EUCAST
EUROPEAN COMMITTEE ON ANTIMICROBIAL SUSCEPTIBILITY TESTING
European Society of Clinical Microbiology and Infectious Diseases

Challenging bugs for AST: e.g. *H. influenzae*

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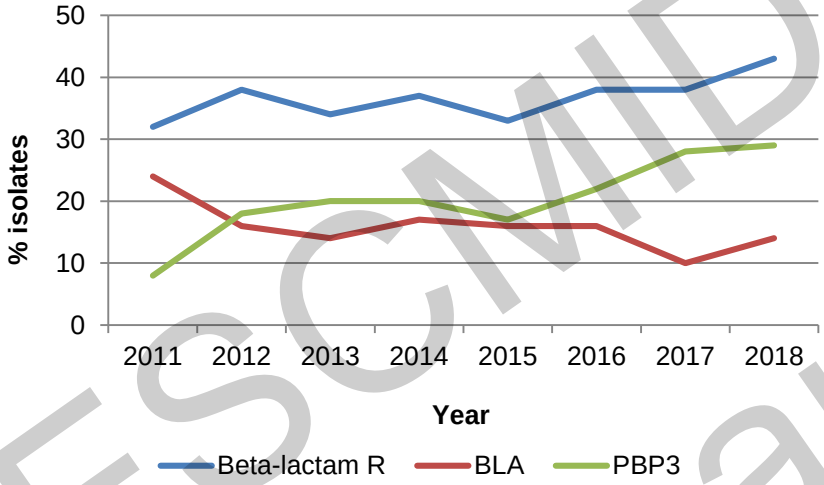
H. influenzae and antimicrobial resistance

- Beta-lactam resistance
  - Beta-lactamase production
    - TEM and ROB
  - PBP3 mutations
    - Groups I, II, III, III-like, III+, III+-like

- Non-beta lactam resistance
  - Fluoroquinolones
  - Tetracyclines
  - Trimethoprim-sulfamethoxazole

Prevalence of PBP3 mutations

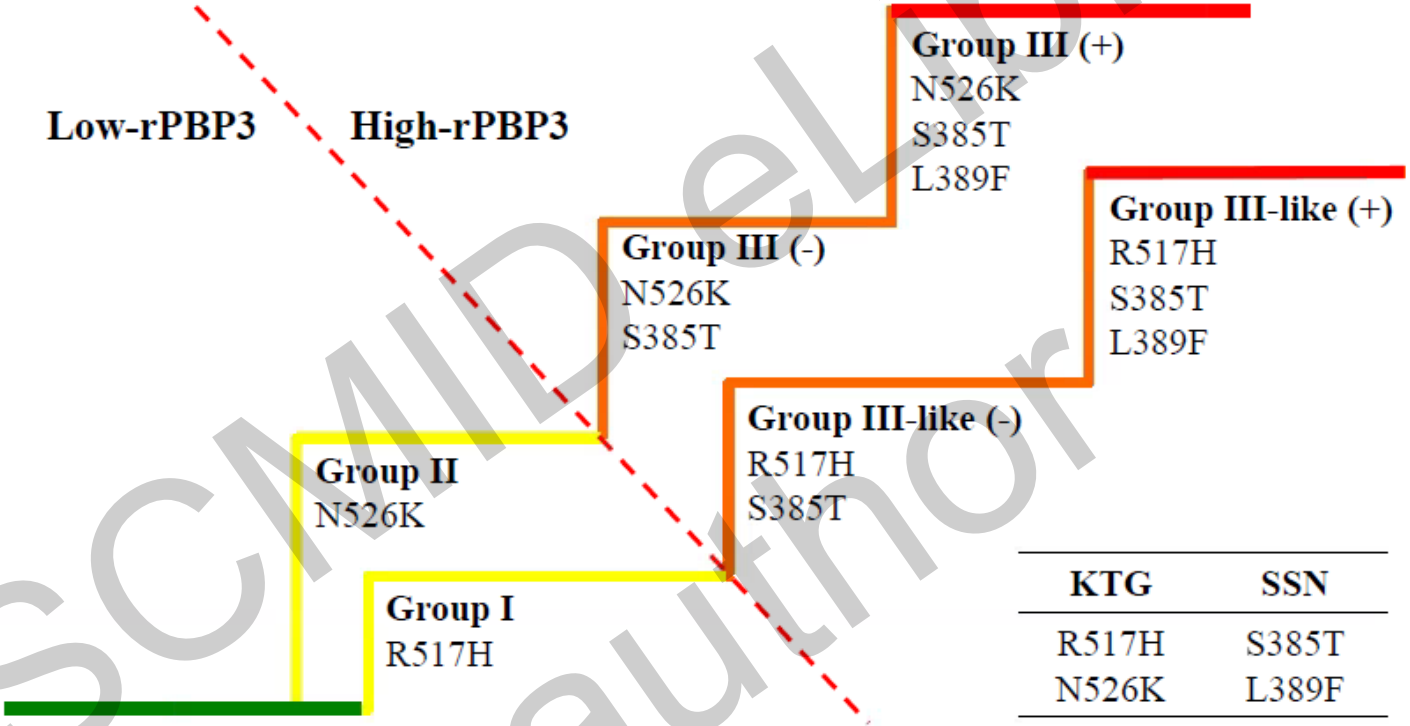
Beta-lactam resistance in *H. influenzae* in Kronoberg county, Sweden 2011-2018



Beta-lactam resistance in *H. influenzae* in European laboratories 2017 (small-scale multi-lab study)

Country	No of isolates	PCG R (%)	BLA (%)	PBP3 (%)
All	**304**	40	13	27
Denmark	**25**	20	4	16
Estonia	**25**	40	12	28
Finland	**25**	40	8	32
France	**25**	48	0	48
Germany	**25**	40	16	24
Iceland	**25**	44	16	28
Ireland	**25**	60	28	32
Norway	**25**	32	12	20
Spain	**25**	48	20	28
Sweden 1	**27**	36	19	17
Sweden 2	**27**	48	11	37
UK	**25**	32	8	24

H. influenzae and PBP3 mutations



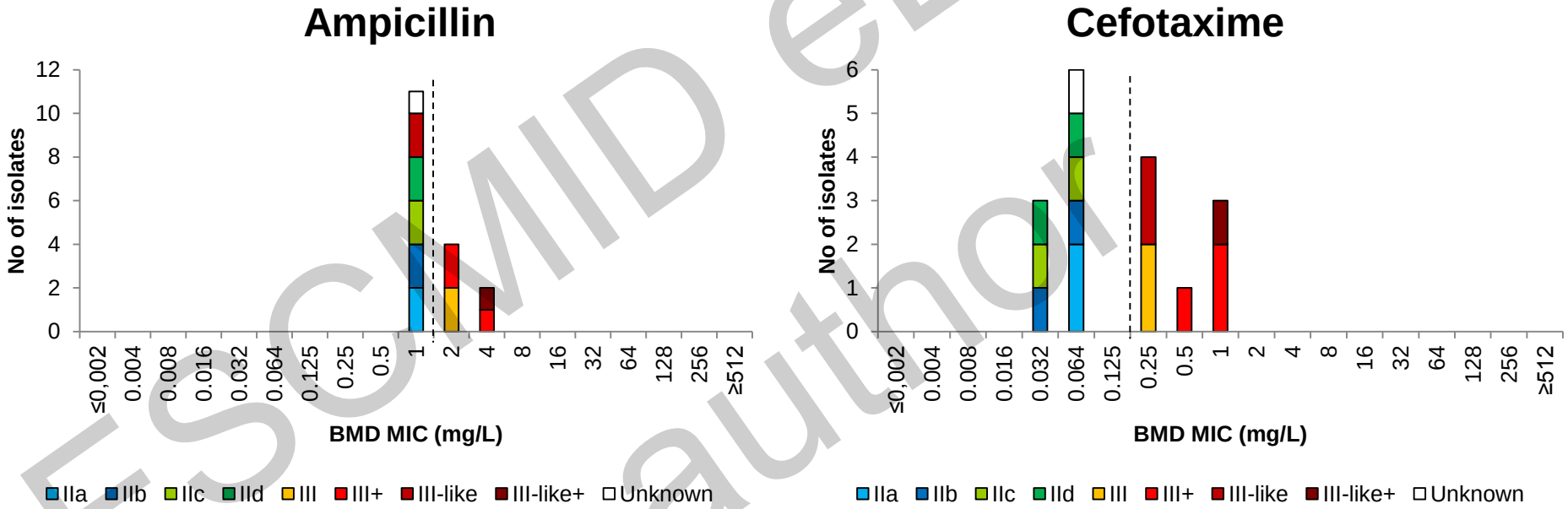
KTG	SSN
R517H	S385T
N526K	L389F

Compiled by Dagfinn Skaare in thesis ”Non-beta-lactamase-mediated beta-lactam resistance in *Haemophilus influenzae*. Mechanisms, epidemiology and susceptibility testing.”

PBP3 mutations and beta-lactam resistance

17 *H. influenzae* – all with PBP3 mutations

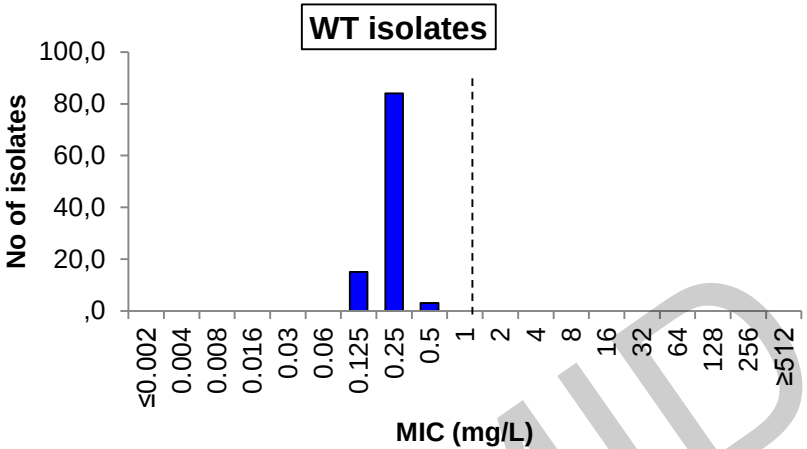
EUCAST breakpoints are shown as dotted lines

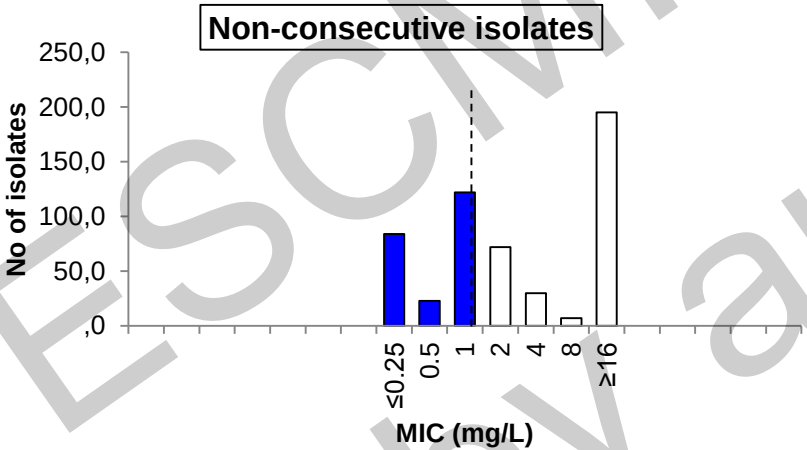


Blue/green bars = PBP3 mutations group II

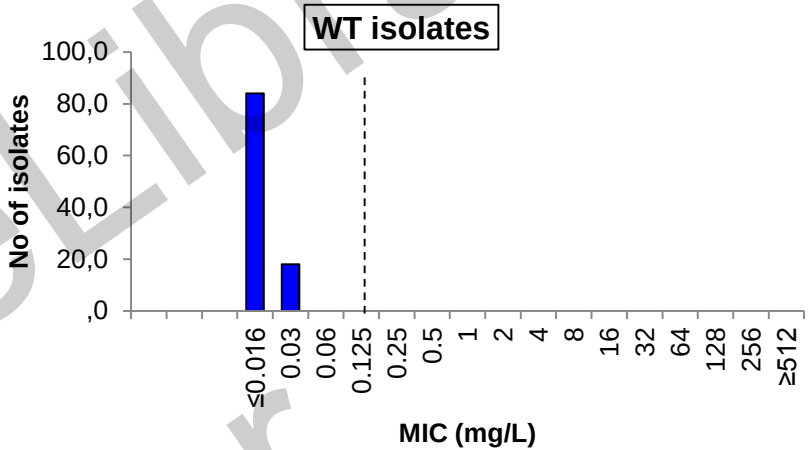
Red/orange bars = PBP3 mutations group III

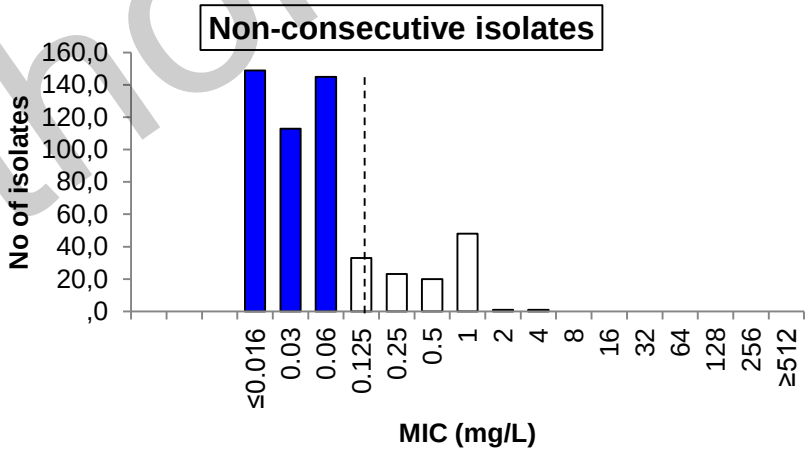
Ampicillin



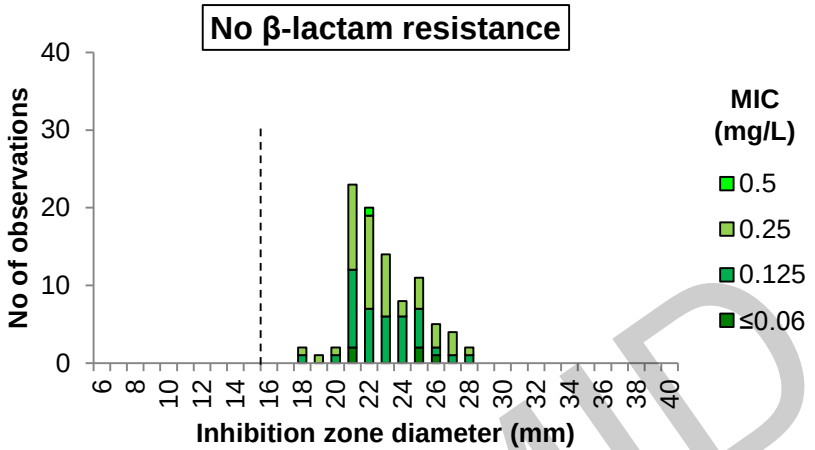


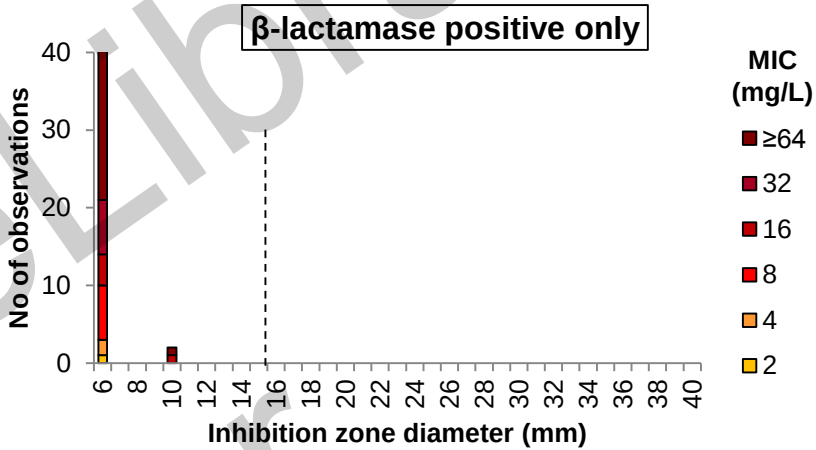
Cefotaxime

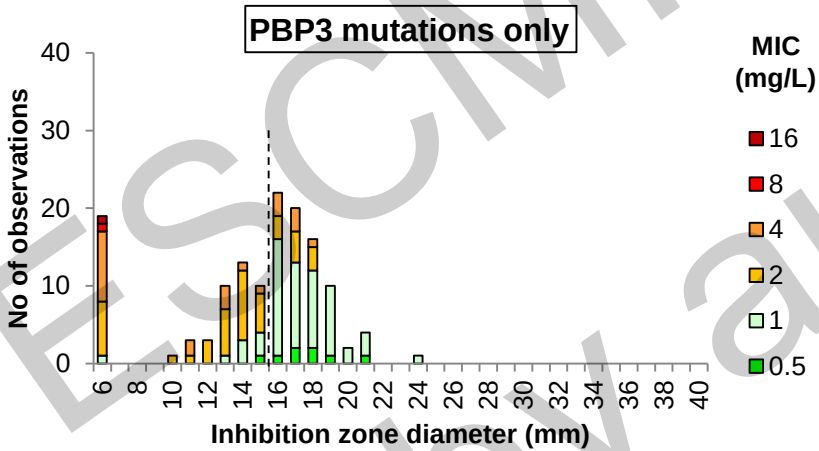


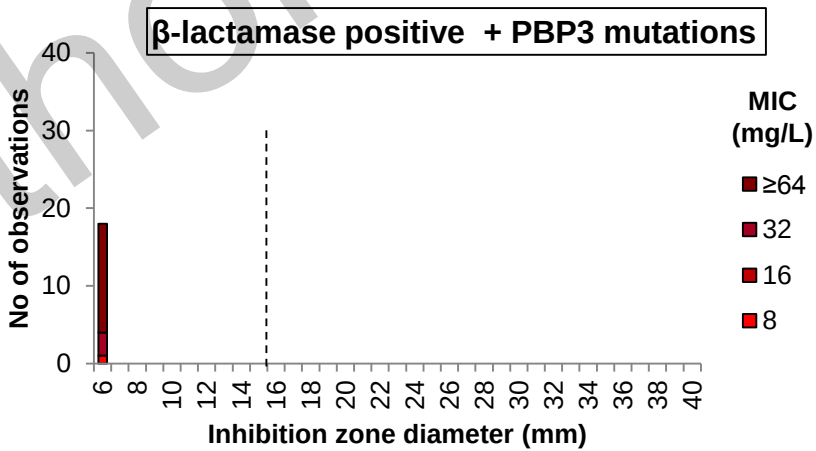


Ampicillin 2 µg disk diffusion per beta-lactam resistance mechanism

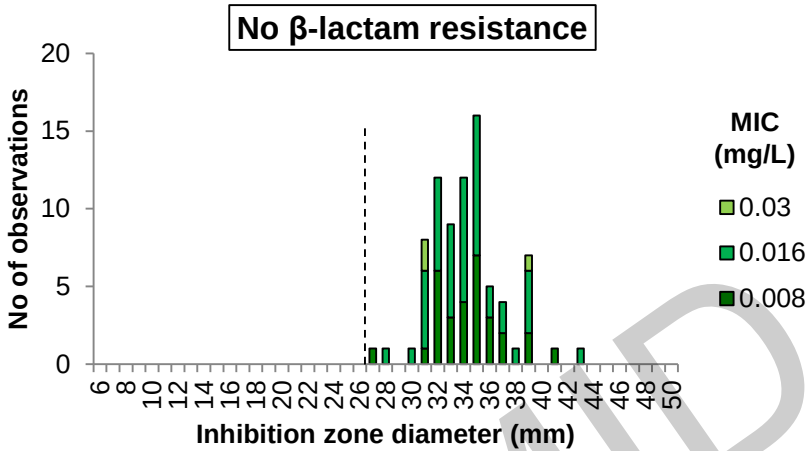


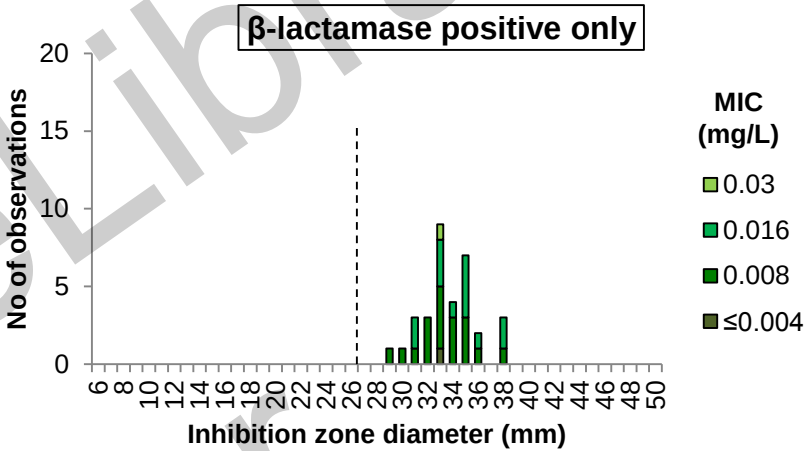


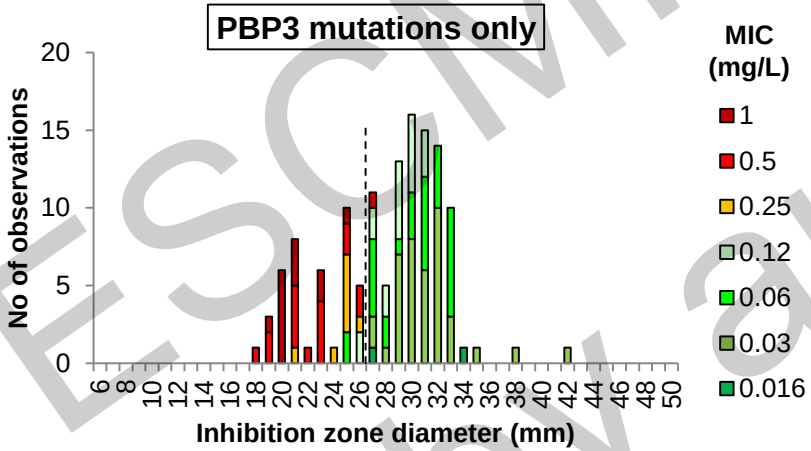


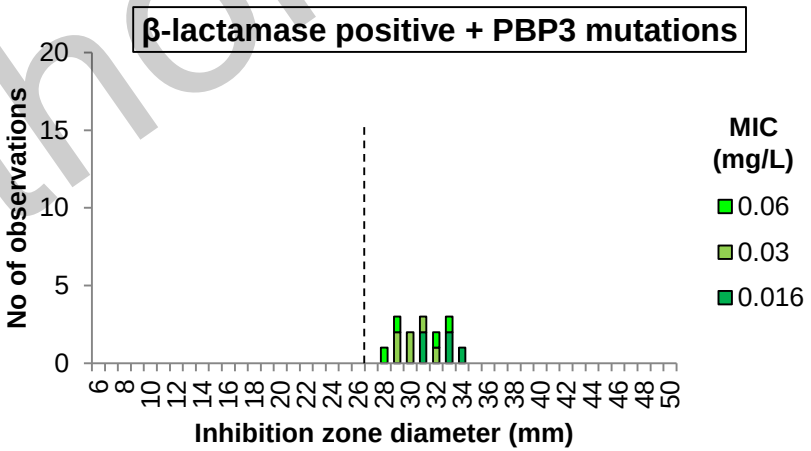


Cefotaxime 5 µg disk diffusion per beta-lactam resistance mechanism

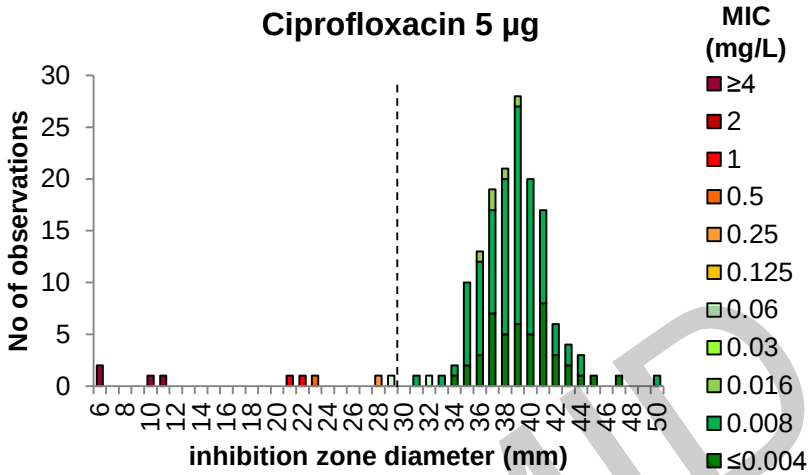


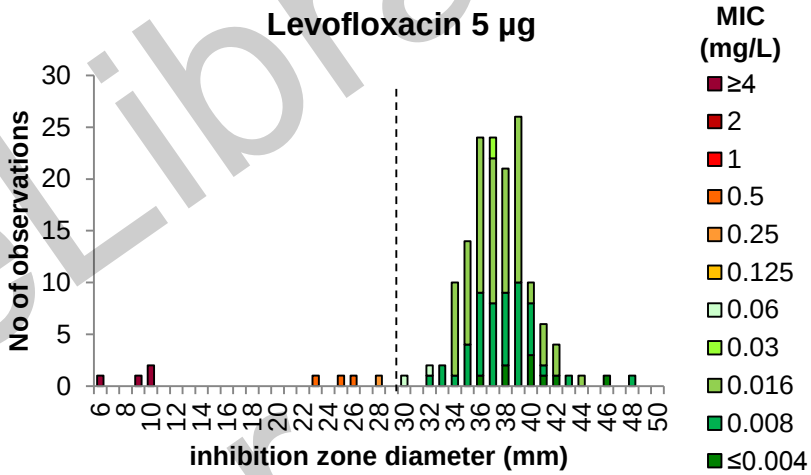


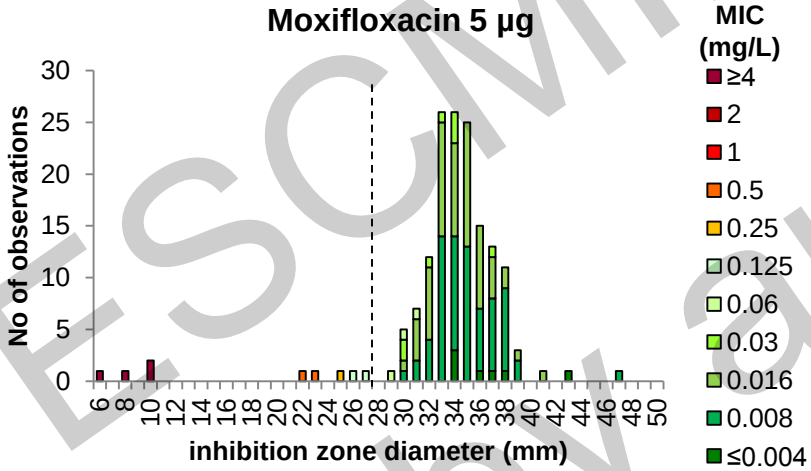


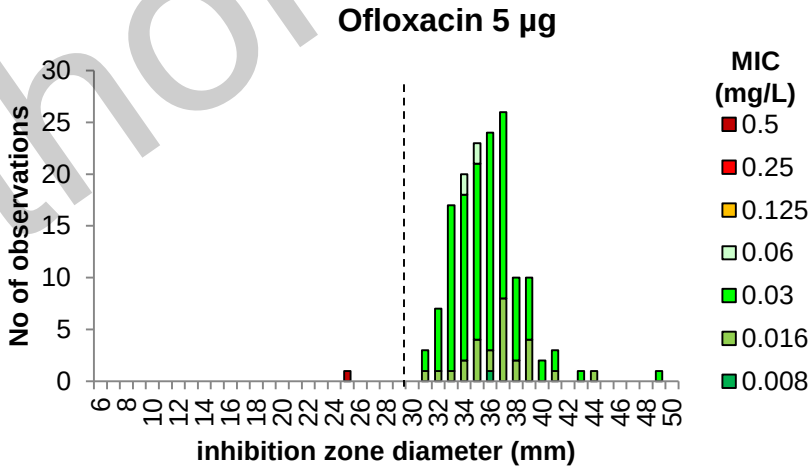


H. influenzae and fluoroquinolones

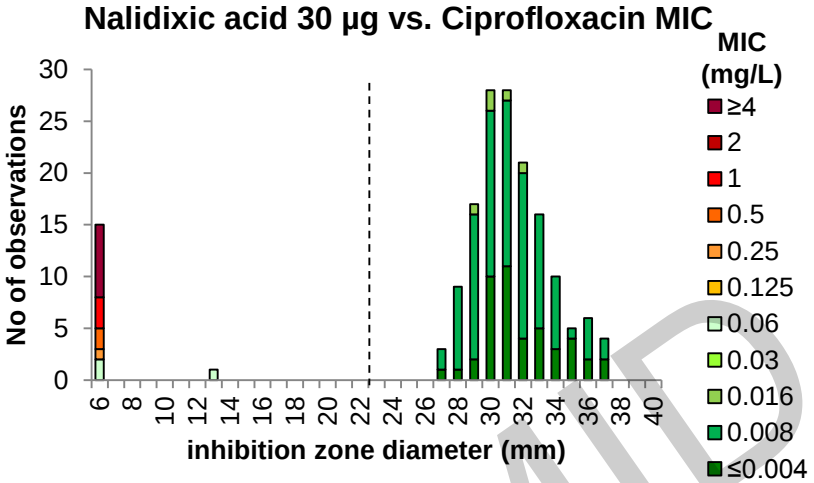


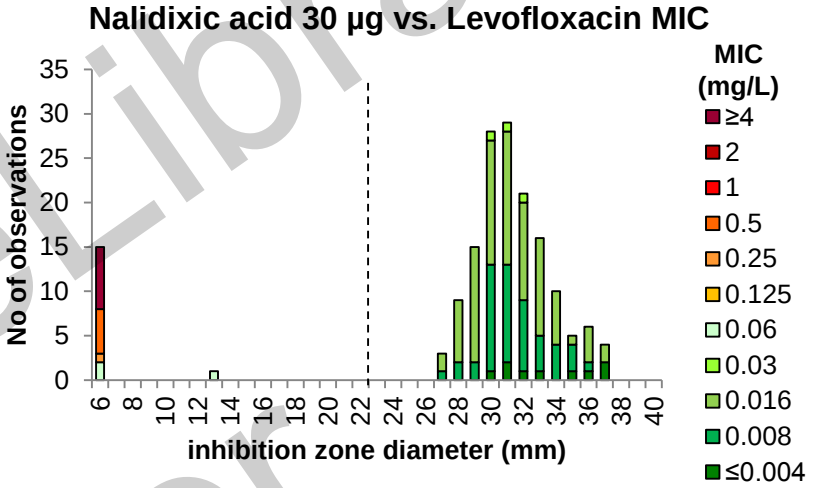


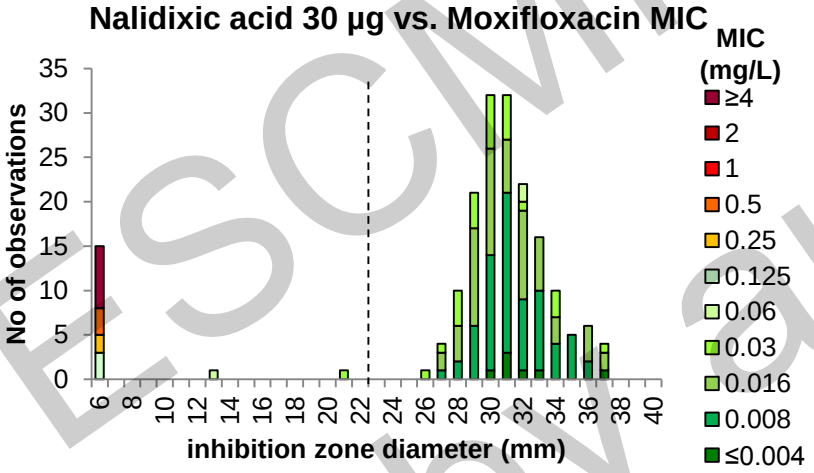


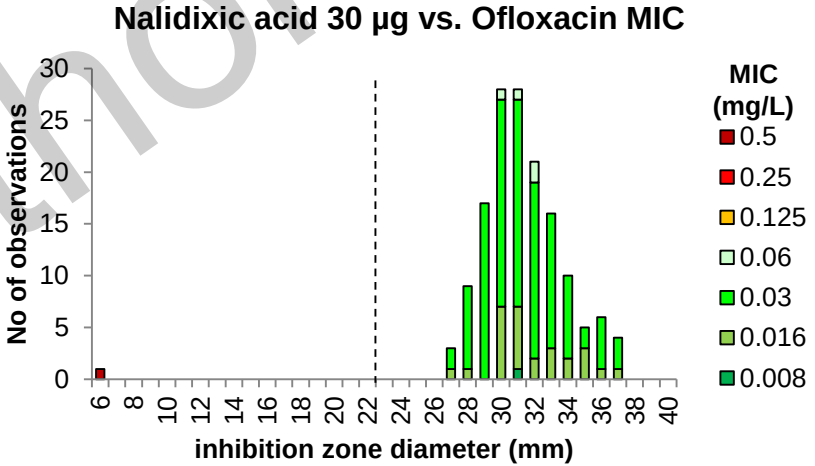


H. influenzae and fluoroquinolones – screening with nalidixic acid

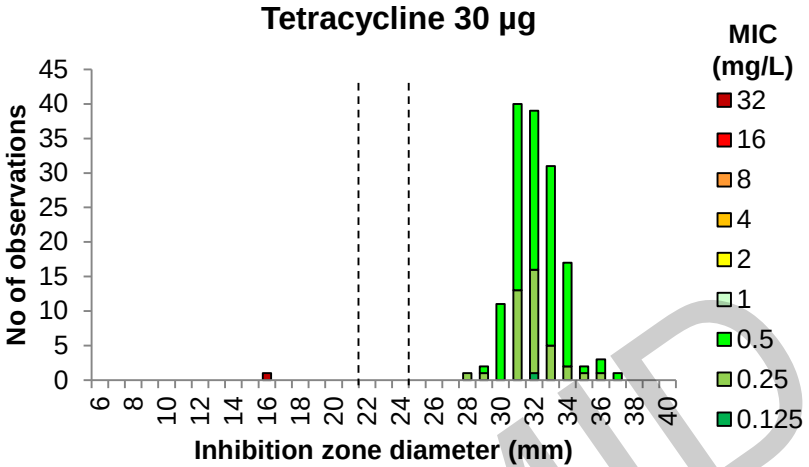


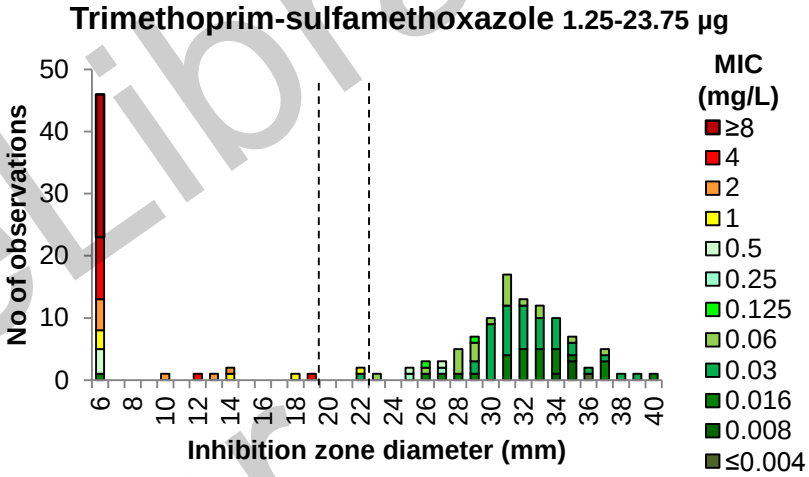


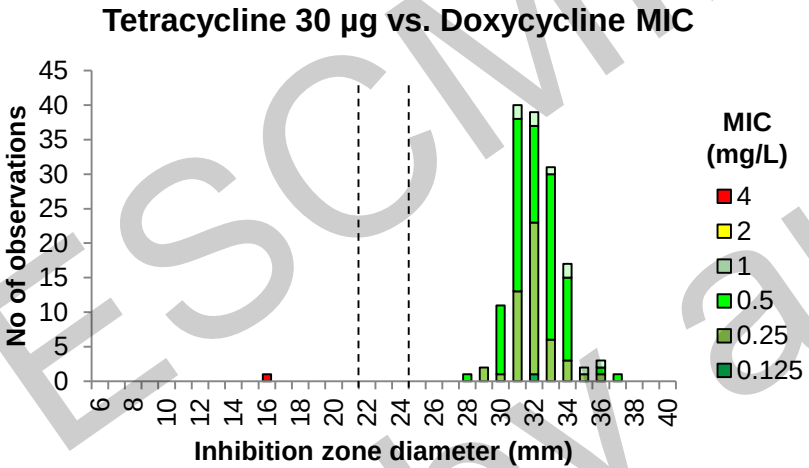




H. influenzae with tetracyclines and trimethoprim-sulfamethoxazole







AST of *H. influenzae*

	Broth microdilution (BMD)*	Disk diffusion
Media	MH-F broth	MH-F agar
Inoculum	5 x 10^5 CFU/mL	McFarland 0.5
Incubation	16-20 h 35±1°C Ambient air (sealed panels)	16-20 h 35±1°C 5% CO_2

* According to ISO 20776-1 but with EUCAST MH-F broth

MH-F broth = MH broth with 5% lysed horse blood and 20 mg/L β-NAD
MH-F agar = MH agar with 5% defibrinated horse blood and 20 mg/L β-NAD

MH-F broth

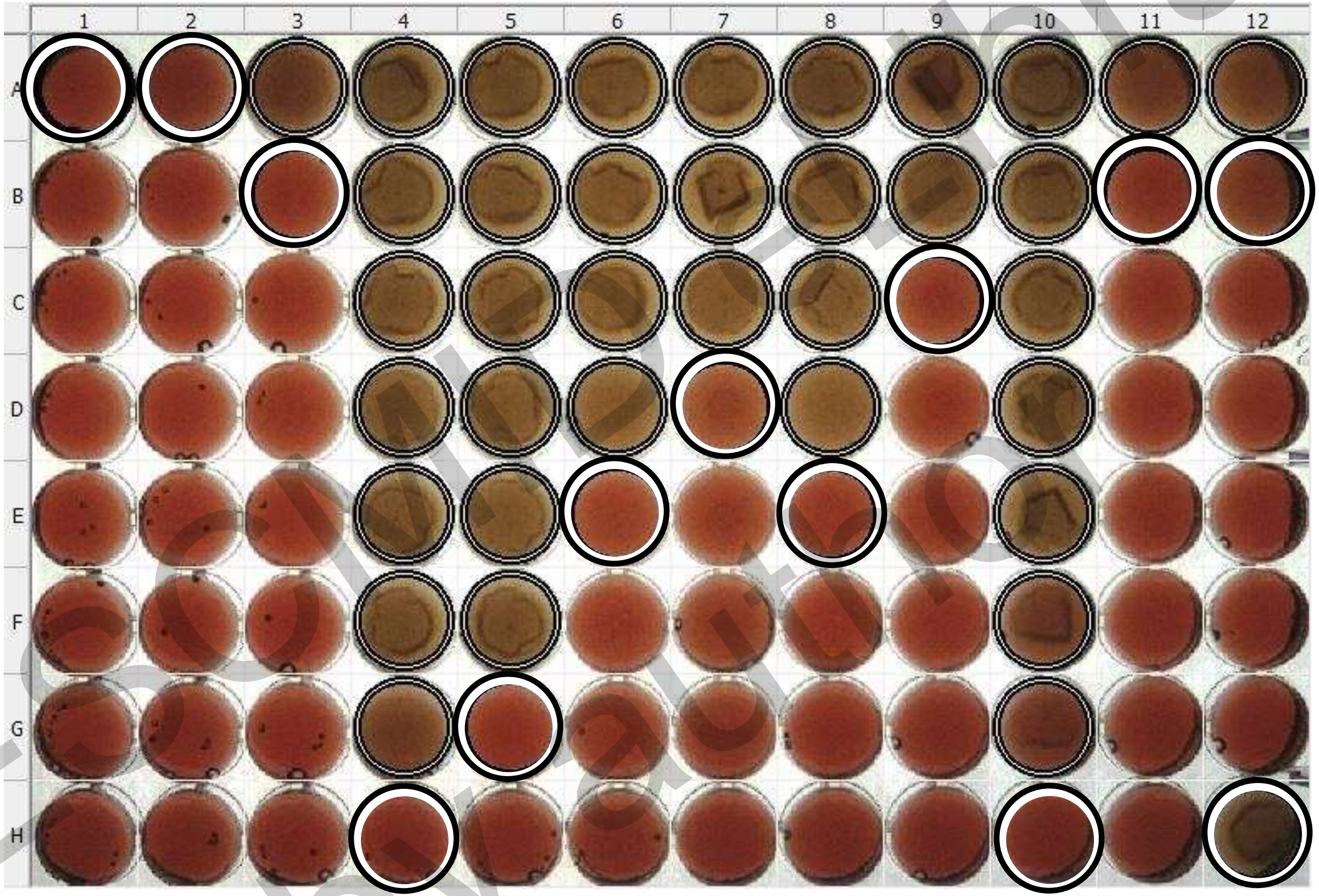
- Mueller-Hinton broth with 5% lysed horse blood and 20 mg/L β-NAD

- Mechanically defibrinated horse blood is lysed by repeated freezing and thawing followed by centrifugation.
  - Important with clear broth to facilitate reading MIC endpoints.

  http://www.eucast.org/ast_of_bacteria/media_preparation/

- Developed by EUCAST to be used for *S. pneumoniae*, other streptococci, *H. influenzae* and several other fastidious organisms.
  - Both 20 mg/L β-NAD and 5% lysed horse blood are needed to support sufficient growth of *H. influenzae*.

Reading BMD for *H. influenzae*



Reproducibility of BMD for *H. influenzae*

Isolate	AMC	AMO	AMP	CTX	CRO	CXM	IMI	MER	CIP	LEV	MOX	TET	TSU
1	2	2	2	0.12	0.03	4	≤0.25	0.06	≤0.015	≤0.015	≤0.015	0.5	≤0.06
	2	2	2	0.06	0.03	4	≤0.25	0.06	≤0.015	≤0.015	≤0.015	0.5	≤0.06
	2	2	2	0.06	0.03	4	≤0.25	0.06	≤0.015	≤0.015	≤0.015	0.5	≤0.06
2	4	4	2	0.25	0.06	4	1	0.12	≤0.015	≤0.015	≤0.015	1	≤0.06
	4	4	2	0.25	0.06	4	0.5	0.12	≤0.015	≤0.015	≤0.015	0.5	≤0.06
	4	4	2	0.25	0.06	4	0.5	0.12	≤0.015	≤0.015	≤0.015	0.5	≤0.06
3	8	8	2	1	0.25	>8	1	0.25	≤0.015	0.03	≤0.015	0.5	2
	4	4	2	1	0.25	>8	1	0.25	≤0.015	≤0.015	≤0.015	0.5	2
	4	8	2	1	0.25	>8	1	0.25	≤0.015	≤0.015	≤0.015	0.5	2
4	2	4	2	0.12	0.03	4	0.5	0.25	≤0.015	≤0.015	≤0.015	0.5	≤0.06
	2	2	1	0.06	0.03	4	0.5	0.12	≤0.015	≤0.015	≤0.015	0.5	≤0.06
	2	2	1	0.06	≤0.015	4	0.5	0.12	≤0.015	≤0.015	≤0.015	0.5	≤0.06
5	4	8	4	0.25	0.06	>8	8	0.5	≤0.015	≤0.015	≤0.015	0.5	≤0.06
	4	8	4	0.12	0.06	>8	8	0.5	≤0.015	≤0.015	≤0.015	0.5	≤0.06
	4	4	2	0.12	0.06	>8	8	0.25	≤0.015	≤0.015	≤0.015	0.5	≤0.06
6	>8	>8	8	1	0.25	>8	4	0.5	≤0.015	≤0.015	≤0.015	0.5	≤0.06
	8	>8	4	1	0.25	>8	4	0.25	≤0.015	≤0.015	≤0.015	0.5	≤0.06
	8	8	2	1	0.25	>8	8	0.25	≤0.015	≤0.015	≤0.015	0.5	≤0.06

S	R

Gradient tests for *H. influenzae* and beta-lactam agents

Essential agreement for gradient tests vs. BMD for *H. influenzae* with various PBP3 mutations and beta-lactam agents.
17 isolates tested on MH-F from two manufacturers (n=34 per antimicrobial agent).

Reading of results	Gradient test brand	Essential agreement (%)	
		All agents	Excluding cefuroxime, imipenem and meropenem
According to manufacturer[1]	Etest	69	83
	MICE	70	87
	MTS	63	80
First inhibition[2]	Etest	83	93
	MICE	89	100
	MTS	80	93

Agents tested
Ampicillin
Cefepime
Cefotaxime
Ceftriaxone
Cefuroxime
Imipenem
Meropenem

1. According to manufacturers' instructions with growth of small colonies within the ellipse and regrowth taken into account
2. First inhibition of growth. Small colonies within the ellipse and regrowth disregarded.

Karlsson C. *et al.* Evaluation of Etest, M.I.C.E. and MIC Test Strip beta-lactam gradient tests for beta-lactamase negative *Haemophilus influenzae* with beta-lactam resistance due to PBP3 substitutions. Poster, ECCMID 2016.

Disk diffusion of *H. influenzae*

- **MH-F:** Mueller-Hinton Fastidious agar
  - 5% defibrinated horse blood and 20 mg/L β-NAD

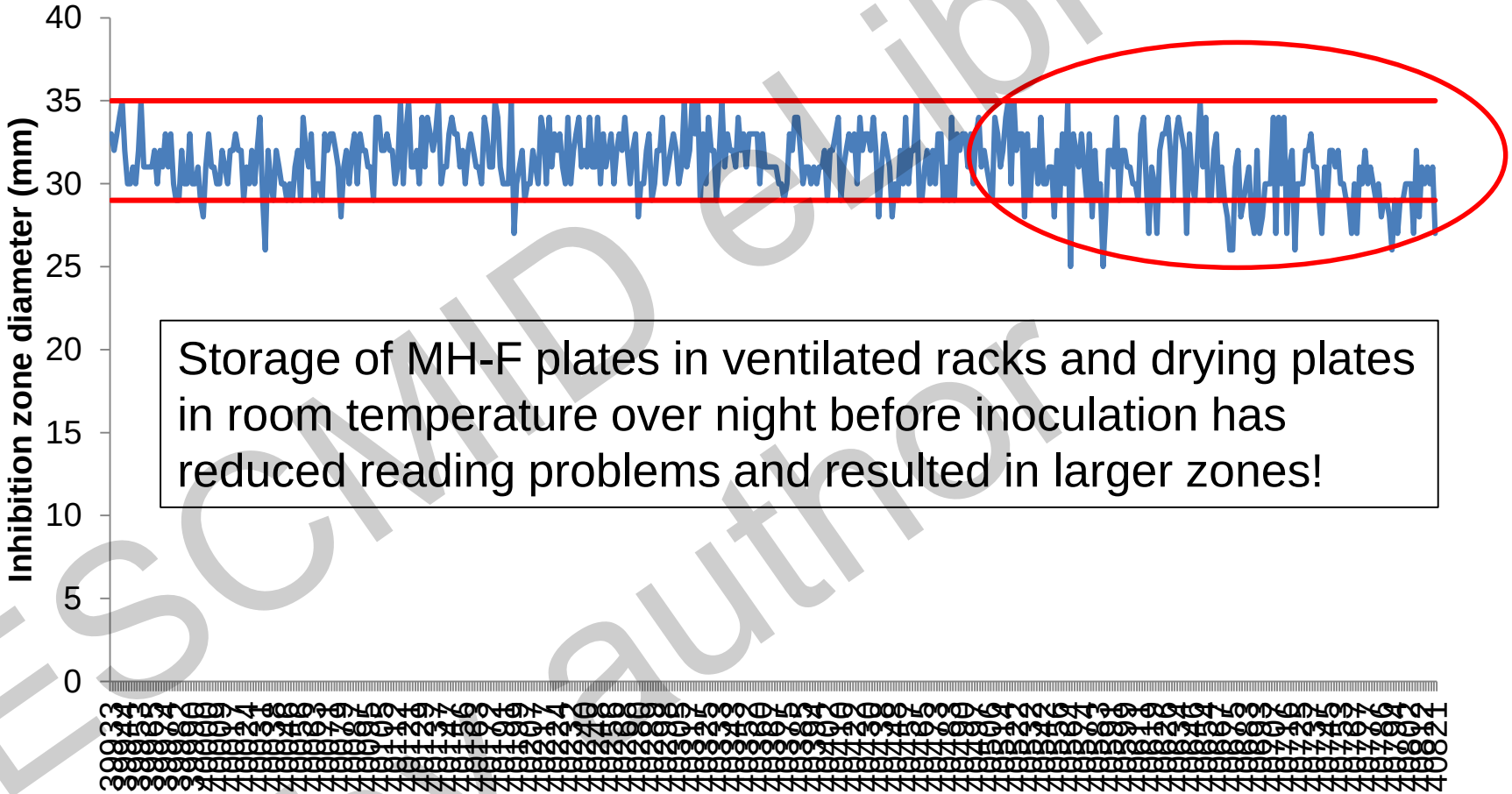
- The MH-F agar was developed to ensure good growth of *S. pneumoniae*, other streptococci and *H. influenzae*
  - Mechanically defibrinated horse blood
  - β-NAD with purity of ≥98%

- Excess humidity in agar plates can cause problems with haze within zones and fuzzy zone edges.
  - No drops of water should be visible on the agar surface or inside the lid

Excess humidity of agar plates - example with MH-F

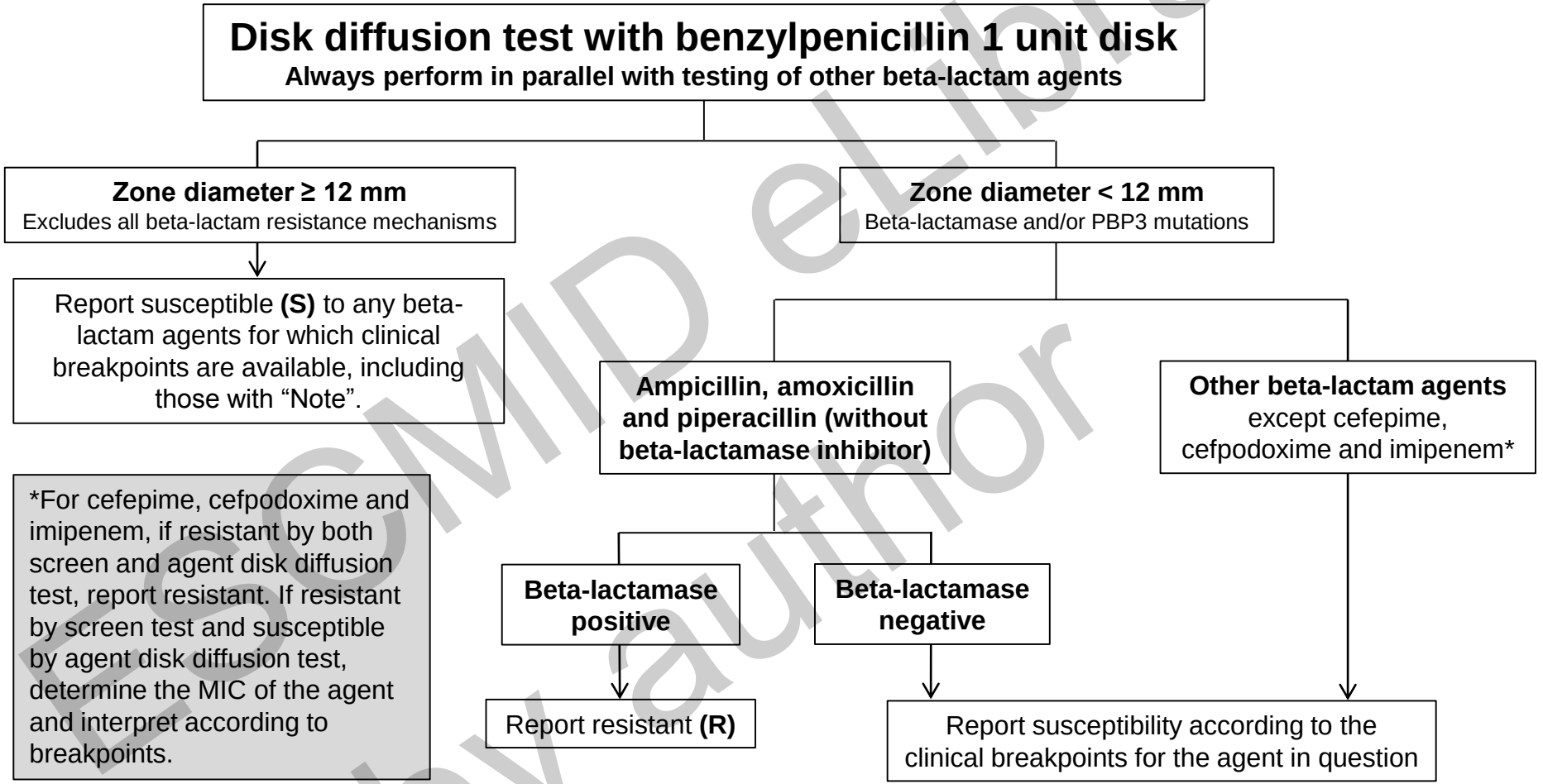
If necessary, dry plates either at 20-25°C overnight, or at 35°C, with the lid removed, for 15 min.

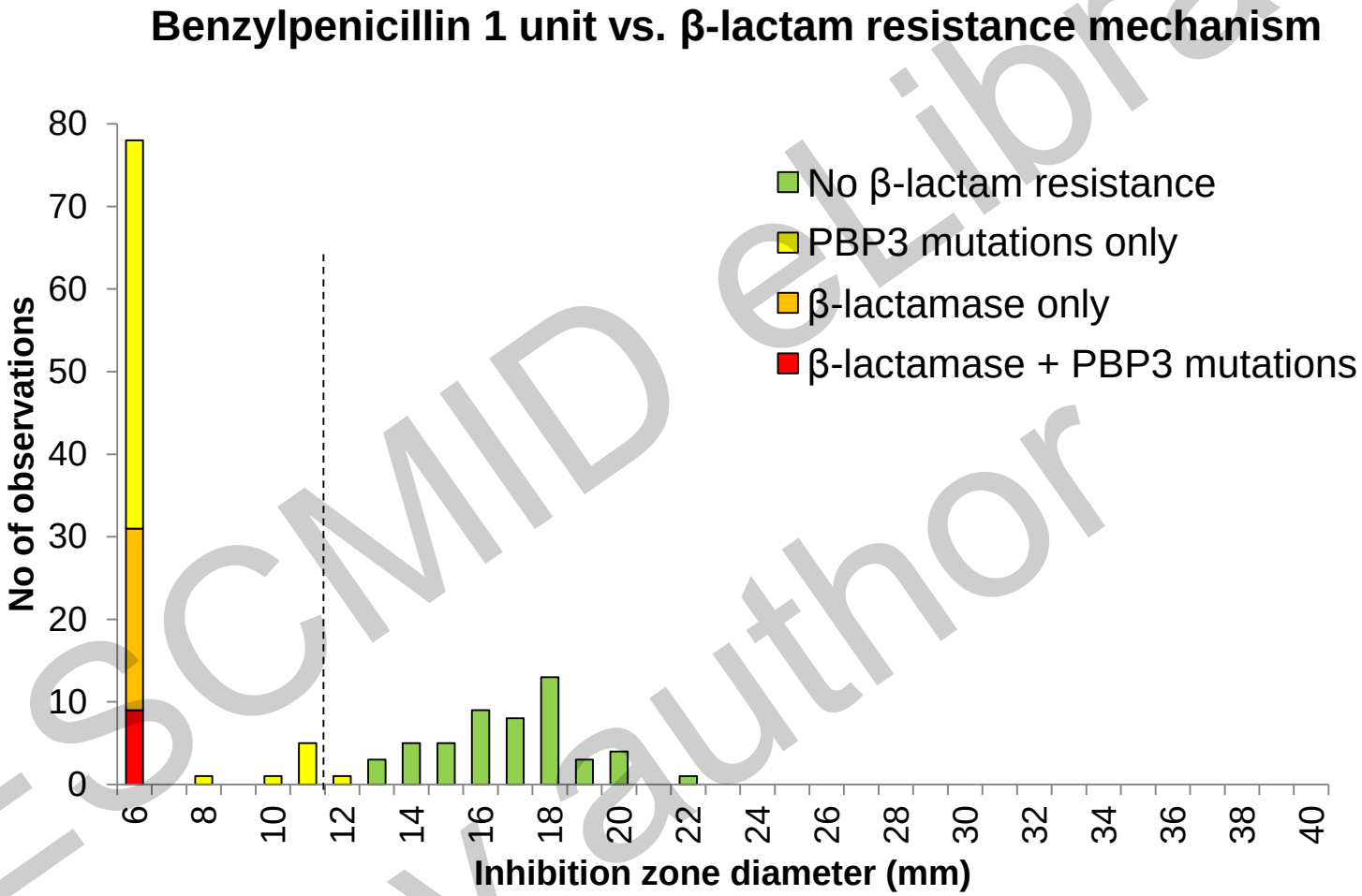
H. influenzae NCTC 8468 with cefotaxime 5 µg



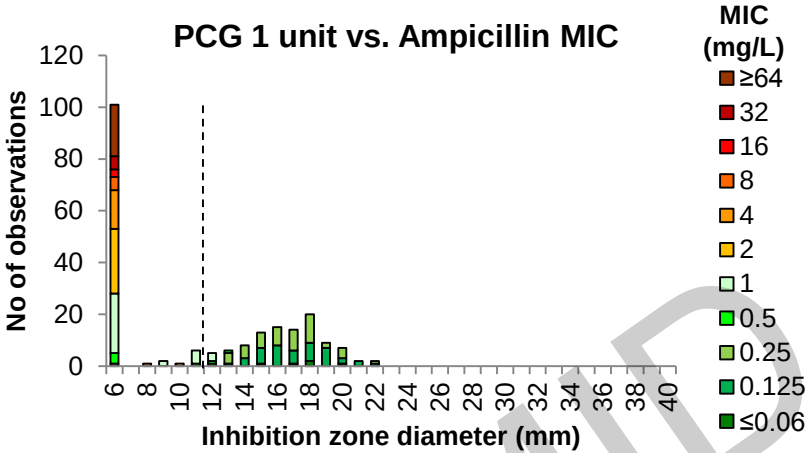
Storage of MH-F plates in ventilated racks and drying plates in room temperature over night before inoculation has reduced reading problems and resulted in larger zones!

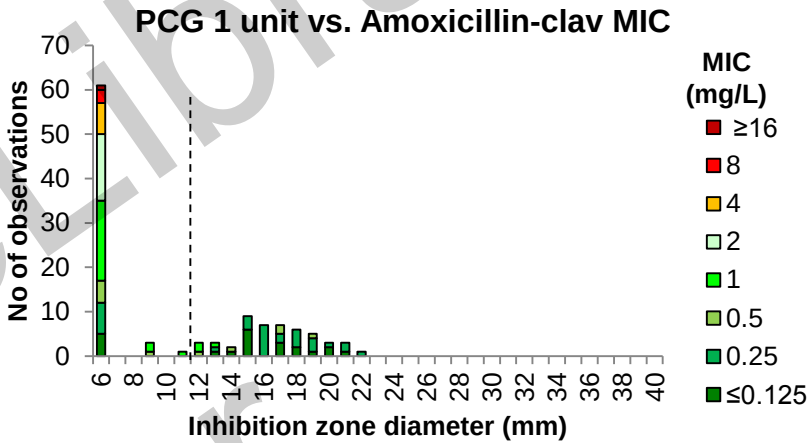
Screening for beta-lactam resistance in *H. influenzae* (EUCAST BP table v 9.0, 2019)

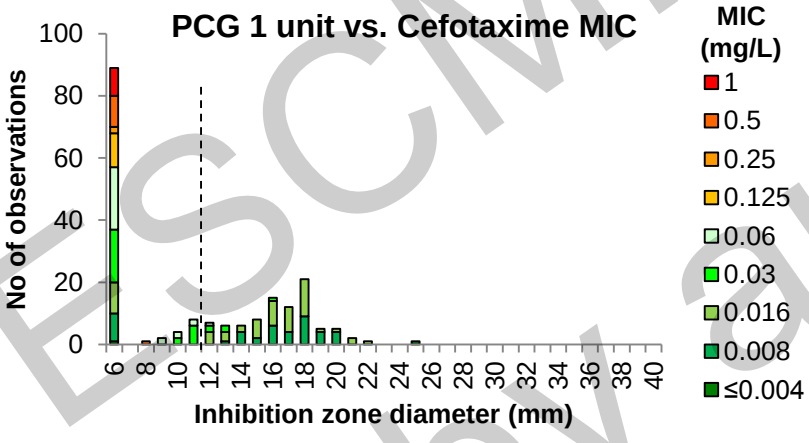


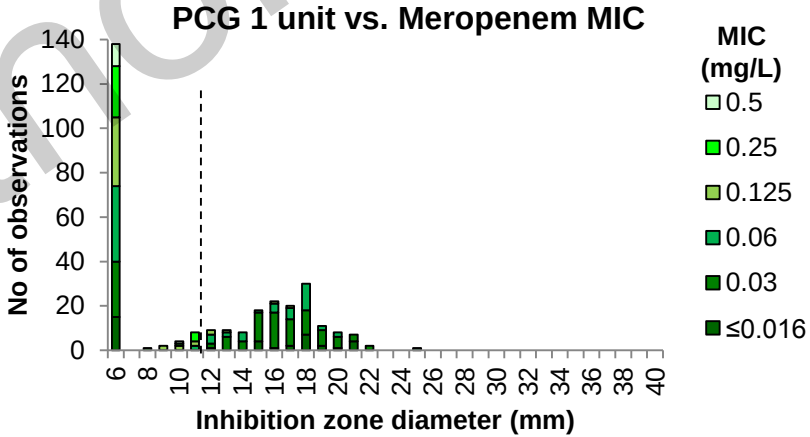


Screening for beta-lactam resistance in *H. influenzae*

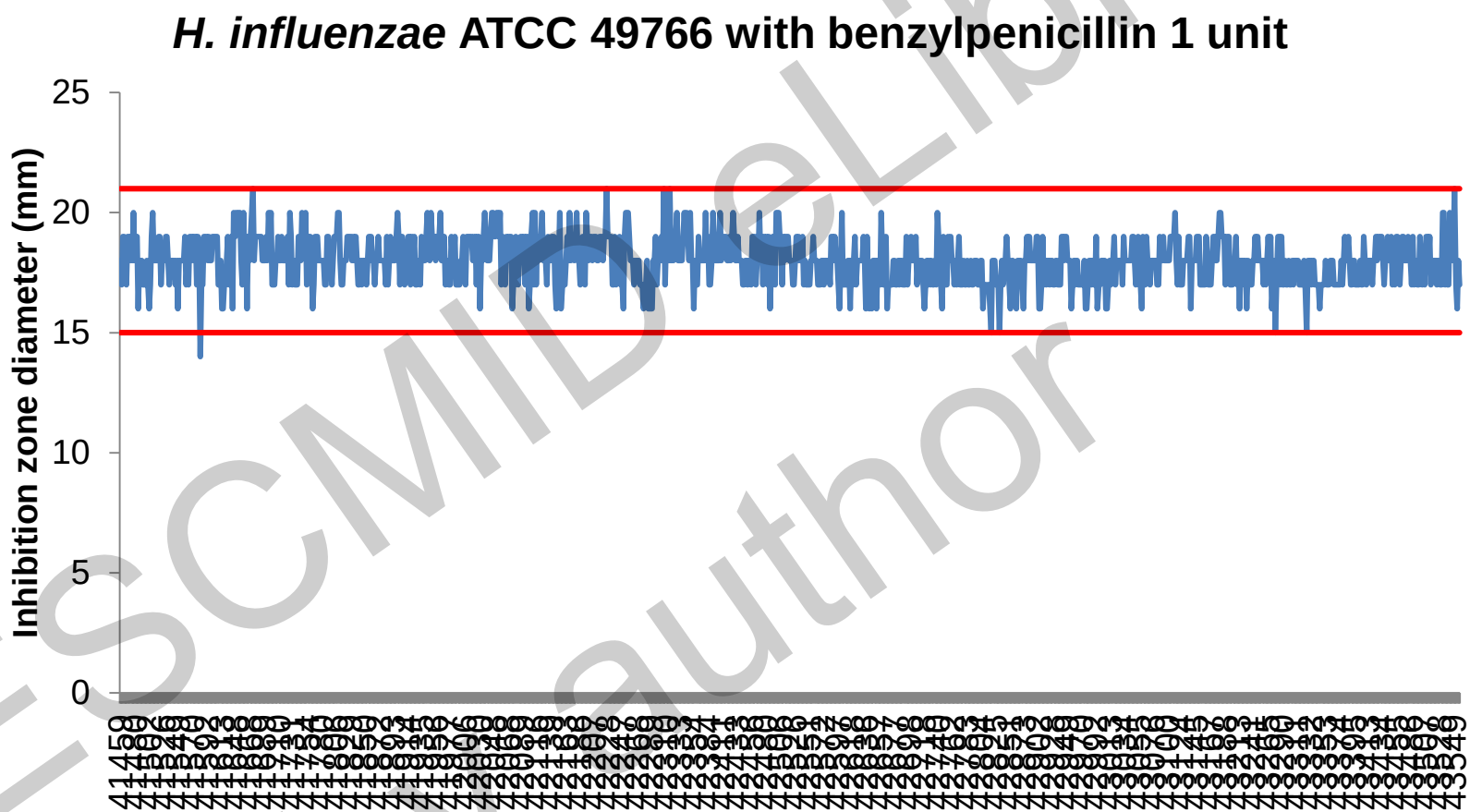




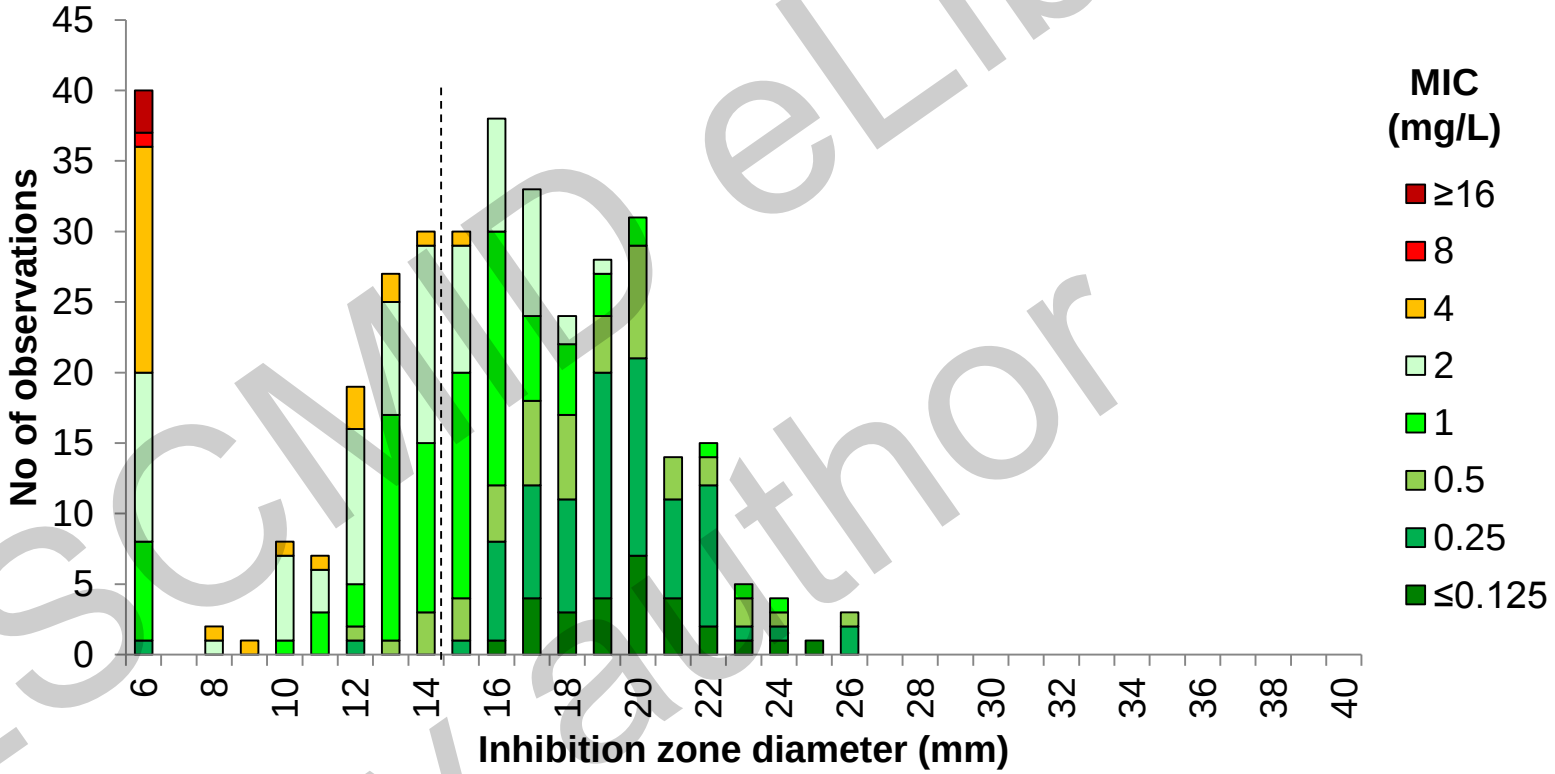




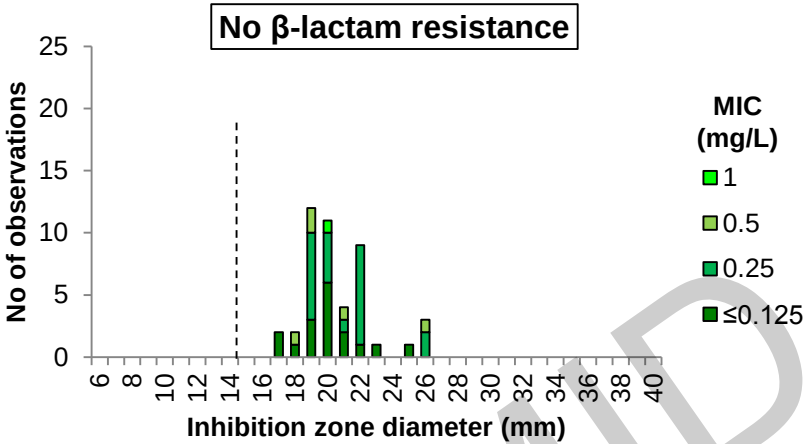
Stability of benzylpenicillin 1 unit disks over more than 5 years

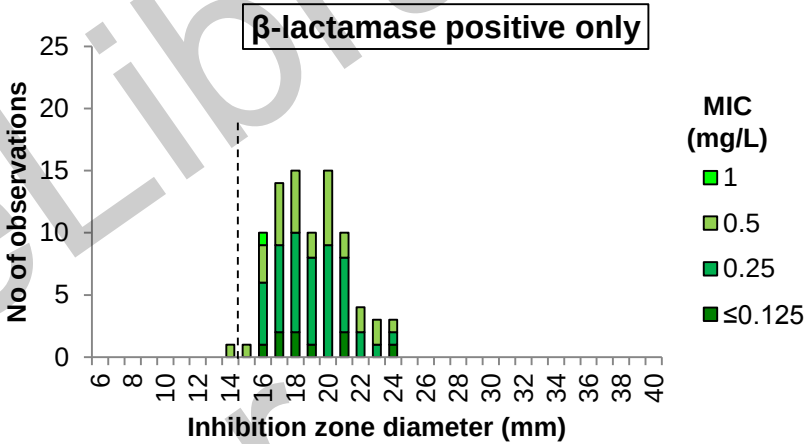


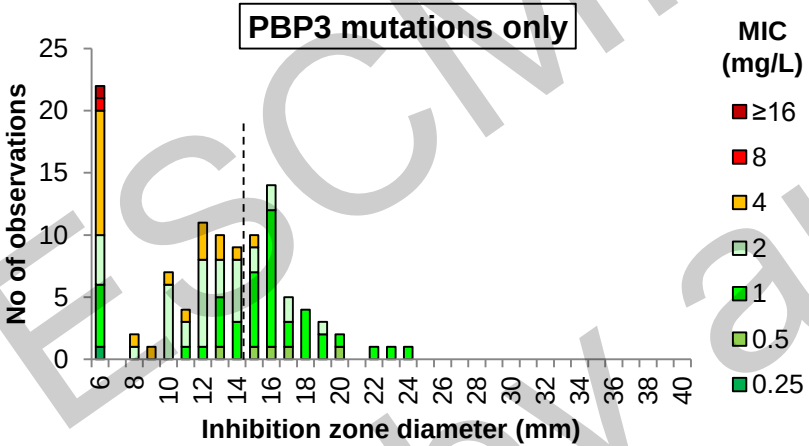
Amoxicillin-clavulanic acid 2-1 µg vs. MIC
H. influenzae, 167 isolates (360 correlates)

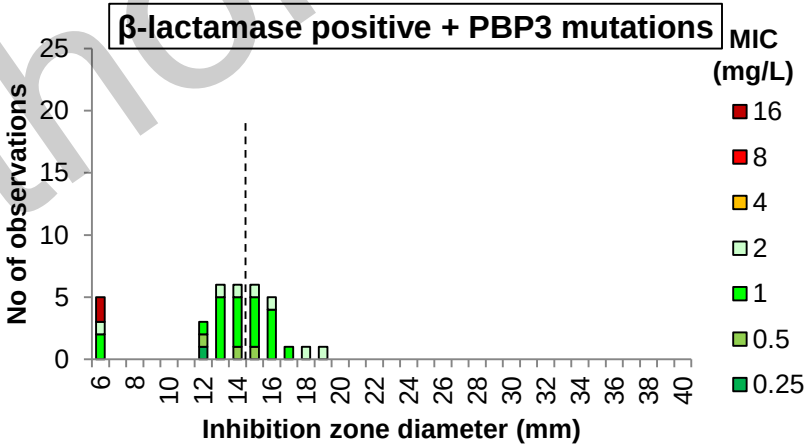


Amoxicillin-clavulanic acid 2-1 µg disk diffusion per beta-lactam resistance mechanism









Amoxicillin-clavulanic acid

- Better separation with other combinations of amoxicillin and clavulanic acid?
  - Standard 20-10 µg disk results in poorer separation
  - Additional combinations of amoxicillin and clavulanic acid under investigation

- Better results with specific reading instructions for amoxicillin-clavulanic acid?
  - Look carefully and take all growth into account
  - Disregard thin growth and read the outer zone

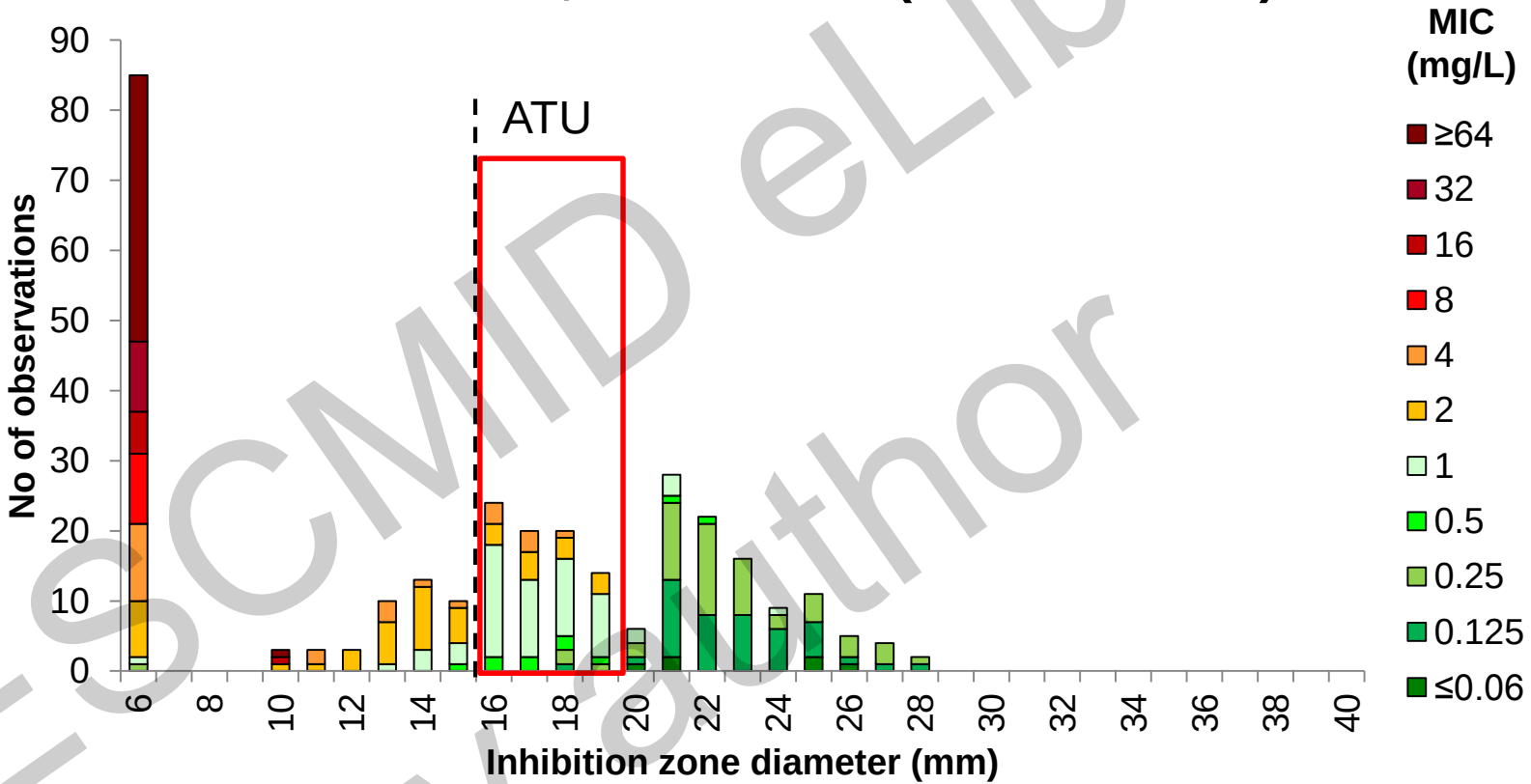
- Improved screening test?
  - To differentiate between isolates with β-lactamase only and those with β-lactamase and PBP3 mutations

H. influenzae and ATU

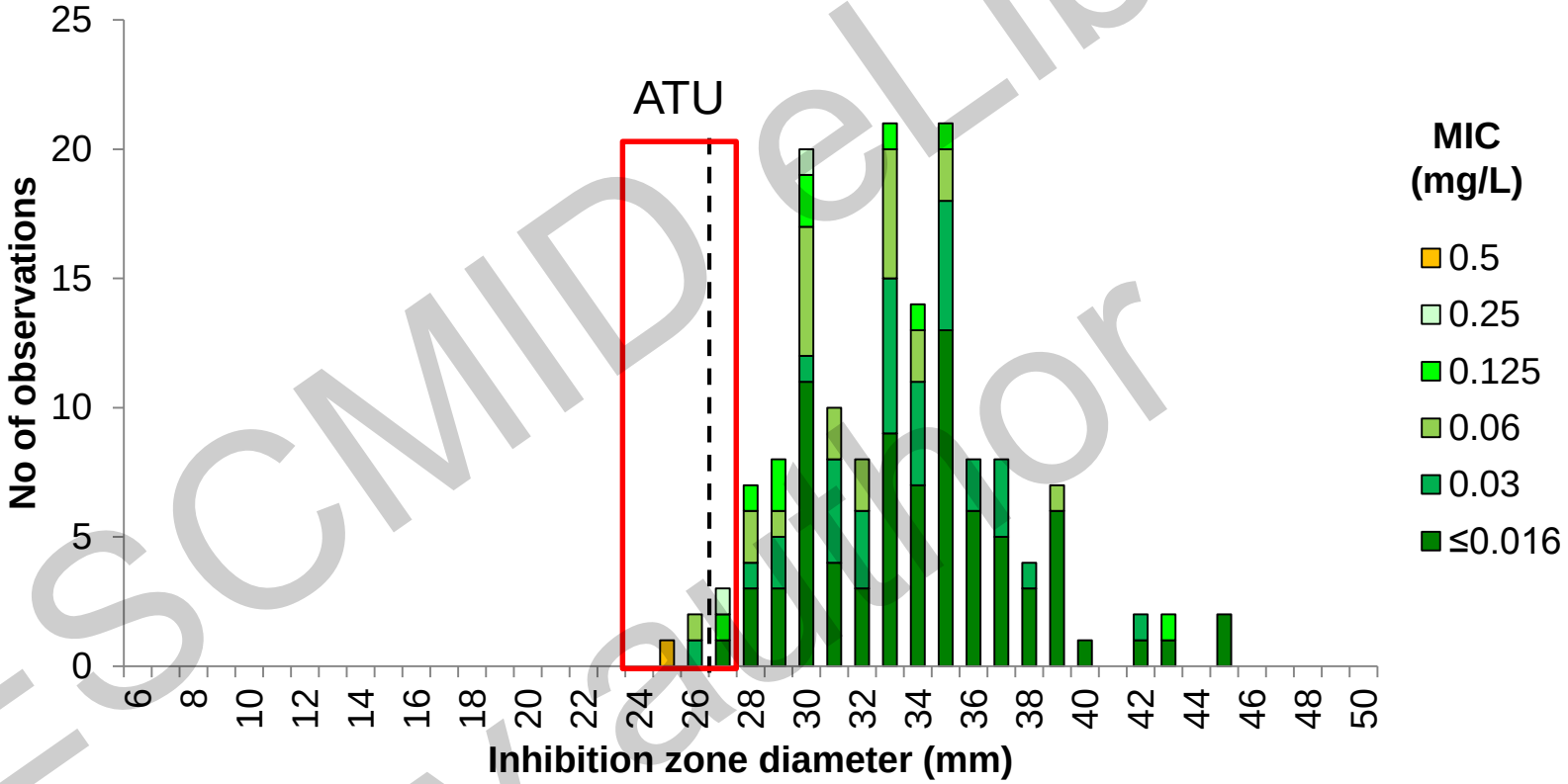
- Only for beta-lactam agents
- Only for PCG screen-positive isolates

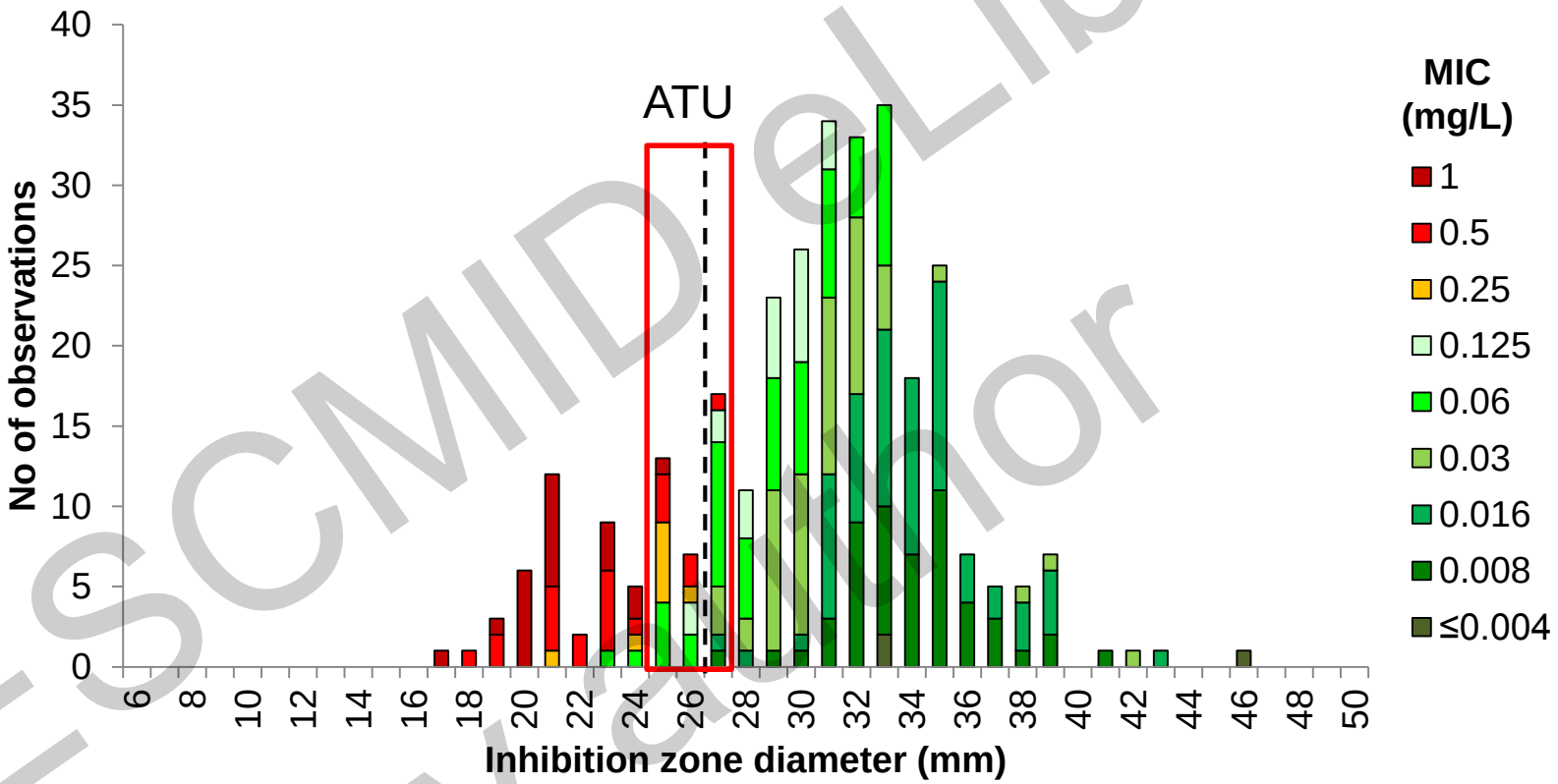
Agents with ATU for *H. influenzae*

- Ampicillin, amoxicillin-clavulanic acid and piperacillin-tazobactam
- Cefepime, cefotaxime, cefpodoxime, ceftriaxone and cefuroxime
- Imipenem

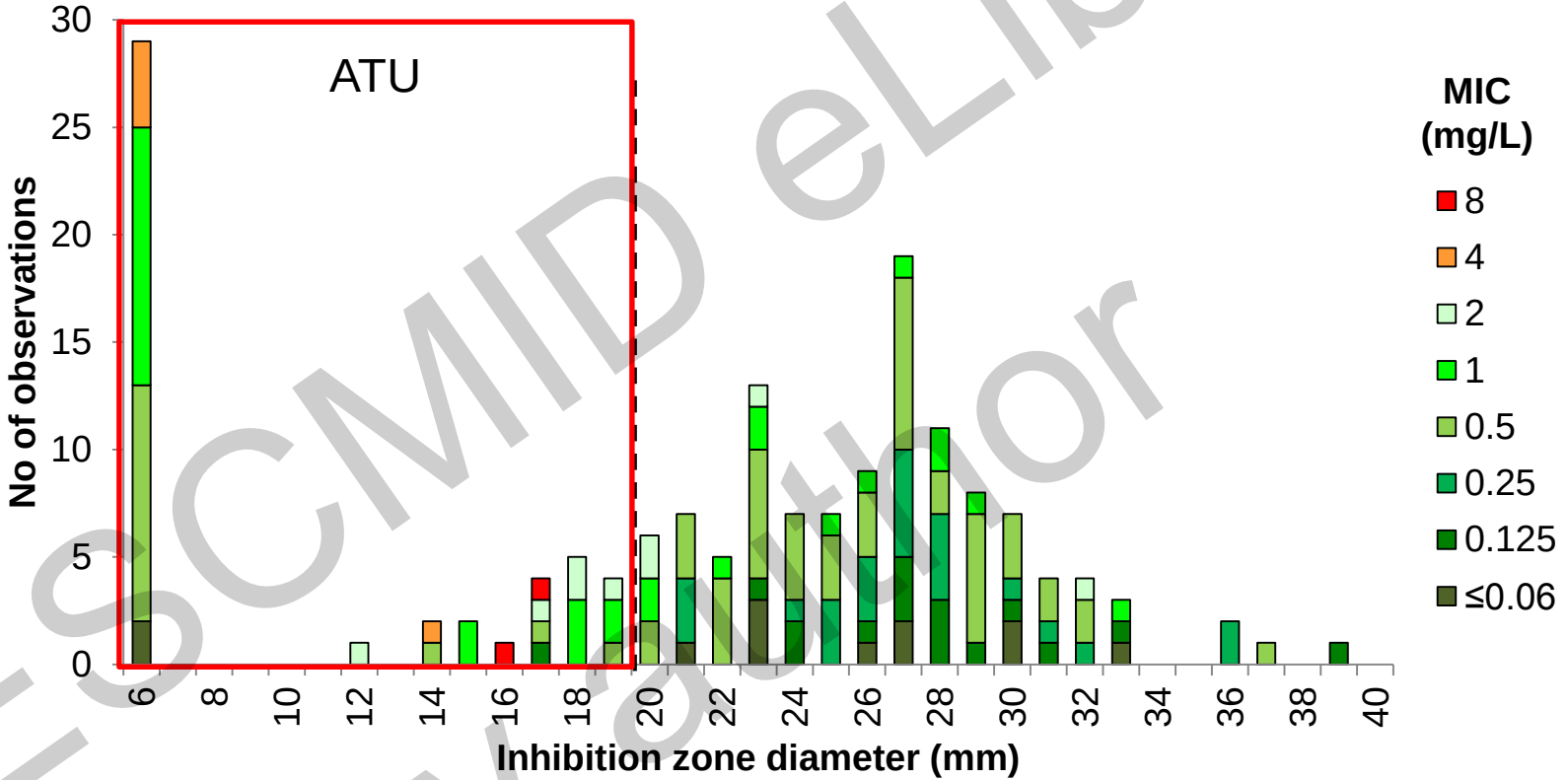


Piperacillin-tazobactam 30-6 µg vs. MIC
H. influenzae, 149 isolates





Imipenem 10 µg vs. MIC
H. influenzae, 150 isolates (162 correlates)



Standardisation and quality of materials

- All AST must be standardised to get reproducible and reliable results!
  - EUCAST methodology documents and instruction videos
  - Frequent quality control

- Use materials of good quality (good performance according to EUCAST QC criteria)
  - Evaluation of antimicrobial disks, Åhman *et al*., CMI 25 (2019):345-352
  - Evaluation of Mueller-Hinton agar, Åhman *et al*. Poster 2772, ECCMID 2019, Tuesday 16 April 12.30-13.30

Thanks!

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- EUCAST Network Laboratories contributing with data and/or isolates.

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- Check the EUCAST website regularly for updates on methodology, QC ranges and breakpoints.

www.eucast.org

- For questions and comments, please contact erika.matuschek@escmid.org or the EUCAST secretariat (see website).