal methylation	R	R
<i>Msr</i>	MS _B	Efflux	R	S
<i>inu</i>	L	Inactivation	S	(S-)R



Positiv D-test



MLS: Macrolides, Lincosamides, Streptogramins

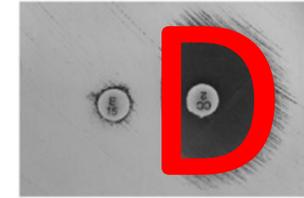
erm: erythromycin ribosome methylation

msr: macrolide streptogramin resistance

Table adapted from: Mandell 9th ed. Table 18.7

Pictures: Steward et al. J Clin Microbiol 2005;43(4):1716–1721.

D-test: clinical relevance



Positiv D-test

Erythromycin R, Clindamycin S, positive D-test

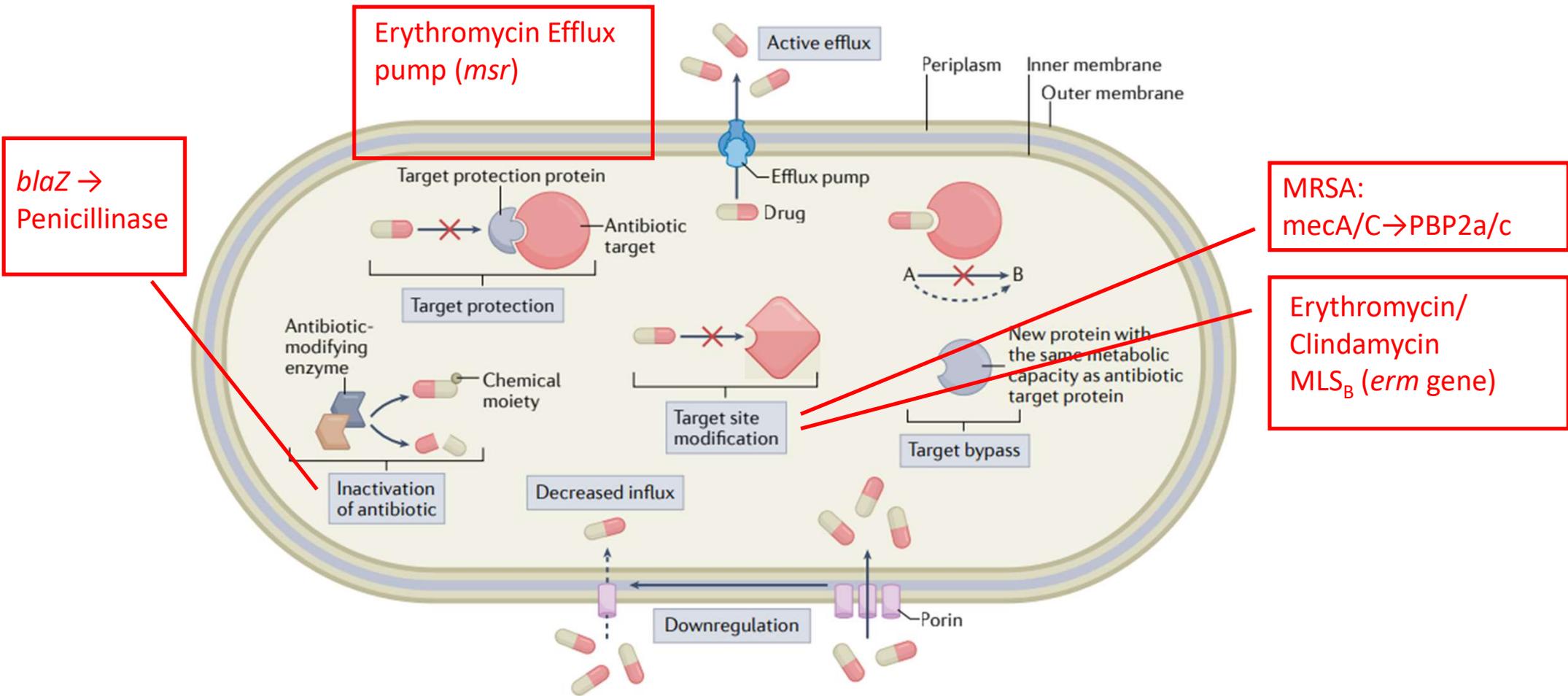
→ Report Clindamycin R

Comment can be added:

"Clindamycin may still be used for short-term therapy of less serious skin and soft tissue infections as constitutive resistance is unlikely to develop during such therapy".

S. aureus

Overview over resistance mechanisms



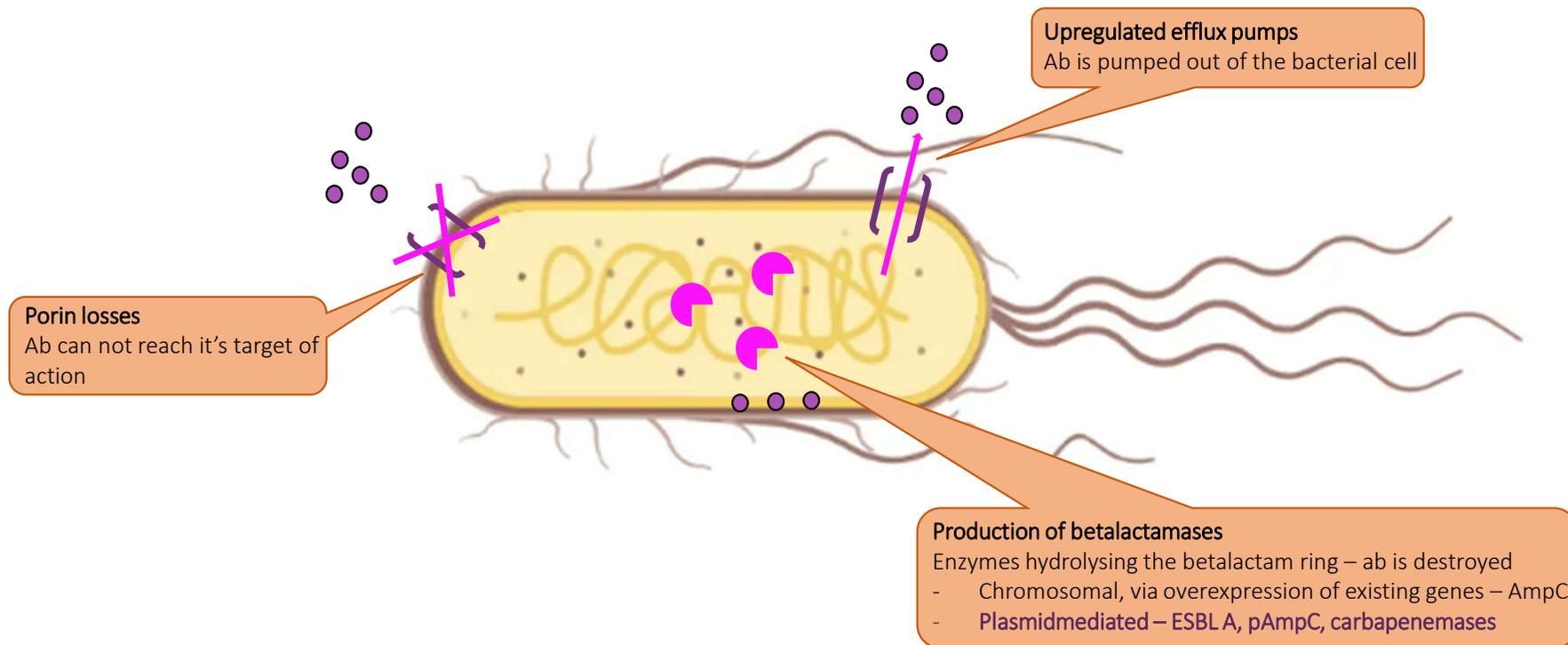
*Enterobacterales and Pseudomonas aeruginosa –
Principles for resistance screening and testing*

Anna Hill



2024 workshop, Malmö
Meet-the-Experts 28.5.2024

Resistance mechanisms in Enterobacteriales



Principles of phenotypic resistance testing

Ambler class	A	C
ESBL group	ESBL _A	Plasmidmediated AmpC (pAmpC, ESBL _M)
Main features	Hydrolyse cephalosporins gen 1-4 NOT cefoxitin	Hydrolyse cephalosporins gen 1-3 AND cefoxitin
Inhibitors	Clavulanic acid	Cloxacillin

Inhibits the effect of ESBL-producing enzymes – hence used in phenotypic ESBL-tests

Screening agents
 Cefotaxim/Ceftazidim (3rd gen)
 Cefpodoxim (3rd gen)
 Cefadroxil (1st gen)
 Cefepime (4th gen, ESBL A in AmpC producers)
 Cefoxitine (Cephamicine, stable towards ESBL A)

What is the most important factor to bear in mind when interpreting your ESBL-test?

MAST-test

DDST-test (Double Disc Synergy Test)

A CPD10
 B CPD10 + ESβL inhibitor
 C CPD10 + AmpC inhibitor
 D CPD10 + ESβL inhibitor + AmpC Inhibitor

Calculator programme is available to download from www.mastgrp.com

ESBL Positive
 $Z_B - Z_A$ and $Z_D - Z_C \geq 5\text{mm}$
 and the differences of each of Z_B and Z_D and Z_A and Z_C are $< 4\text{mm}$

AmpC Positive
 $Z_D - Z_B$ and $Z_C - Z_A \geq 5\text{mm}$
 and the differences of each of Z_A and Z_B and $Z_C - Z_D$ are $< 4\text{mm}$

AmpC and ESBL Positive
 $Z_D - Z_C \geq 5\text{mm}$ and the difference of Z_A and Z_B is $< 4\text{mm}$

AmpC and ESBL Negative
 All zones differ by $\leq 2\text{mm}$

CPD - Cefpodoxime

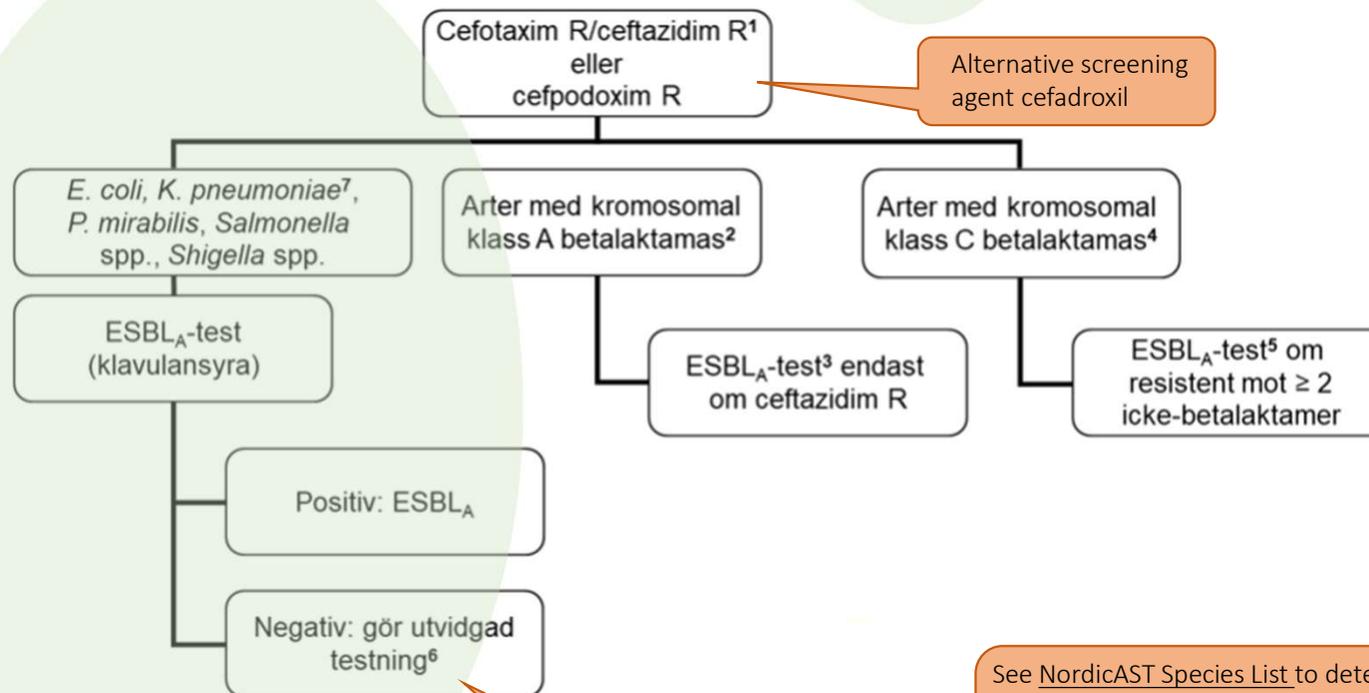
Clavulanic acid effect = ESBL A+

1036
 ESBL

Cloxacillin added = no synergy, pAmpC/ESBL_M⁻

Cefotaxim/Ceftriaxone	R	R	R
Ceftazidim	S	R	R
Piperacillin/tazobactam	S	S	S
Meropenem	S	S	S
Ciprofloxacin	S	R	R
Tobramycin	S	S	S
Trimethoprim/sulfamethoxazol	R	S	R

Enterobacterales
ALGORITM FÖR DETEKTION AV KLASSISKA ESBL (ESBLA)
NordicAST v. 14.0, 2024-01-19



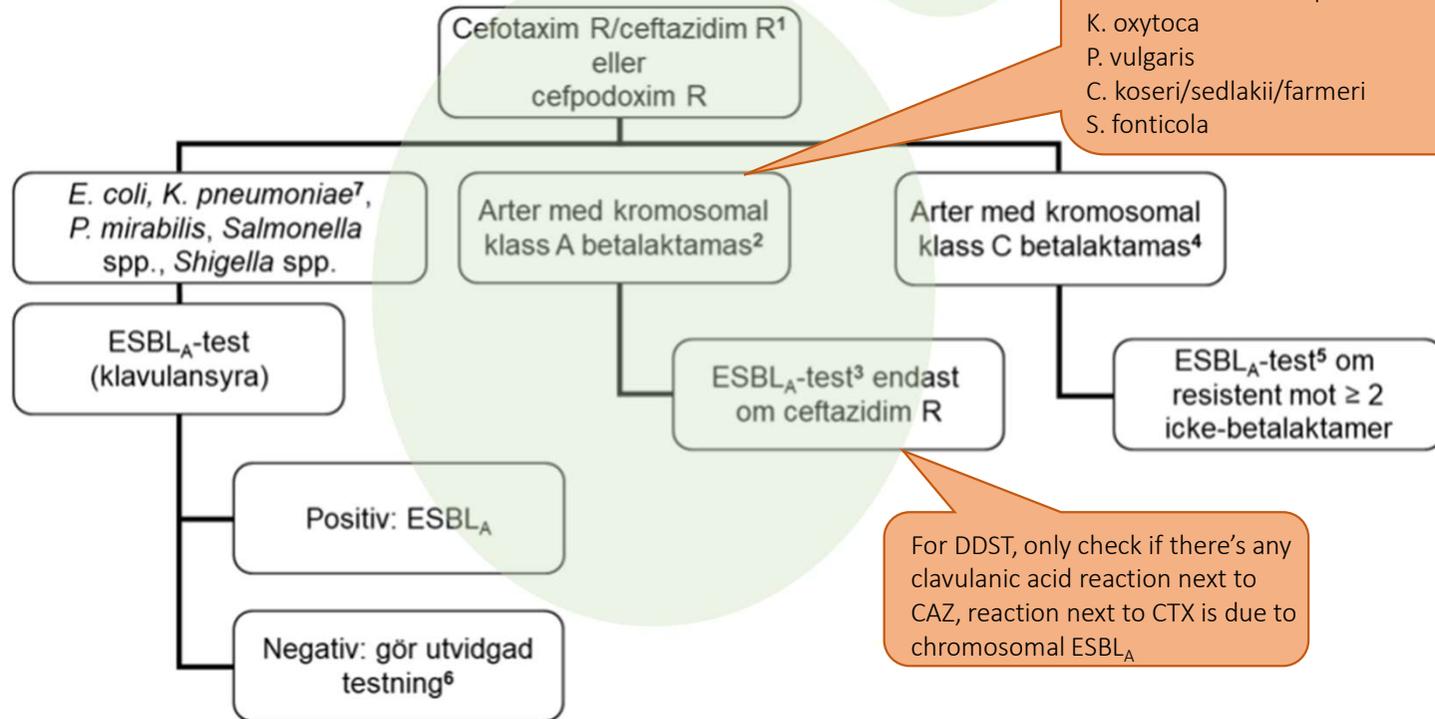
Alternative screening agent cefadroxil

AmpC?
AmpC + A?
Other?

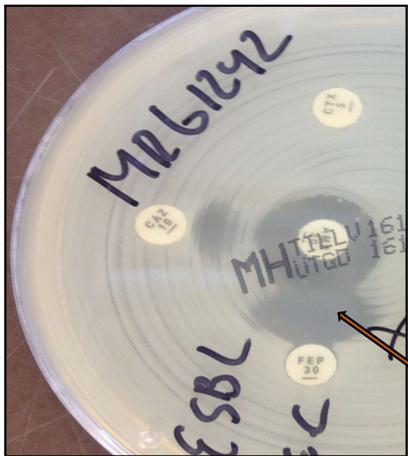
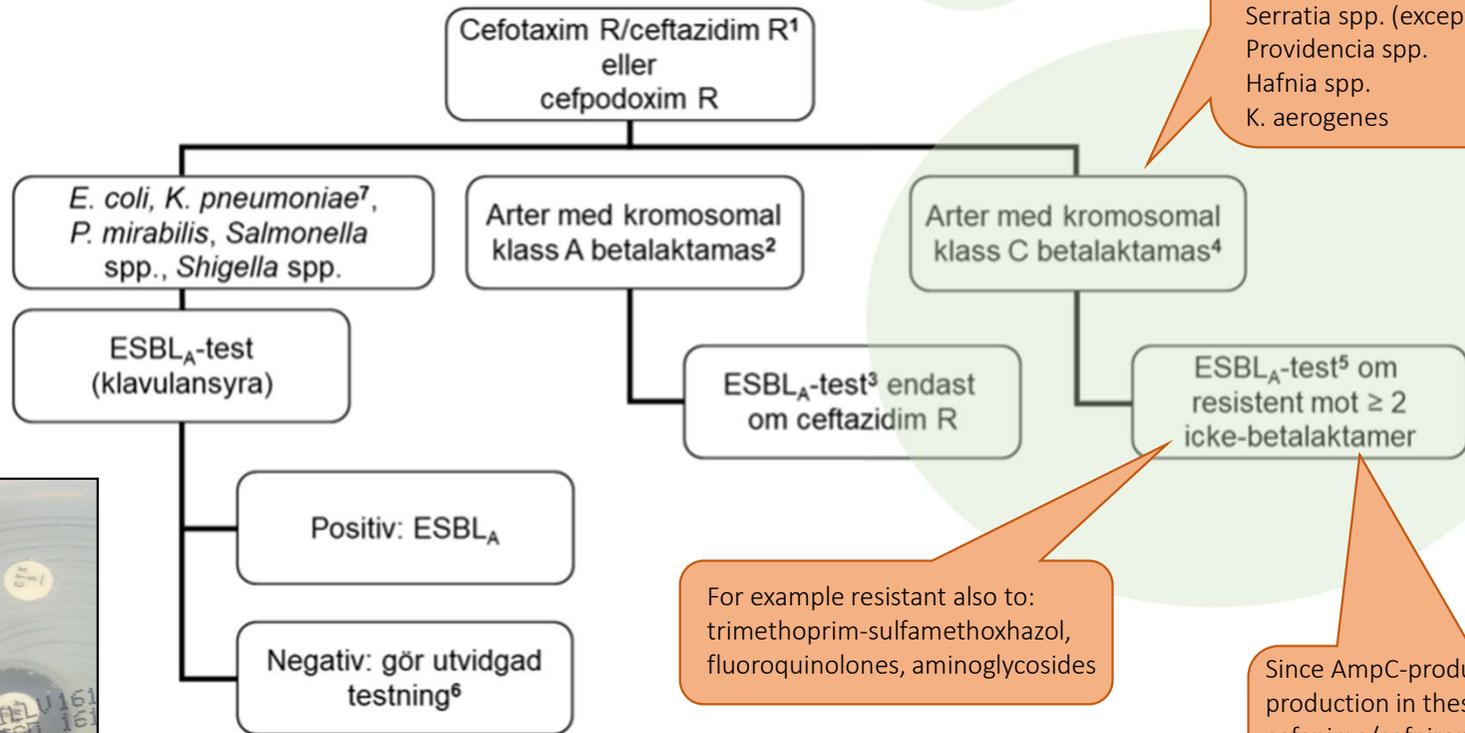
See [NordicAST Species List](#) to determine which species have chromosomal class A and C beta lactamases

NordicAST webpage – Breakpoints and Dosages

Enterobacterales
ALGORITM FÖR DETEKTION AV KLASSISKA ESBL (ESBLA)
NordicAST v. 14.0, 2024-01-19



Enterobacterales
ALGORITM FÖR DETEKTION AV KLASSISKA ESBL (ESBL_A)
 NordicAST v. 14.0, 2024-01-19



Cefepime disc (4th gen), clavulanic acid shows inhibitory effect = ESBL_A

Picture from AK Smekal

Algorithm for detection of plasmid-mediated AmpC (ESBL_M)

Most Enterobacterales harbour chromosomal/inducible AmpC. These species come with exceptions

Cefotaxime R or ceftazidime R AND cefoxitin R¹
in *E. coli*, *K. pneumoniae*², *P. mirabilis*,
Salmonella spp, *Shigella* spp

Reduced susceptibility to cefoxitin is taken as indicator of AmpC production

ESBL_M-test (cloxacillin) positive

ESBL_M-test (cloxacillin) negative

E. coli and *Shigella* spp: PCR should be used to separate chromosomal and plasmid-mediated AmpC

K. pneumoniae, *P. mirabilis*, *Salmonella* lack chromosomal AmpC and feature ESBL_M

Other mechanism (porin loss, PBP-changes)

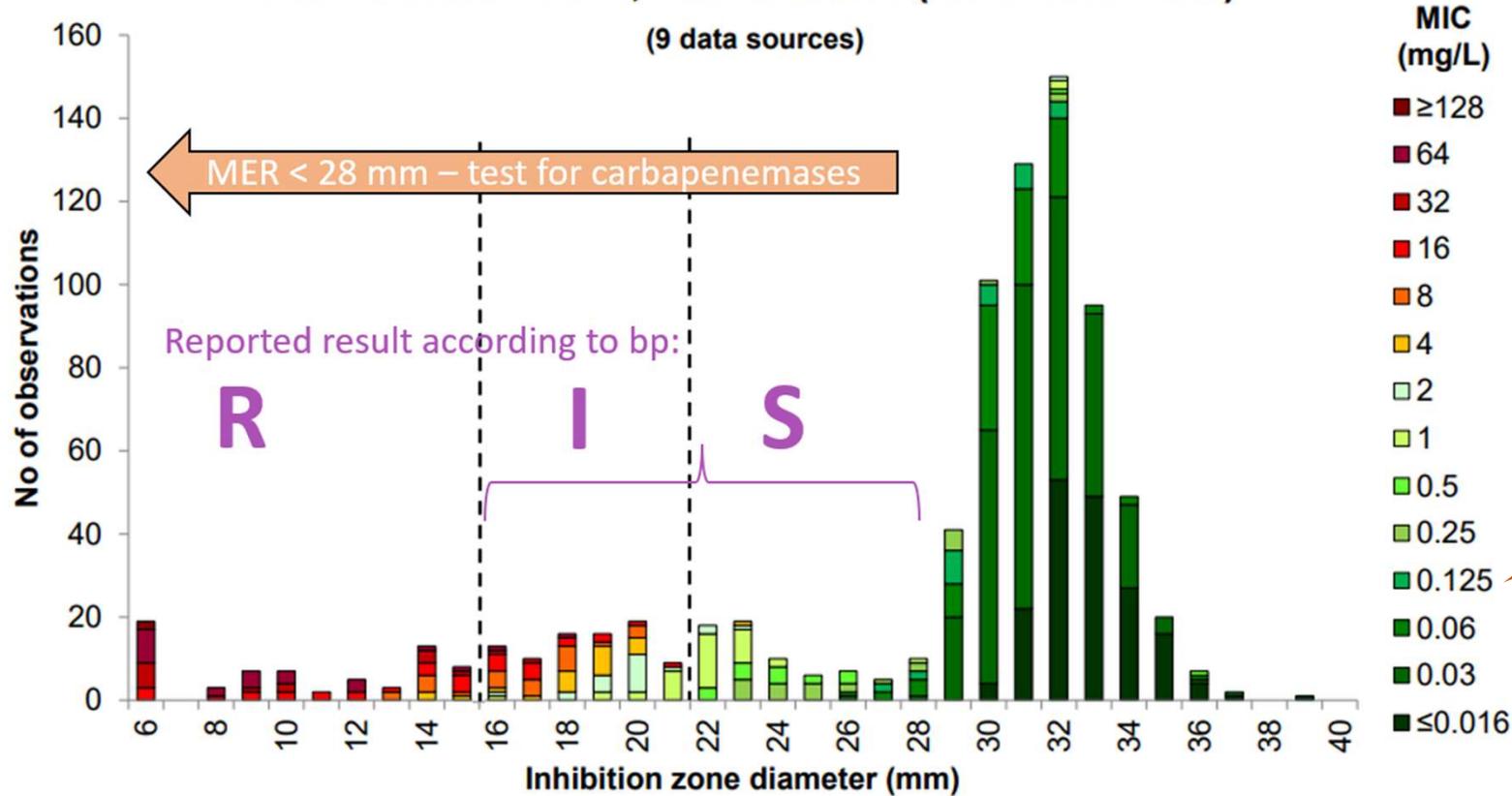
Phenotypic testing is enough

Screening for carbapenemases

Carbapenems	MIC breakpoints (mg/L)			Disk content (µg)	Zone diameter breakpoints (mm)		
	S ≤	R >	ATU		S ≥	R <	ATU
Ertapenem	0.5	0.5		10	23	23	
Imipenem, <i>Enterobacterales</i> except <i>Morganellaceae</i>	2	4		10	22	19	
Imipenem, <i>Morganellaceae</i>	0.001	4		10	50	19	
Imipenem-relebactam, <i>Enterobacterales</i> except <i>Morganellaceae</i>	2	2		10-25	22	22	20-22
Meropenem (indications other than meningitis)	2	8		10	22	16	
Meropenem (meningitis)	2	2		10	22	22	
Meropenem (screen)	0.125	Note		10	28	Note	

Screening bp is not equal to clinical bp

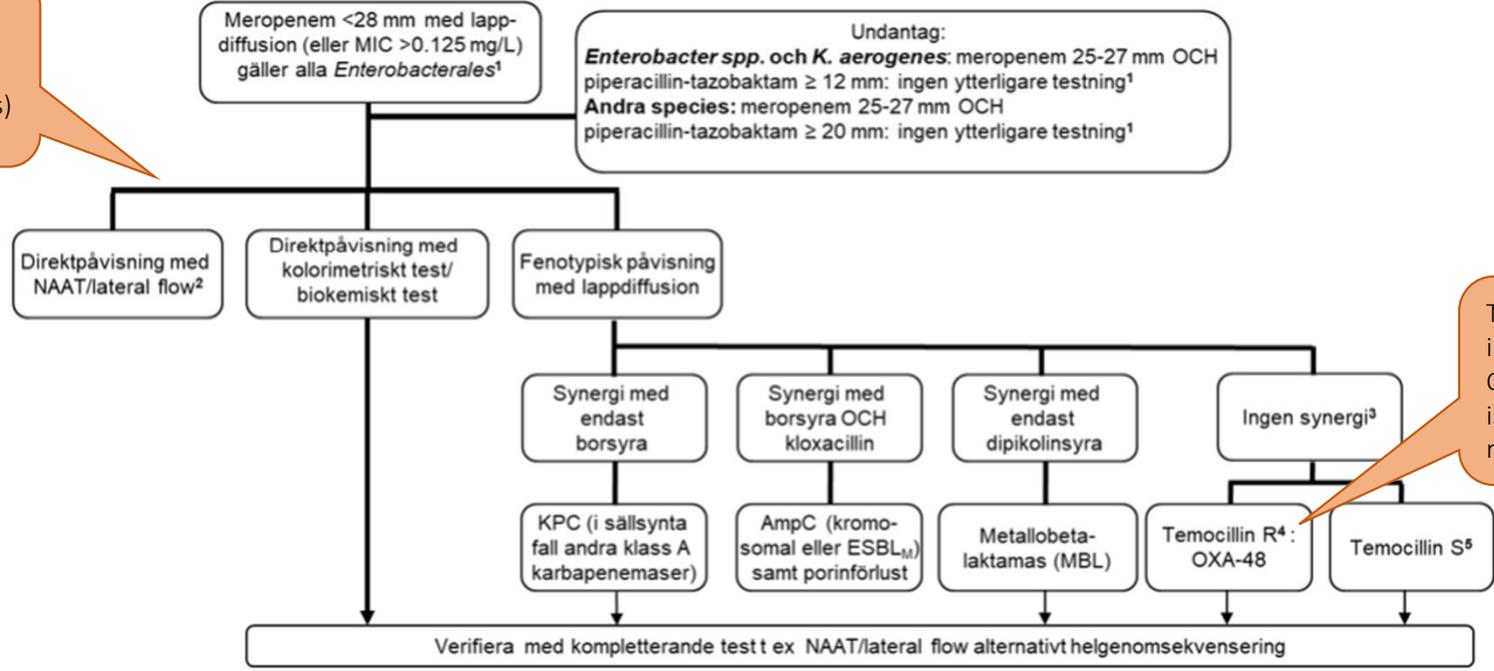
Meropenem 10 µg vs. MIC *Enterobacterales*, 576 isolates (820 correlates)



Breakpoints (meningitis)	
MIC	S ≤ 2, R > 2 mg/L
Zone diameter	S ≥ 22, R < 22 mm

Enterobacterales
ALGORITM FÖR DETEKTION AV KARBAPENEMASER (ESBL_{CARBA})
 NordicAST v. 14.0, 2024-01-19

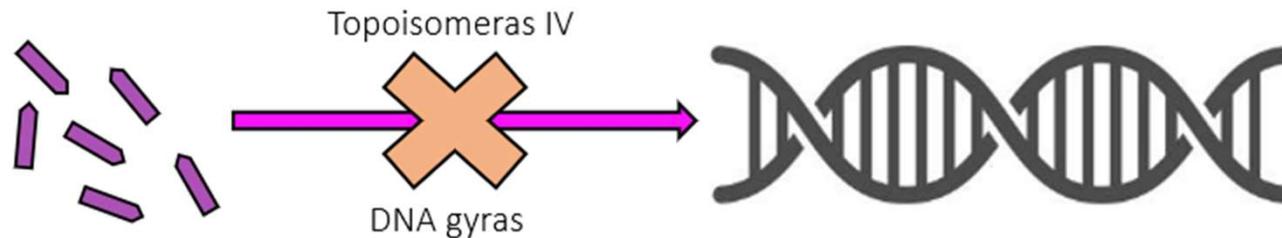
Tests should at least include these
 A KPC
 B NDM, VIM (IMP) (MBLs)
 D OXA-48 like



Undantag:
Enterobacter spp. och K. aerogenes: meropenem 25-27 mm OCH piperacillin-tazobaktam ≥ 12 mm: ingen ytterligare testning¹
Andra species: meropenem 25-27 mm OCH piperacillin-tazobaktam ≥ 20 mm: ingen ytterligare testning¹

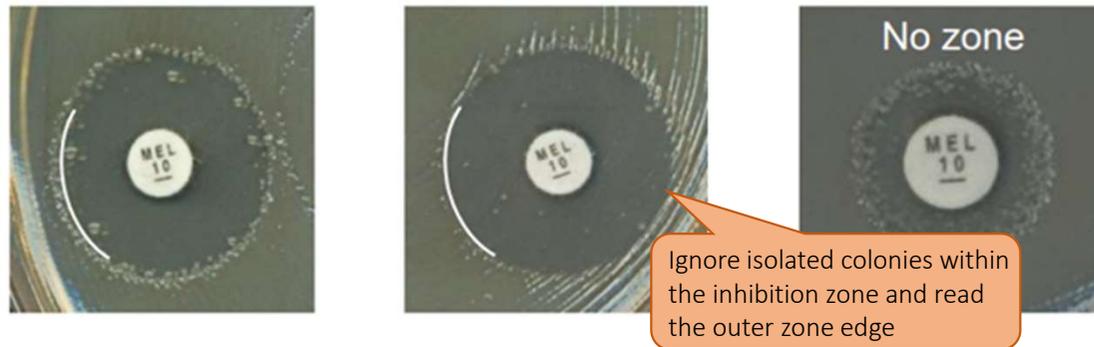
Temocillin R is indicative of OXA-48. OXA-48 producing isolates are also resistant to AMC/PTZ

Quinolone resistance screening in Enterobacterales



- Stepwise mutations lead to increasing MICs
- Pefloxacin has high sensitivity for all resistance mechanisms
- Low level resistance could be missed in *Salmonella* spp using CIP/NAL as screening agents
- Pefloxacin could also be used for other Enterobacterales and is the screening agent of choice regarding meningitis

Mecillinam and satellite colonies



Satellite colonies are due to slow growing, non-stable resistant variants of the bacteria

Usually outgrown by the normal bacterial cells and shown not to be of clinical relevance

Not reproducible and do not correlate with clinical outcome

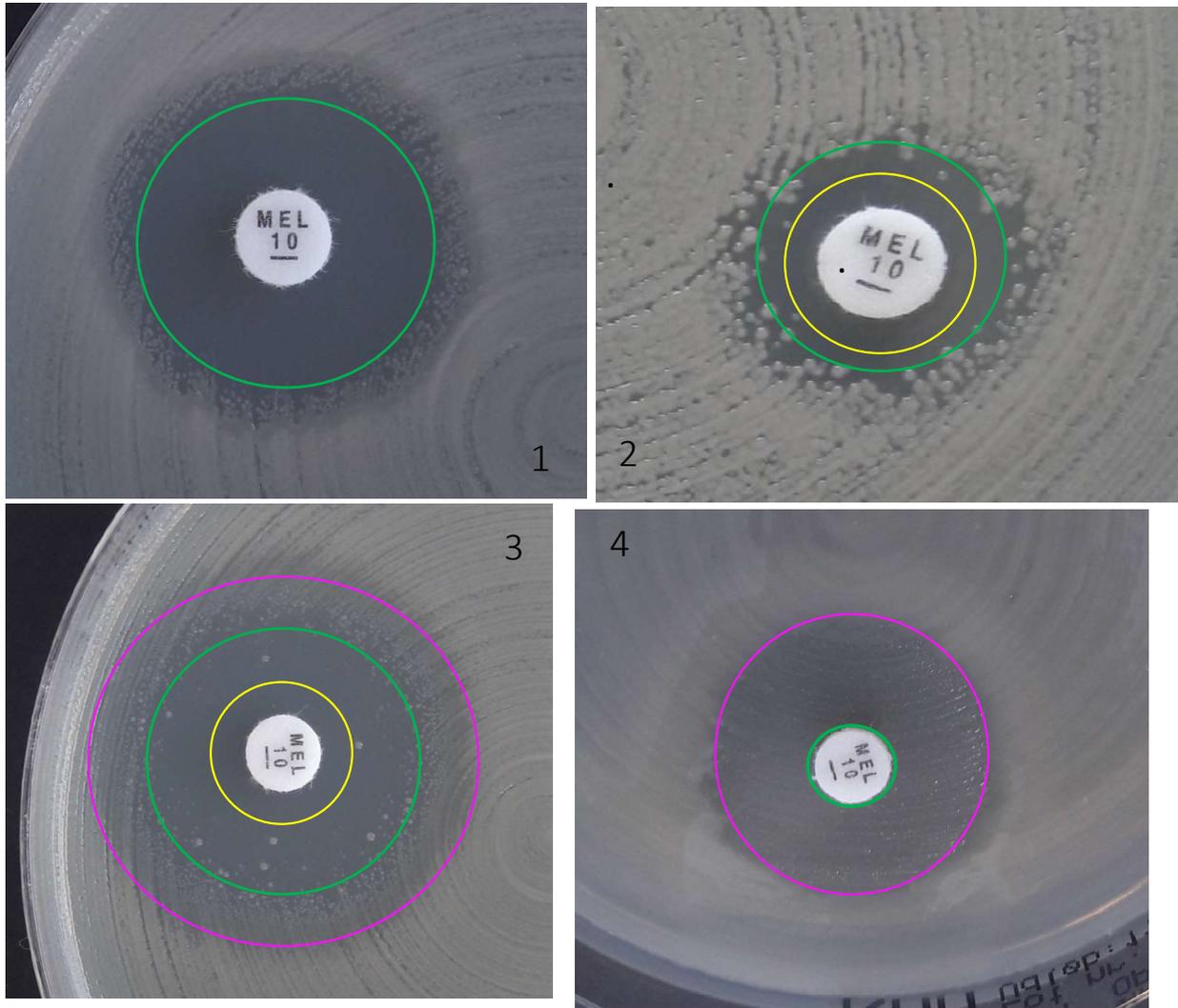
Mecillinam is known to be sensitive to differences regarding sodium chloride concentration in the agar hence especially affected by batch-to-batch variation

Reading_guide_v_10.0_EUCAST_Disk_Test_2023.pdf

"Mecillinam(FL1060), a 6f-Amidinopenicillanic Acid Derivative: In Vitro Evaluation", Thybring, 1975

"Susceptibility testing of urinary isolates of *Escherichia coli* to mecillinam using NCCLS methodology", R Skov 2005

Mecillinam and satellite colonies



Resistance

monas



ux pumps
ut of the bacterial cell

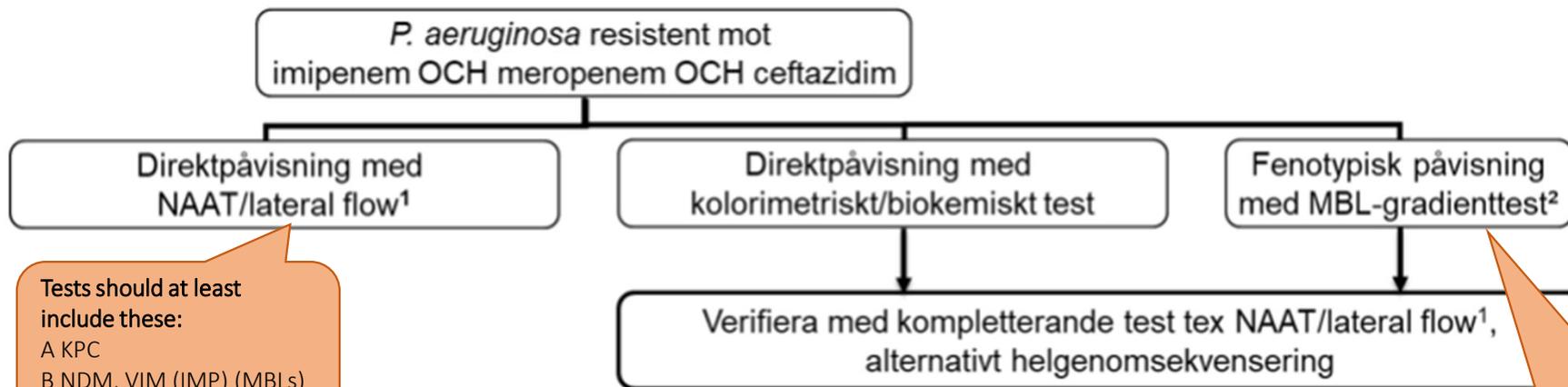


n ring – ab is destroyed
nases

Porin losses
Ab can not reach it's
target of action

Is the isolate carbapenem resistant OR carbapenemase producing?

Pseudomonas aeruginosa
ALGORITM FÖR DETEKTION AV KARBAPENEMASER
v. 14.0, NordicAST v. 14.0, 2024-01-19



Tests should at least include these:

- A KPC
- B NDM, VIM (IMP) (MBLs)
- D OXA-48 like

MBLs (NDM, VIM, IMP) are always ceftolozane-tazobactam R
If tested isolate is S and there are no epidemiological reason/low likelihood of aquired resistance, further testing may not be required

Haemophilus influenzae – A case-based guide to β -lactam resistance screening

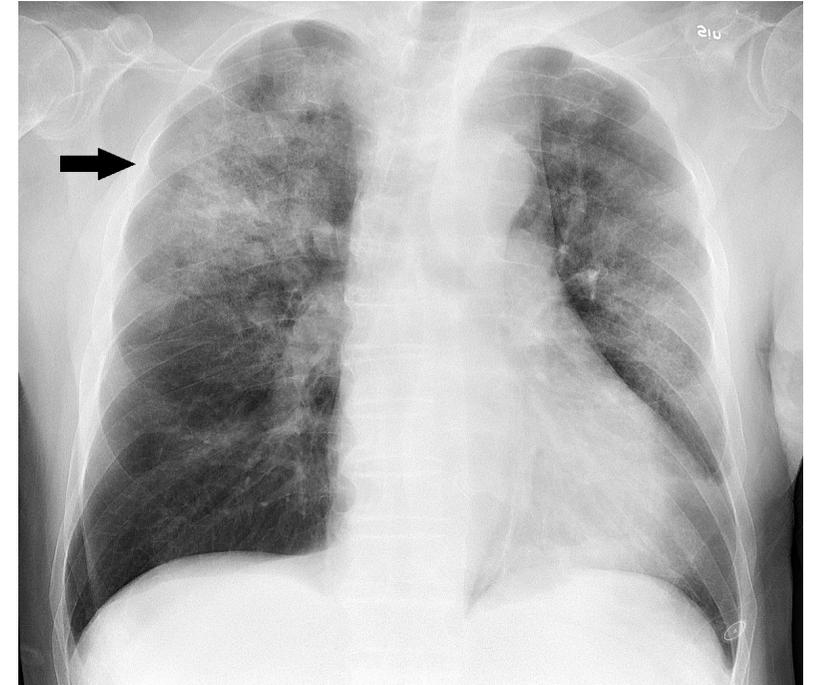
Dennis Back Holmgaard



2024 workshop, Malmö
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Case presentation

- 75 year man with COPD
- i.v. Ampicillin 2 g x 4 + Gentamicin 5 mg/kg
- X-ray with a right sided pneumonia
- *Haemophilus influenzae* in a tracheal sample



<https://en.wikipedia.org/wiki/Pneumonia>

Is the treatment sufficient for pneumonia with Haemophilus influenzae?

2 different mechanisms confers β -lactam resistance:

- PBP 3 changes - amino acid substitutions in the transpeptidase region of penicillin binding protein 3 (PBP3) due to mutations in the *ftsI* gene
- Acquired plasmid β -lactamase – *bla*_{TEM-1} and *bla*_{ROB-1}

*Which antibiotic would you choose for primary screening for β –
lactam resistance ?*

Haemophilus influenzae: Flow chart based on the benzylpenicillin (PCG) screen test for beta-lactam resistance mechanisms to reduce the number of specific tests for beta-lactam agents

To take full advantage of the procedure, include the amoxicillin-clavulanic acid 2-1 µg disk, but read and interpret only on beta-lactamase positive isolates.

PCG 1 unit zone diameter ≥12 mm

Mechanism: excludes all beta-lactam resistance mechanisms

Report susceptible (S) to beta-lactam agents for which clinical breakpoints are available, including those with "Note", and those with meningitis breakpoints.

Exception: Oral amoxicillin, oral amoxicillin-clavulanic acid and oral cefuroxime are reported "susceptible, increased exposure" (I).

No further testing required.

PCG 1 unit zone diameter <12 mm

Mechanism: beta-lactamase and/or PBP3 mutations

Further testing: test for beta-lactamase.

In meningitis, determine the MIC for the agent considered for clinical use and interpret according to the clinical breakpoints.

Beta-lactamase positive

Mechanisms: beta-lactamase with or without PBP3 mutations

Report resistant (R) to ampicillin, amoxicillin and piperacillin (without beta-lactamase inhibitor).

For other beta-lactam agents, read the amoxicillin-clavulanic acid 2-1 µg disk and interpret as below.

Beta-lactamase negative

Mechanism: PBP3 mutations

Perform susceptibility testing for the relevant agents and interpret according to breakpoints.

For cefepime, cefpodoxime and imipenem, if PCG 1 unit <12 mm and susceptible by agent disk diffusion test, determine the MIC of the agent and interpret according to the clinical breakpoints.

Amoxicillin-clavulanic acid 2-1 µg ≥15 mm

Mechanism: beta-lactamase only

Report susceptible (S) to agents (other than ampicillin, amoxicillin and piperacillin) for which clinical breakpoints are available, including those with "Note", and those with meningitis breakpoints.

Exception: Oral amoxicillin-clavulanic acid and oral cefuroxime are reported "susceptible, increased exposure" (I).

Amoxicillin-clavulanic acid 2-1 µg <15 mm

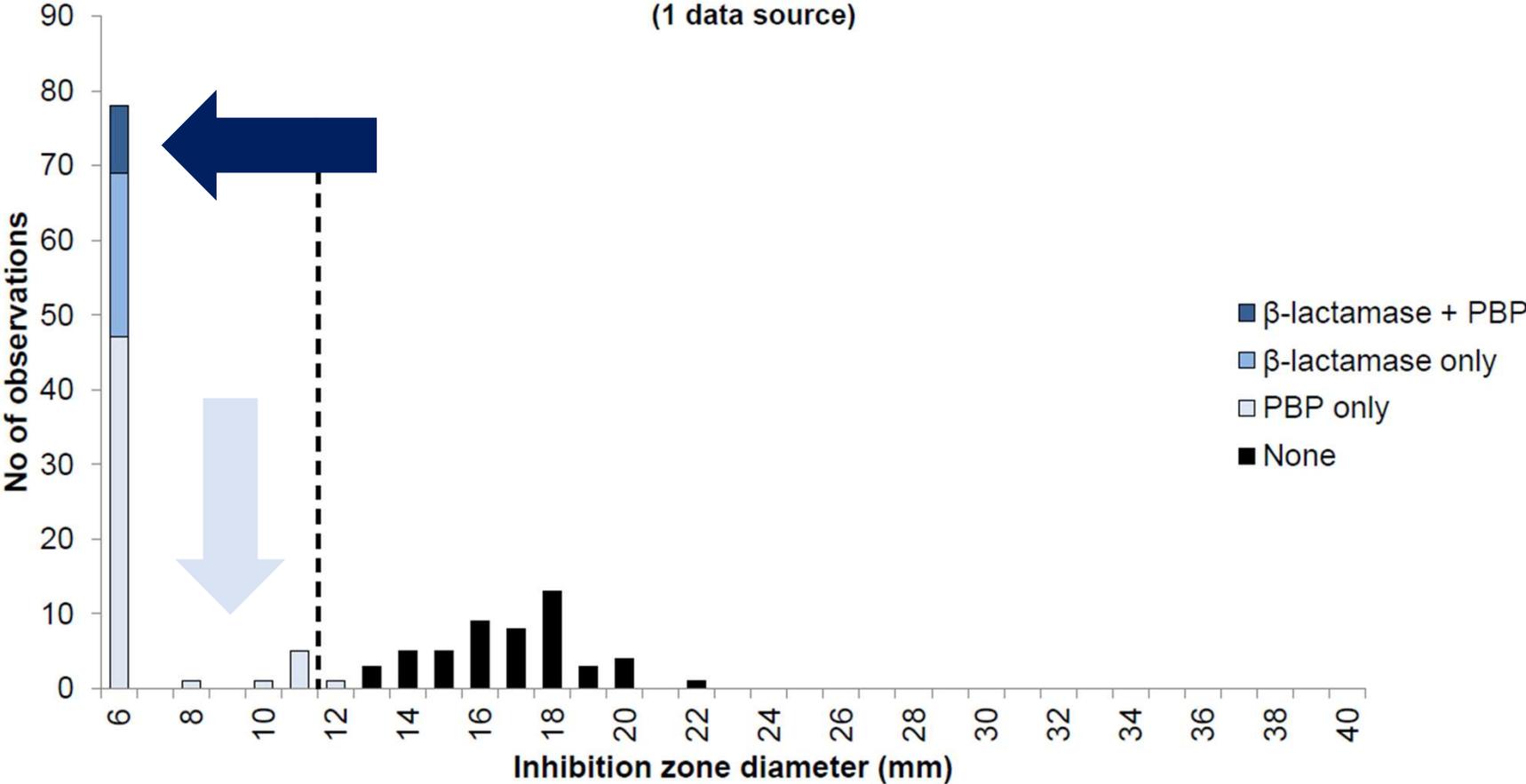
Mechanisms: beta-lactamase and PBP3 mutations

Perform susceptibility testing for the relevant agents and interpret according to breakpoints.

For cefepime, cefpodoxime and imipenem, if PCG 1 unit <12 mm and susceptible by agent disk diffusion test, determine the MIC of the agent and interpret according to the clinical breakpoints.

Benzylpenicillin 1 unit vs. β -lactam resistance mechanism *H. influenzae*, 137 clinical isolates

(1 data source)



Benzylpenicillin screening negative isolates

- Report susceptible for all β -lactams for which there are breakpoints

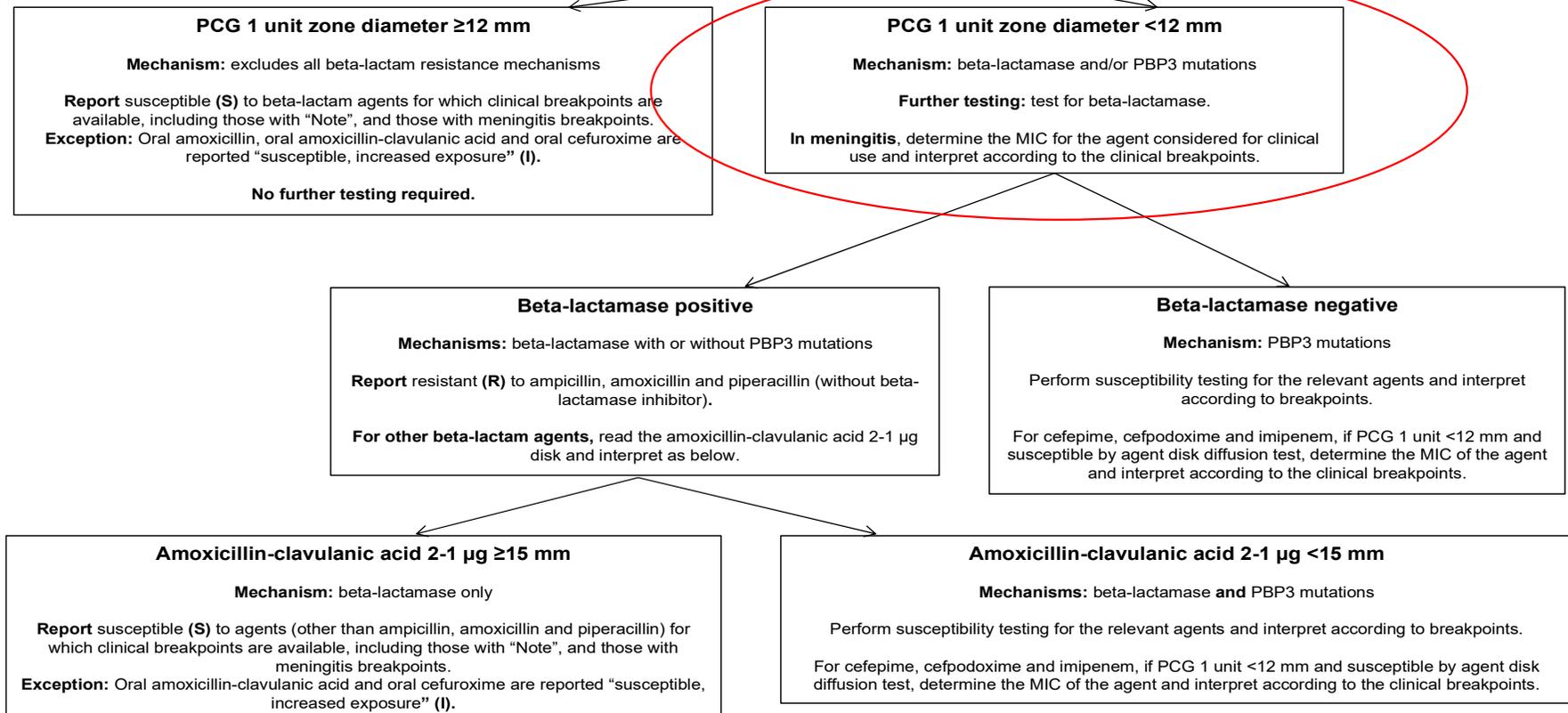
In this case a zone of 6 mm for benzylpenicillin is detected

What is the next step ?

Screening positive isolates

***Haemophilus influenzae*: Flow chart based on the benzylpenicillin (PCG) screen test for beta-lactam resistance mechanisms to reduce the number of specific tests for beta-lactam agents**

To take full advantage of the procedure, include the amoxicillin-clavulanic acid 2-1 µg disk, but read and interpret only on beta-lactamase positive isolates.





- A β -lactamase test is performed
- Our isolate is positive for β -lactamase
- What is our next move, are we done ?

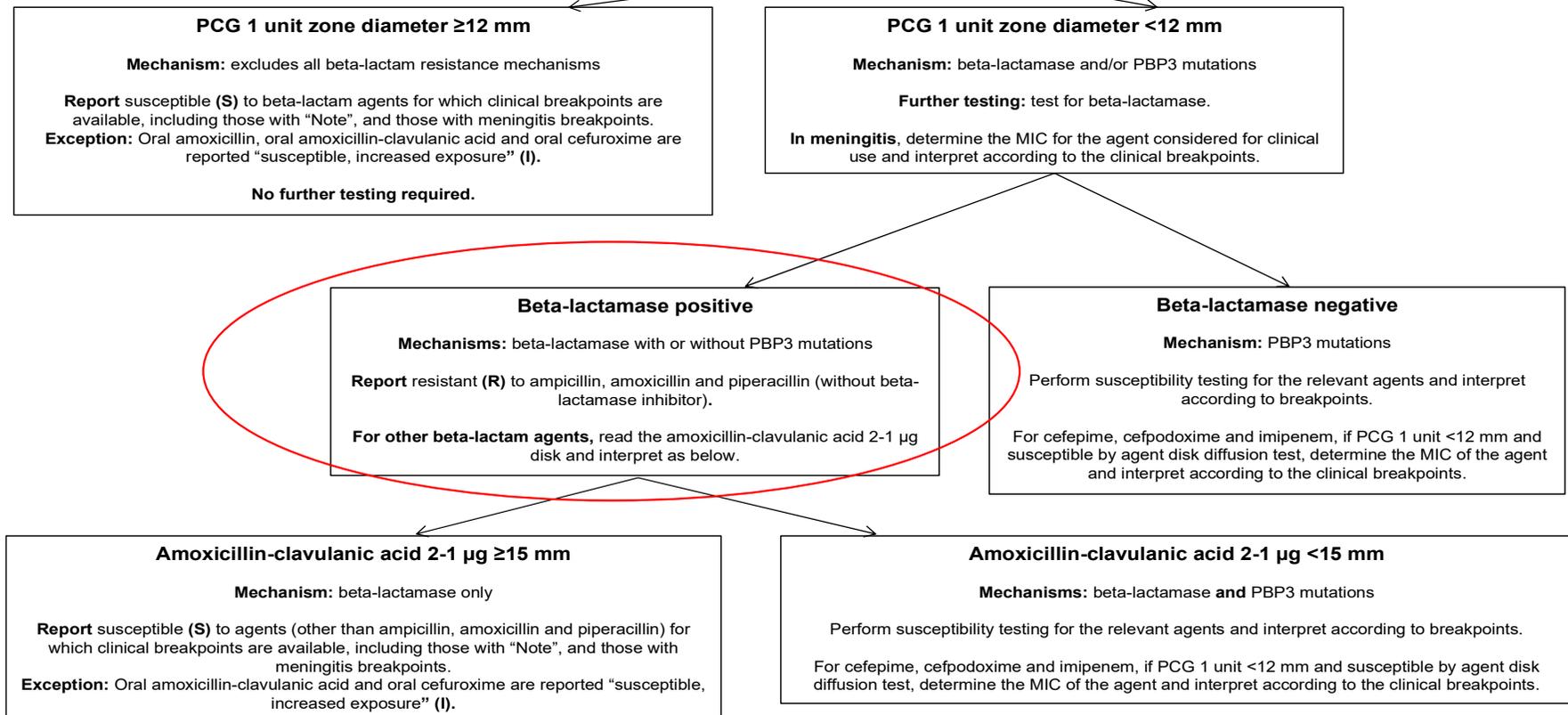
β -lactamase screening negative isolates

- The isolate is resistant due to PBP-3 changes alone
- Perform susceptibility testing for relevant agents individually

β -lactamase screening positive isolates

***Haemophilus influenzae*: Flow chart based on the benzylpenicillin (PCG) screen test for beta-lactam resistance mechanisms to reduce the number of specific tests for beta-lactam agents**

To take full advantage of the procedure, include the amoxicillin-clavulanic acid 2-1 μ g disk, but read and interpret only on beta-lactamase positive isolates.



- Since our isolate produces β -lactamase we can immediately report it resistant to penicillins i.e. ampicillin, amoxicillin and piperacillin
- We need to assess whether the resistance to β -lactam is a result of a β -lactamase alone or in conjunction with PBP-3 changes

How do we determine whether the isolate contains both PBP-changes and β -lactamase?

***Haemophilus influenzae*: Flow chart based on the benzylpenicillin (PCG) screen test for beta-lactam resistance mechanisms to reduce the number of specific tests for beta-lactam agents**

To take full advantage of the procedure, include the amoxicillin-clavulanic acid 2-1 µg disk, but read and interpret only on beta-lactamase positive isolates.

PCG 1 unit zone diameter ≥12 mm

Mechanism: excludes all beta-lactam resistance mechanisms

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Exception: Oral amoxicillin, oral amoxicillin-clavulanic acid and oral cefuroxime are reported "susceptible, increased exposure" (I).

No further testing required.

PCG 1 unit zone diameter <12 mm

Mechanism: beta-lactamase and/or PBP3 mutations

Further testing: test for beta-lactamase.

In meningitis, determine the MIC for the agent considered for clinical use and interpret according to the clinical breakpoints.

Beta-lactamase positive

Mechanisms: beta-lactamase with or without PBP3 mutations

Report resistant (R) to ampicillin, amoxicillin and piperacillin (without beta-lactamase inhibitor).

For other beta-lactam agents, read the amoxicillin-clavulanic acid 2-1 µg disk and interpret as below.

Beta-lactamase negative

Mechanism: PBP3 mutations

Perform susceptibility testing for the relevant agents and interpret according to breakpoints.

For cefepime, cefpodoxime and imipenem, if PCG 1 unit <12 mm and susceptible by agent disk diffusion test, determine the MIC of the agent and interpret according to the clinical breakpoints.

Amoxicillin-clavulanic acid 2-1 µg ≥15 mm

Mechanism: beta-lactamase only

Report susceptible (S) to agents (other than ampicillin, amoxicillin and piperacillin) for which clinical breakpoints are available, including those with "Note", and those with meningitis breakpoints.

Exception: Oral amoxicillin-clavulanic acid and oral cefuroxime are reported "susceptible, increased exposure" (I).

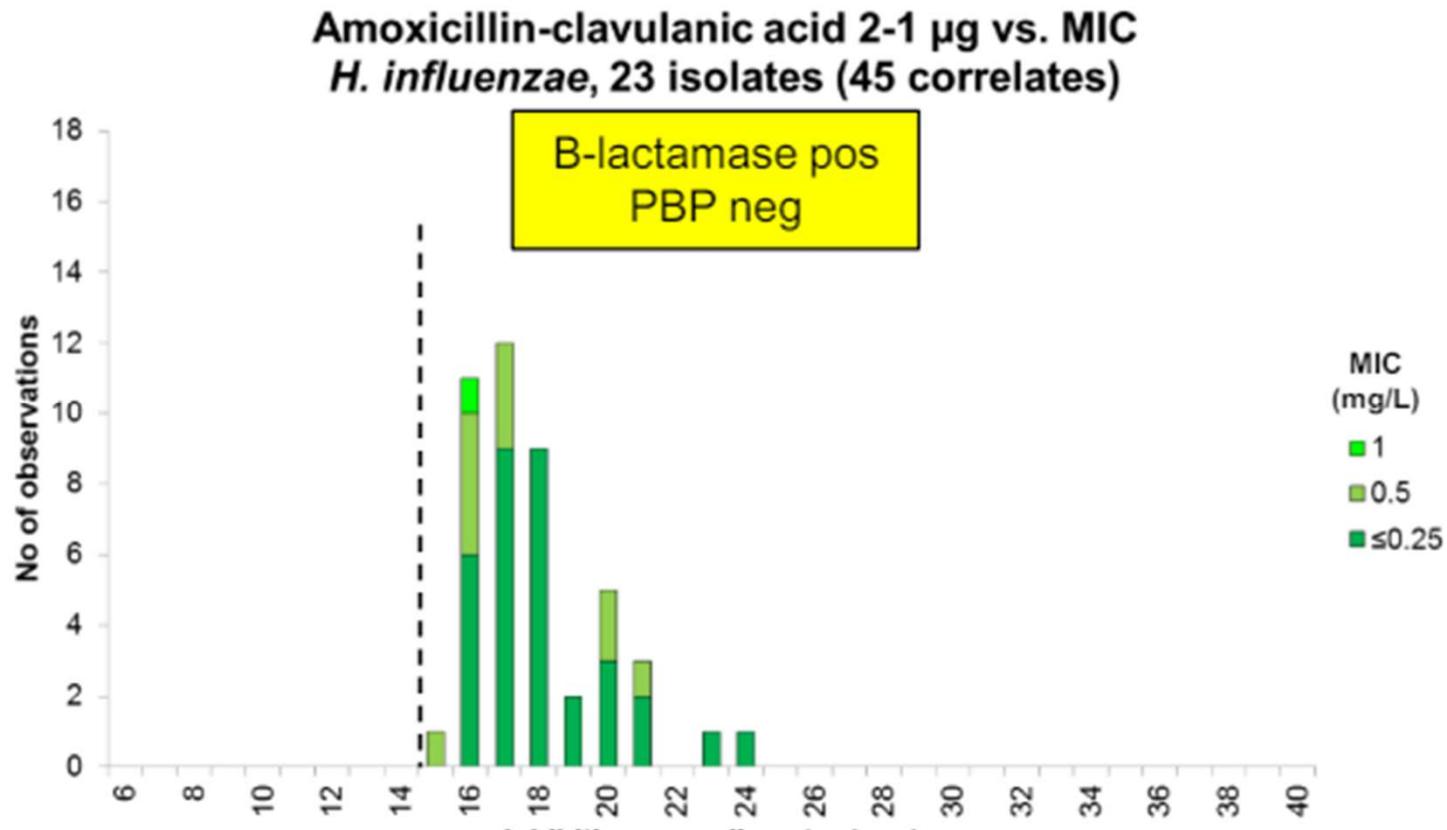
Amoxicillin-clavulanic acid 2-1 µg <15 mm

Mechanisms: beta-lactamase and PBP3 mutations

Perform susceptibility testing for the relevant agents and interpret according to breakpoints.

For cefepime, cefpodoxime and imipenem, if PCG 1 unit <12 mm and susceptible by agent disk diffusion test, determine the MIC of the agent and interpret according to the clinical breakpoints.

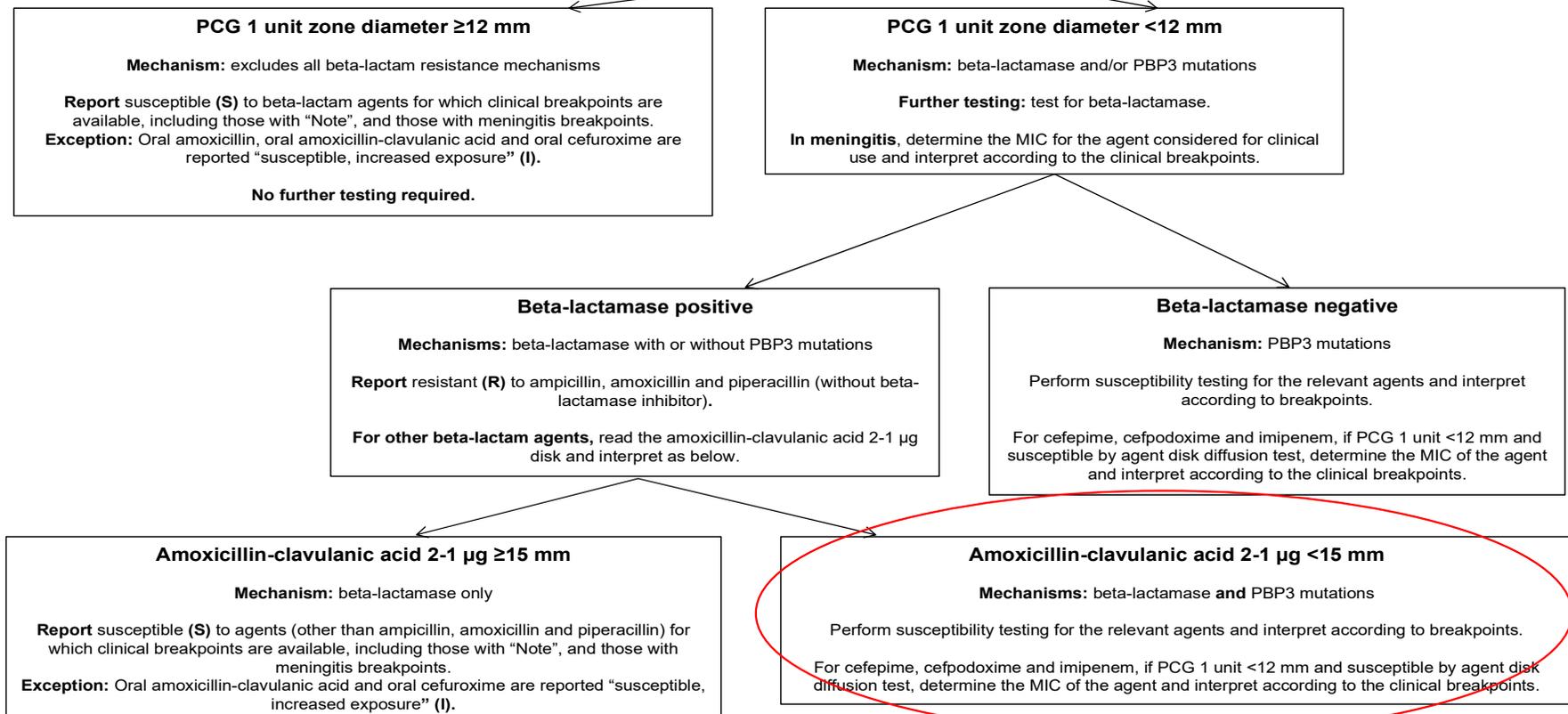
Amoxicillin/Clavulanic acid ≥ 15 mm
Resistance is solely due to a β -lactamase



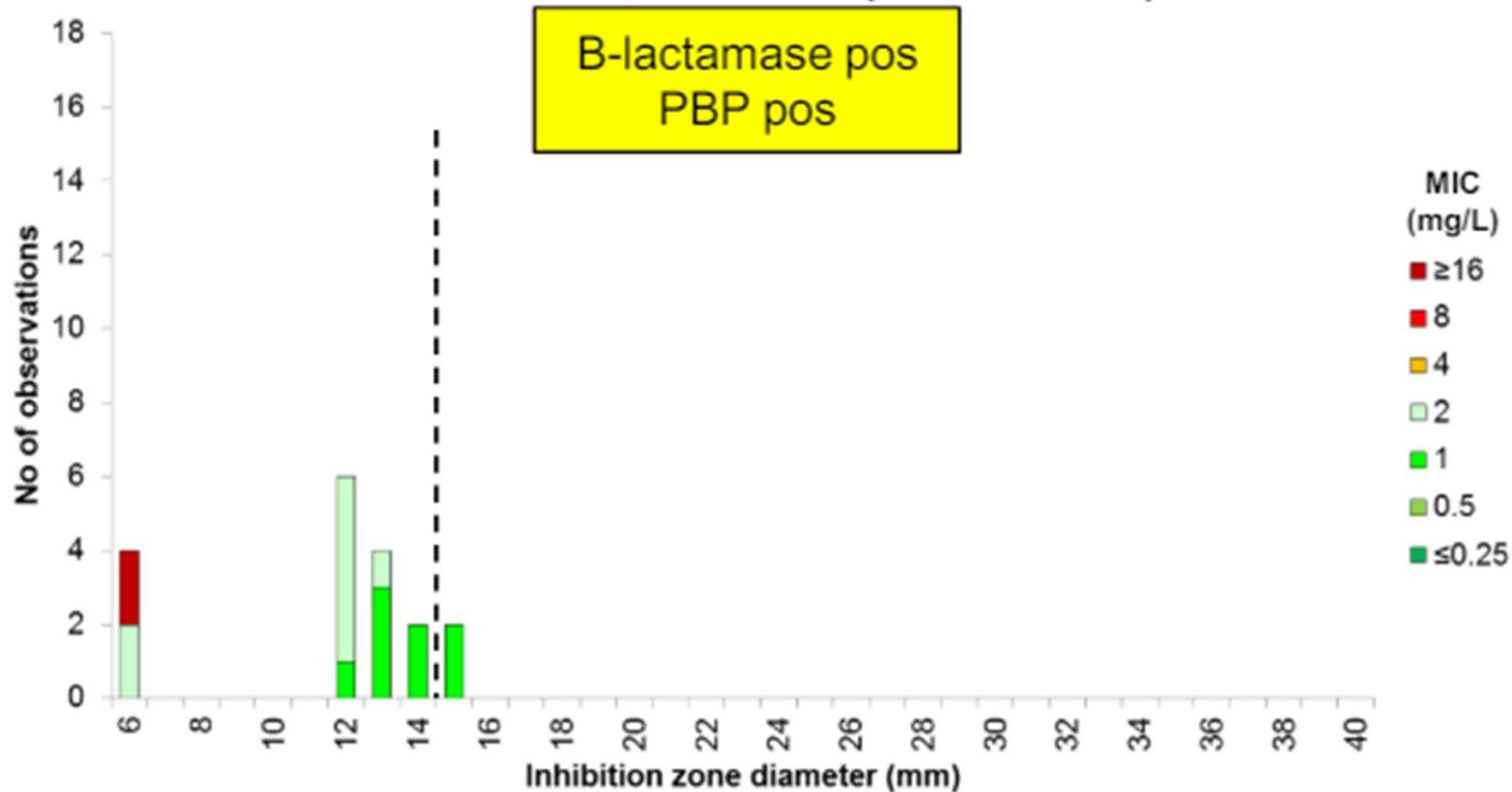
Screening positive isolates

***Haemophilus influenzae*: Flow chart based on the benzylpenicillin (PCG) screen test for beta-lactam resistance mechanisms to reduce the number of specific tests for beta-lactam agents**

To take full advantage of the procedure, include the amoxicillin-clavulanic acid 2-1 µg disk, but read and interpret only on beta-lactamase positive isolates.



**Amoxicillin-clavulanic acid 2-1 μ g vs. MIC
H. influenzae, 9 isolates (18 correlates)**



AMC screening positive isolates

- In our case the isolate has a zone of 12 mm for AMC
- The isolate is resistant due to β -lactamase and PBP-3 changes
- Perform susceptibility testing for relevant agents individually
- Report resistant to penicillins i.e. ampicillin, amoxicillin and piperacillin
- A subsequent inspection of the piperacillin/tazobactam zone revealed a zone of 29 mm and it was reported as susceptible

In conclusion

- Isolates are tested for presence of β -lactam resistance using benzylpenicillin
- β -lactam resistant isolates are tested for β -lactamase using a β -lactamase test
- β -lactamase producing strains are subsequently screened with AMC to determine whether β -lactamase producing strains also contain changes to PBP-3

Unexpected phenotypes

- Ceftriaxone/Cefotaxime resistant isolates is unexpected in Scandinavia and resistant strains should be retested
- Ciprofloxacin resistant isolates is also unexpected and a test should be repeated to confirm resistance

General considerations # 1

- Breakpoints for meningitis are exclusively provided for cefotaxime, ceftriaxone and meropenem and individual MIC determination should be performed in all isolates exhibiting beta-lactam resistance
- Amoxicillin, amoxicillin-clavulanic acid and cefuroxime in oral formulations should always be reported as “susceptible, increased exposure (I)” when deemed susceptible.

General considerations # 2

- The stepwise character of PBP3 resistance leads to close or overlapping susceptible and resistant populations when testing beta-lactam susceptibility in these isolates. Hence, an area of technical uncertainty (ATU) is needed in many situations.