



Anaerobic disk diffusion and beyond

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AST of anaerobic bacteria

- Agar dilution (reference method by EUCAST and CLSI)
 - EUCAST: FAA with 5% defibrinated horse blood (FAA-HB)
 - CLSI: Brucella Blood Agar with hemin and vitamin K (BBA)
- Broth microdilution
 - CLSI: Only recommended for *Bacteroides* spp.
- Disk diffusion
 - EUCAST: FAA-HB

Correlation between agar dilution MICs on FAA-HB and BBA

Benzylopenicillin

212 isolates of various species

		Brucella																
		<0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	≥512
FAA	<0.008	12	11	2														
	0.016	11	12	15	1													
	0.03	30	1	10	7													
	0.06	9	1	4	10	12												
	0.125				2	21	5											
	0.25					3	7	1										
	0.5							1	1									
	1								2	1	1							
	2								1	5	2							
	4										2	2						
	8										1	2	2					
	16												1	1				
	32													1	1			
	64														1	1		
	128															1	1	
	256																1	1
≥512																	1	

Identical MICs

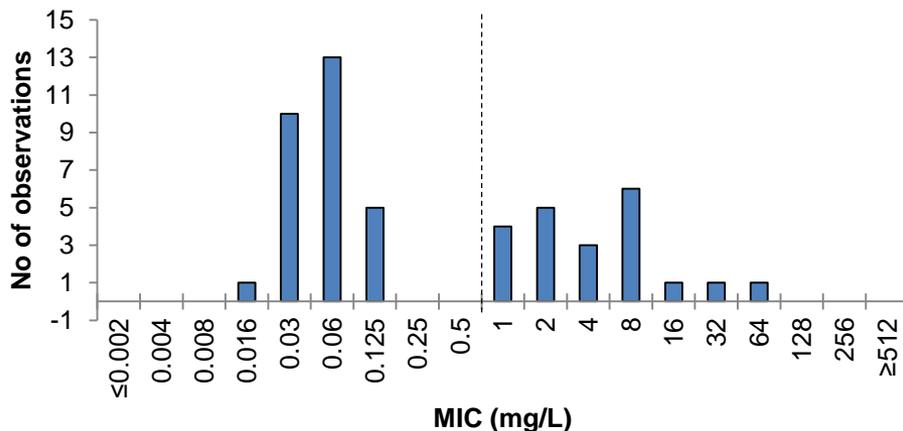
MICs 1 dilution higher or lower

Lower MICs on BBA for some *F. necrophorum*, probably due to poorer growth on BBA.

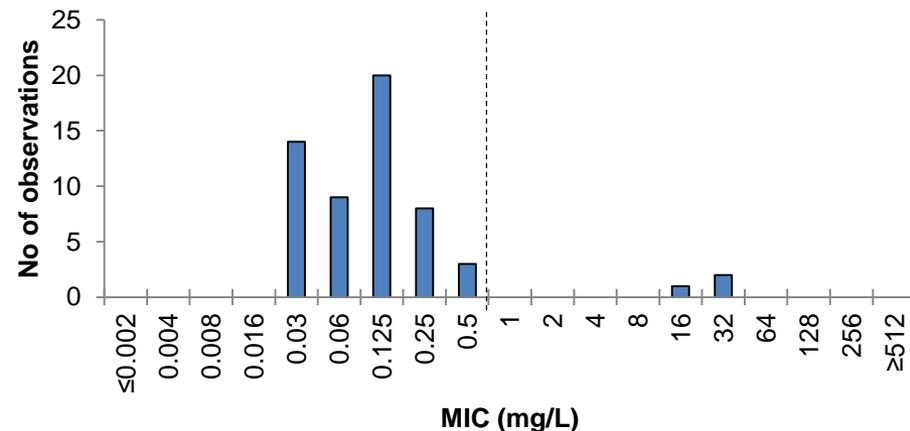
Revised MIC breakpoints

- EUCAST has revised MIC breakpoints for anaerobic bacteria and established organism-specific breakpoints based on agar dilution on FAA-HB (Breakpoint Table v. 12.0, 2022).

Benzylpenicillin MIC FAA vs. BBA
Prevotella spp., 50 isolates



Piperacillin-tazobactam MIC FAA vs. BBA
C. perfringens, 58 isolates



Disk diffusion methodology

Inoculum	McF 1.0 (0.9-1.1)
Medium	Fastidious Anaerobe Agar with 5% mechanically defibrinated horse blood (FAA-HB) – dried plates
Incubation	Anaerobic environment 35-37°C, 16-20 h
Reading of zones	Read zone diameters from the front of the plate See specific Reading Guide for anaerobic bacteria

AST of bacteria

- IVDR
- Media preparation
- MIC determination
- Disk diffusion methodology**
- Disk diffusion implementation
- Breakpoint tables
- Quality Control
- Strains with defined susceptibility
- Calibration and validation
- Warnings!
- MIC testing services from EUCAST
- Previous versions of documents

www.eucast.org

- [Disk diffusion manual v 12.0](#) (1 January, 2024)
- [Disk diffusion - Slide show v 12.0](#) (1 January, 2024)
- [Disk diffusion - Reading guide v 10.0](#) (2 January, 2023).
- [Anaerobic bacteria - disk diffusion methodology v 2.0](#) (2 January 2023). QC recommendations are now included in the general [QC document](#). Disk diffusion breakpoints for anaerobic bacteria are valid for FAA with 5% mechanically defibrinated horse blood as the only additive.
- [Anaerobic bacteria - disk diffusion reading guide v 2.0](#) (2 January 2023). Disk diffusion breakpoints for anaerobic bacteria are valid for FAA with 5% mechanically defibrinated horse blood as the only additive.

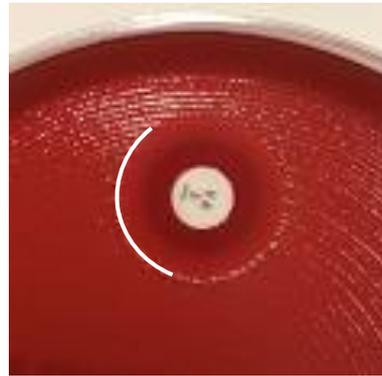
For translations to other languages - contact National AST committees (NAC).

Important aspects of methodology

- Dry plates before inoculation
- *Bacteroides* spp. and *C. perfringens*
 - Remove excess fluid from the cotton swab before inoculation to avoid over-inoculation
- *Prevotella* spp., *F. necrophorum* and *C. acnes*
 - Spread the inoculum evenly over the entire agar surface, ensuring that there are no gaps between streaks
- Limit the number of disks on each plate to allow good growth and to avoid overlapping of zones
- Follow the specific reading instructions

Reading of zones

Specific instructions



- Ignore any faint haze within the inhibition zone and read the most obvious zone. **Tilt the plate towards you to better define the obvious zone edge.**
- Ignore haemolysis and swarming and read inhibition of growth.
- Isolated colonies within the inhibition zone should be taken into account. **For clindamycin, it is particularly important to examine zones carefully for colonies growing within the zone.**

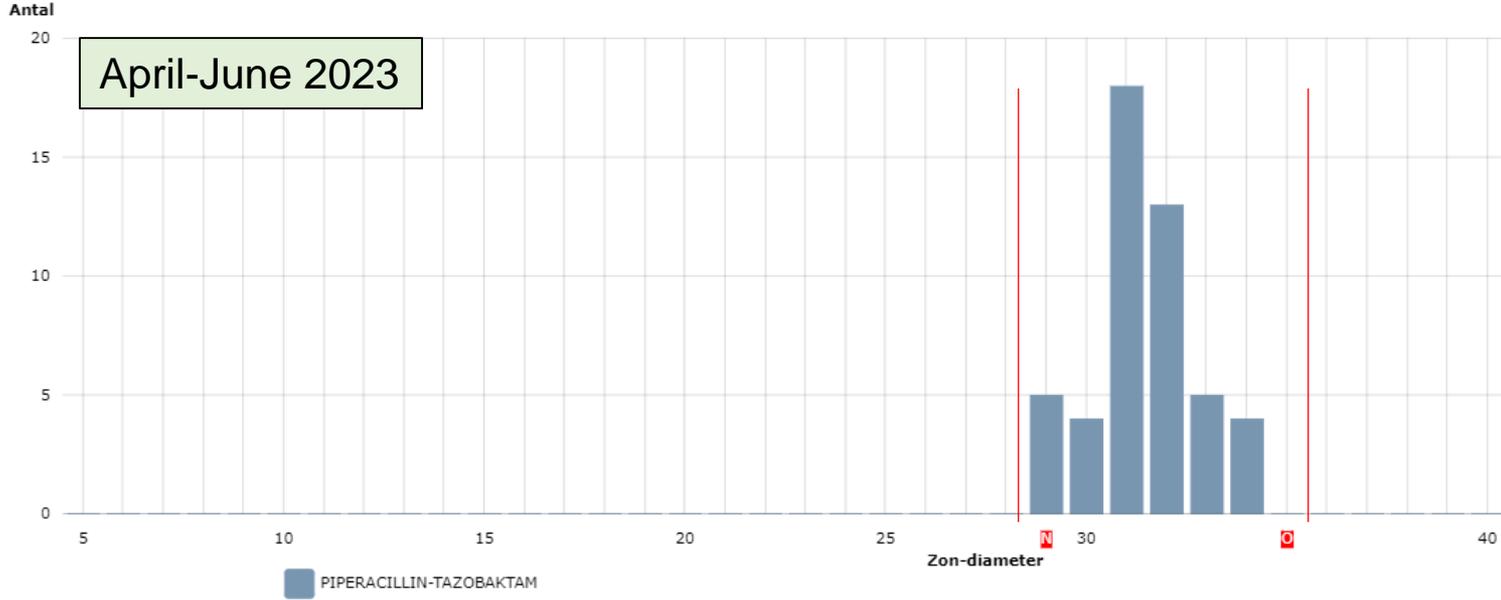
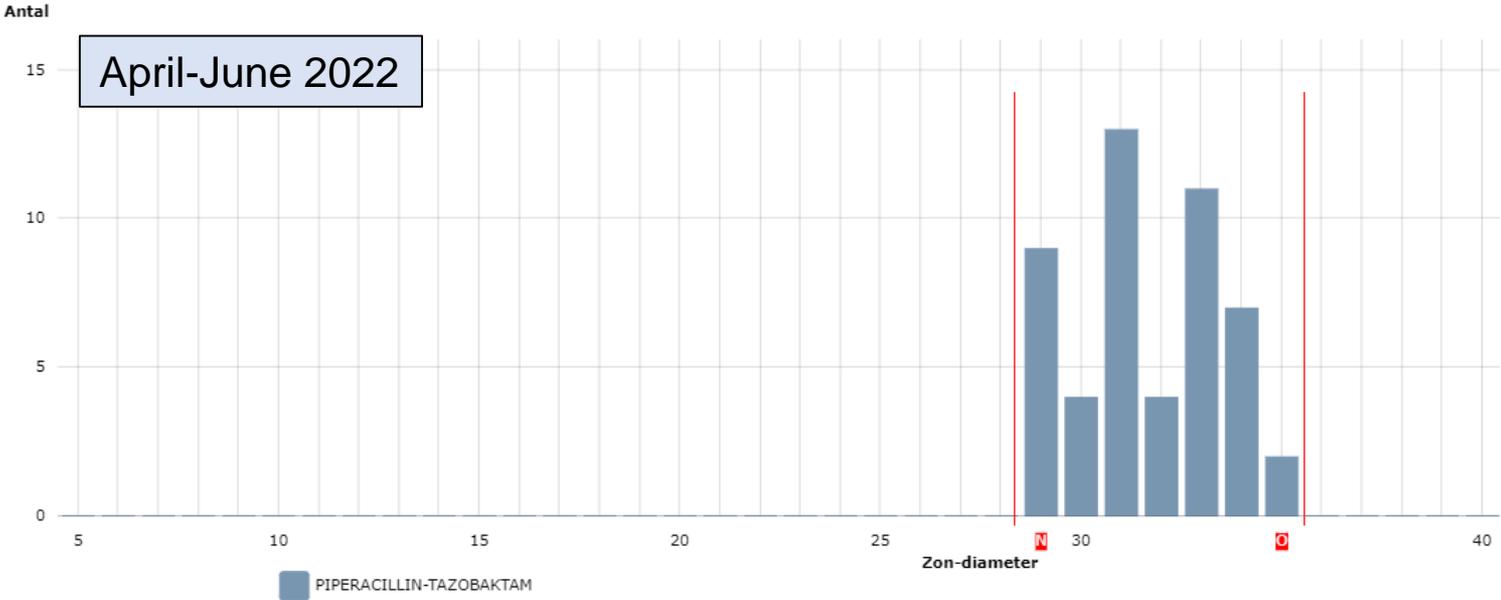
Implementation in our routine laboratory, March 2022

- Theoretical presentation of methodology
- Reading exercise
 - *Bacteroides fragilis* ATCC 25285
 - *Clostridium perfringens* ATCC 13124
 - 5 clinical isolates
 - *Bacteroides fragilis* (*cfiA* positive, MER R, CLI R)
 - *Prevotella bivia*
 - *Fusobacterium necrophorum*
 - *Clostridium perfringens* (CLI R)
 - *Cutibacterium acnes* (CLI R)

Implementation in our routine laboratory, March 2022

- Routines for preparation, storage and handling of FAA-HB plates
 - Storage in fridge
 - 3 weeks shelf life
 - Drying in room temperature over night before testing
- Routines for quality control
 - Same frequency as for the other QC strains (4 times a week)
 - The 2 anaerobic QC strains are tested at each occasion
 - Control of the anaerobic environment with *C. perfringens* DSM 25589

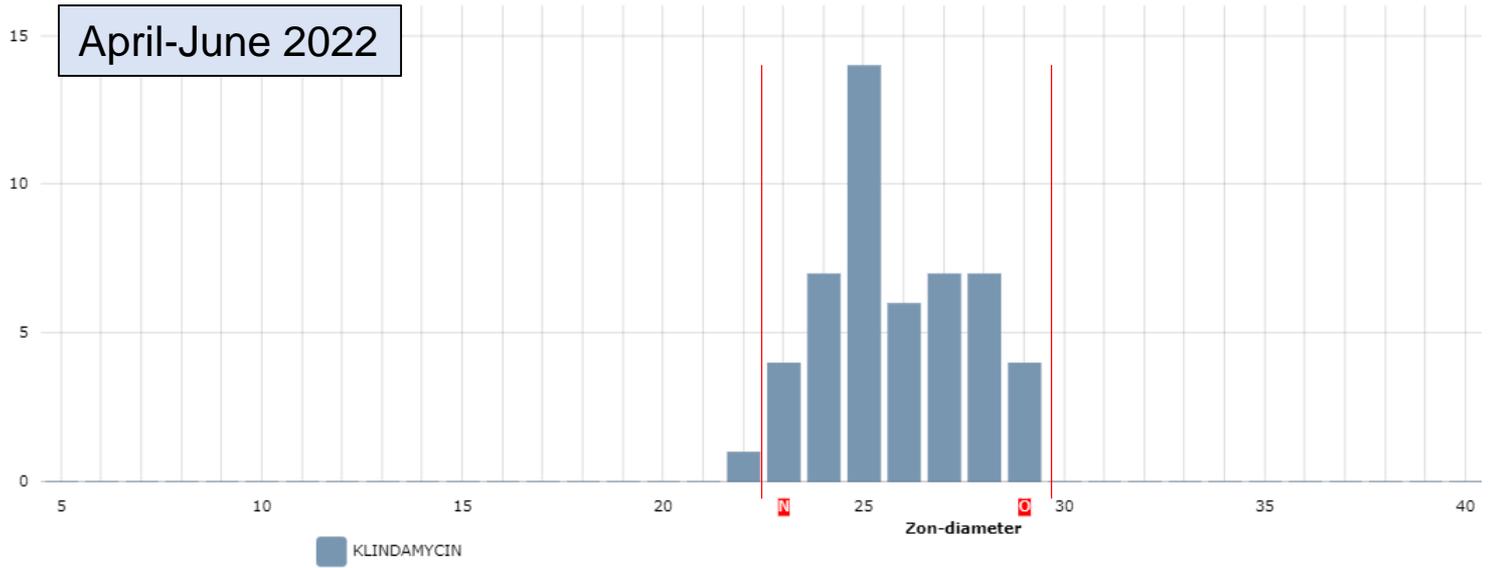
Bacteroides fragilis ATCC 25285 and piperacillin-tazobactam 30-6 µg



Bacteroides fragilis ATCC 25285 and clindamycin 2 µg

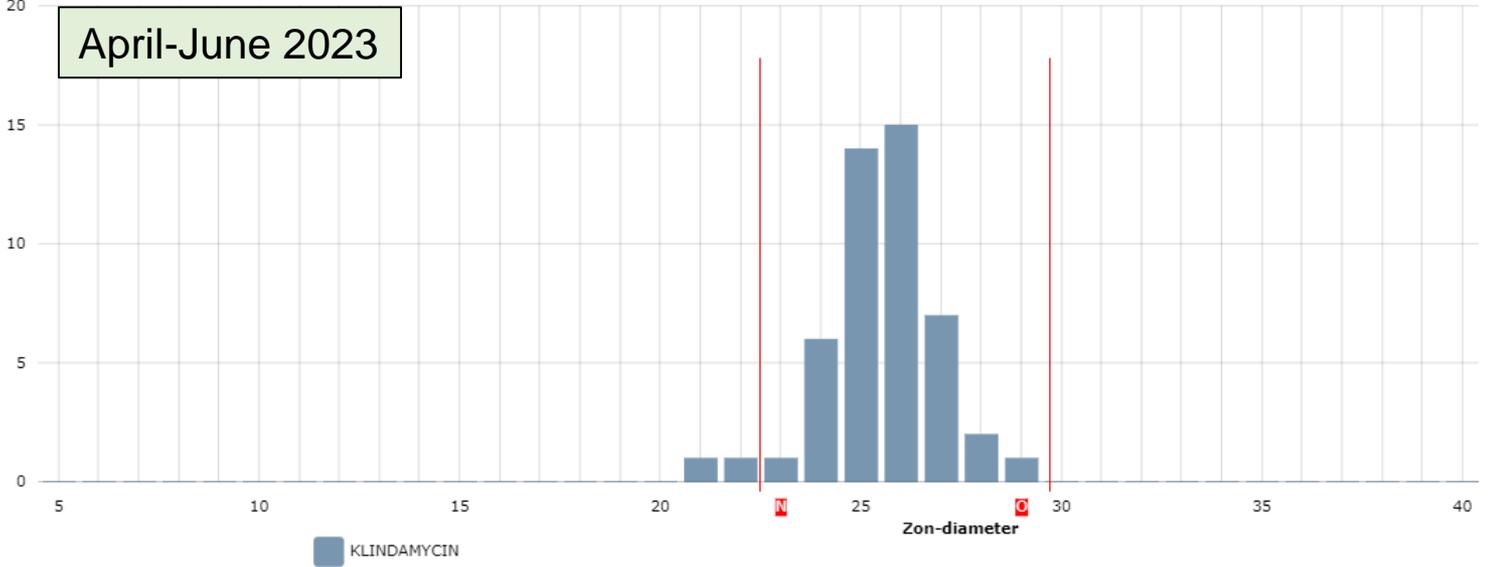
Antal

April-June 2022



Antal

April-June 2023



Bacteroides fragilis ATCC 25285 and metronidazole 5 µg

Antal

April-June 2022

15

10

5

0

5

10

15

20

25

30

35

40

Zon-diameter

METRONIDAZOL

Antal

April-June 2023

20

15

10

5

0

5

10

15

20

25

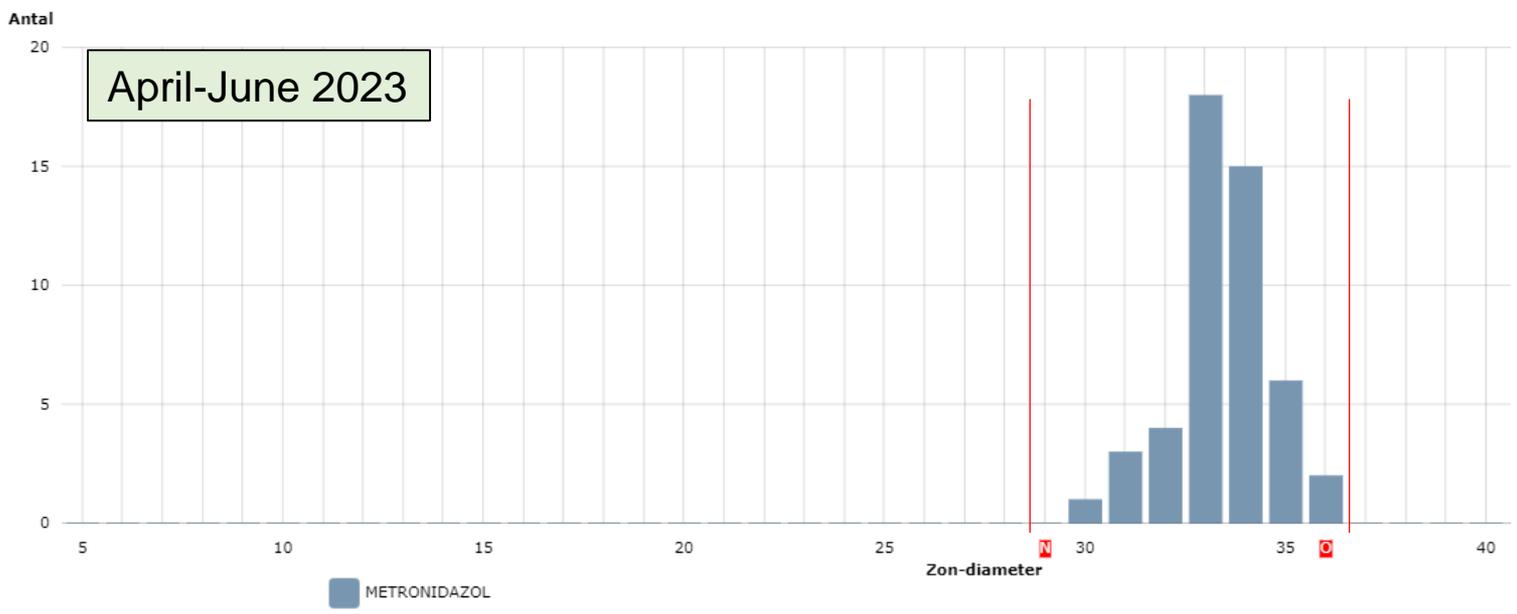
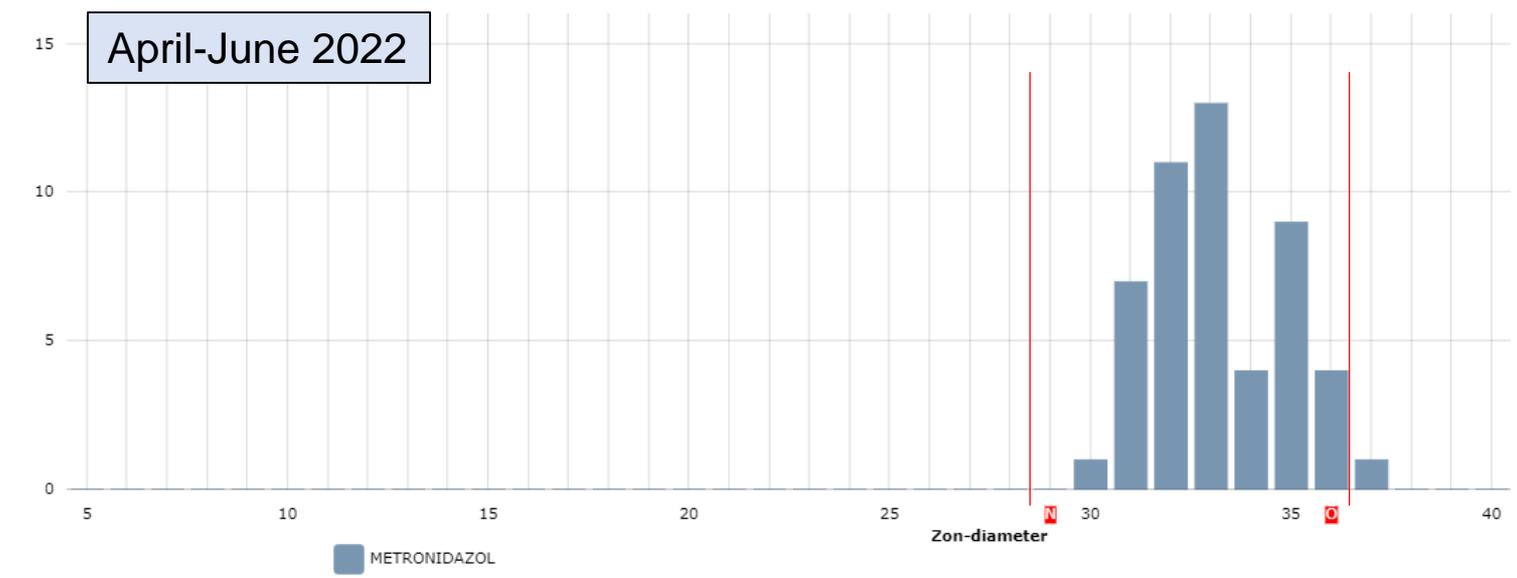
30

35

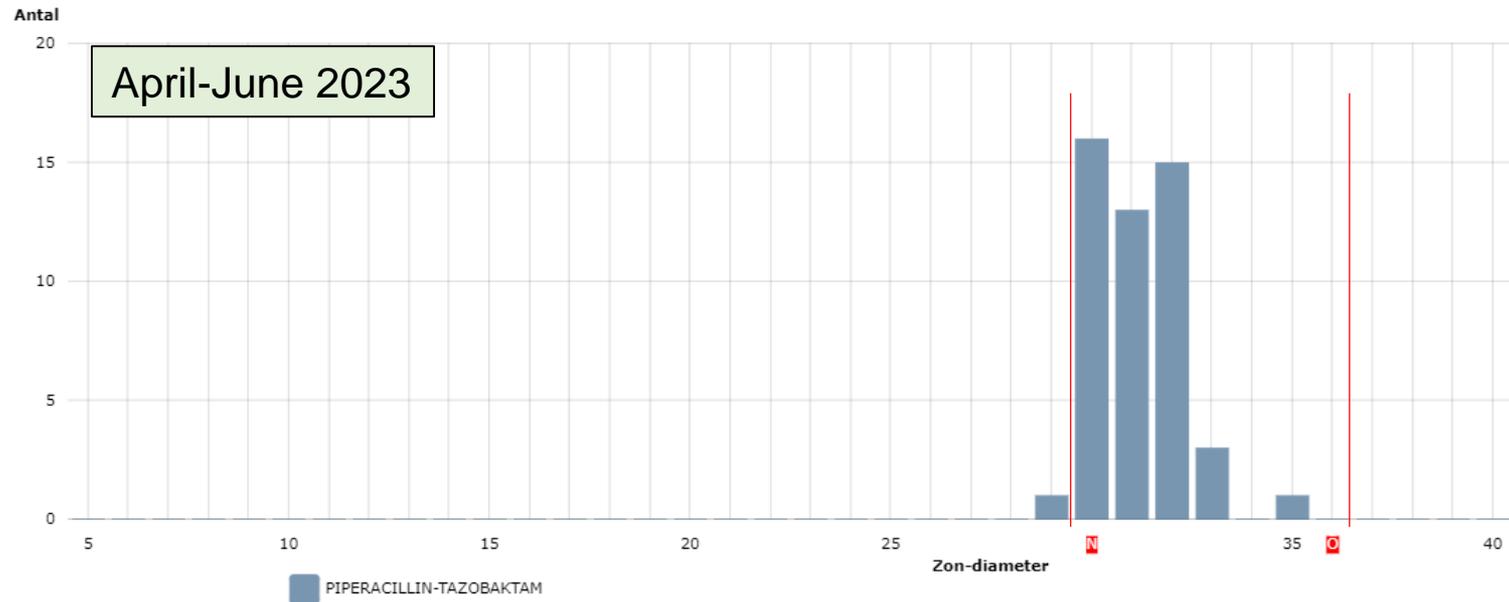
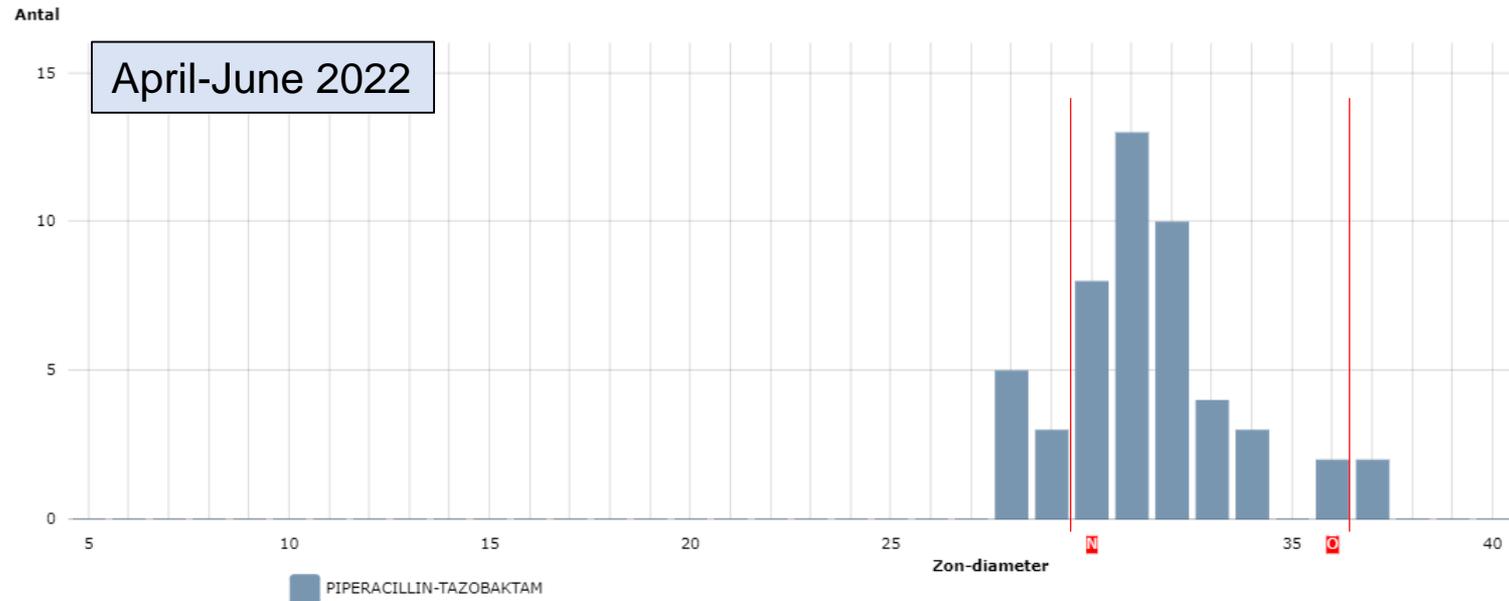
40

Zon-diameter

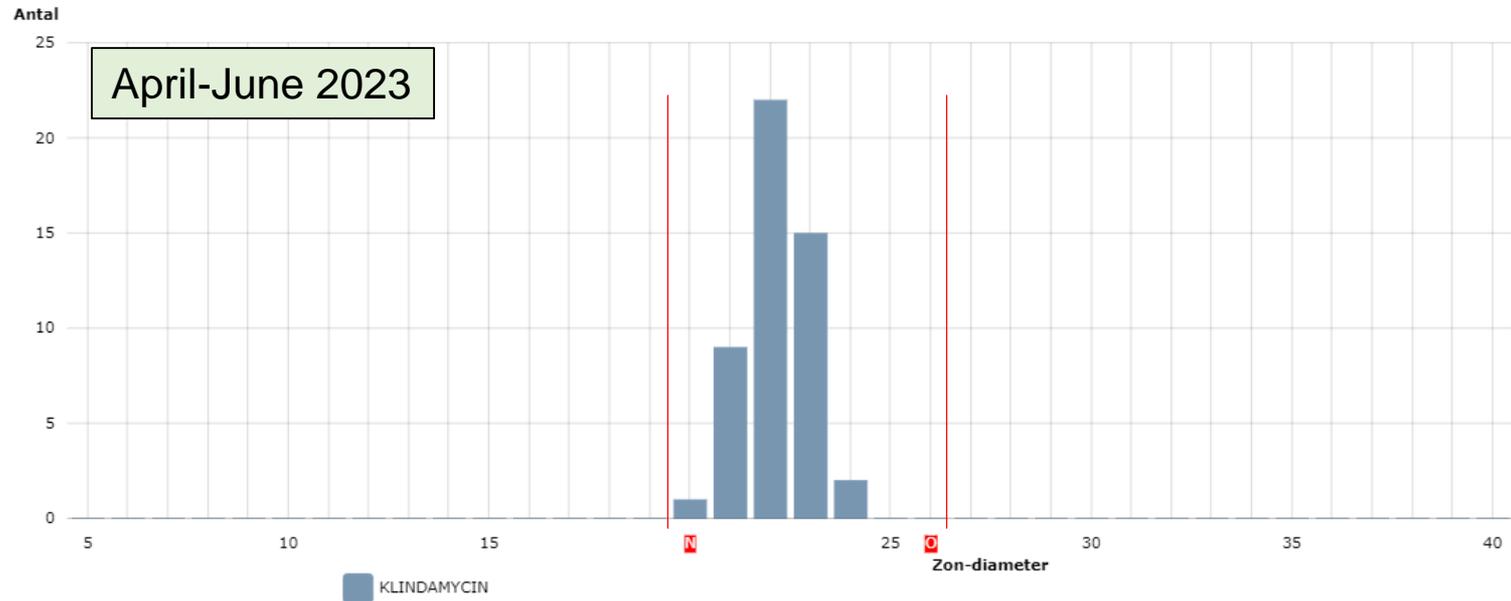
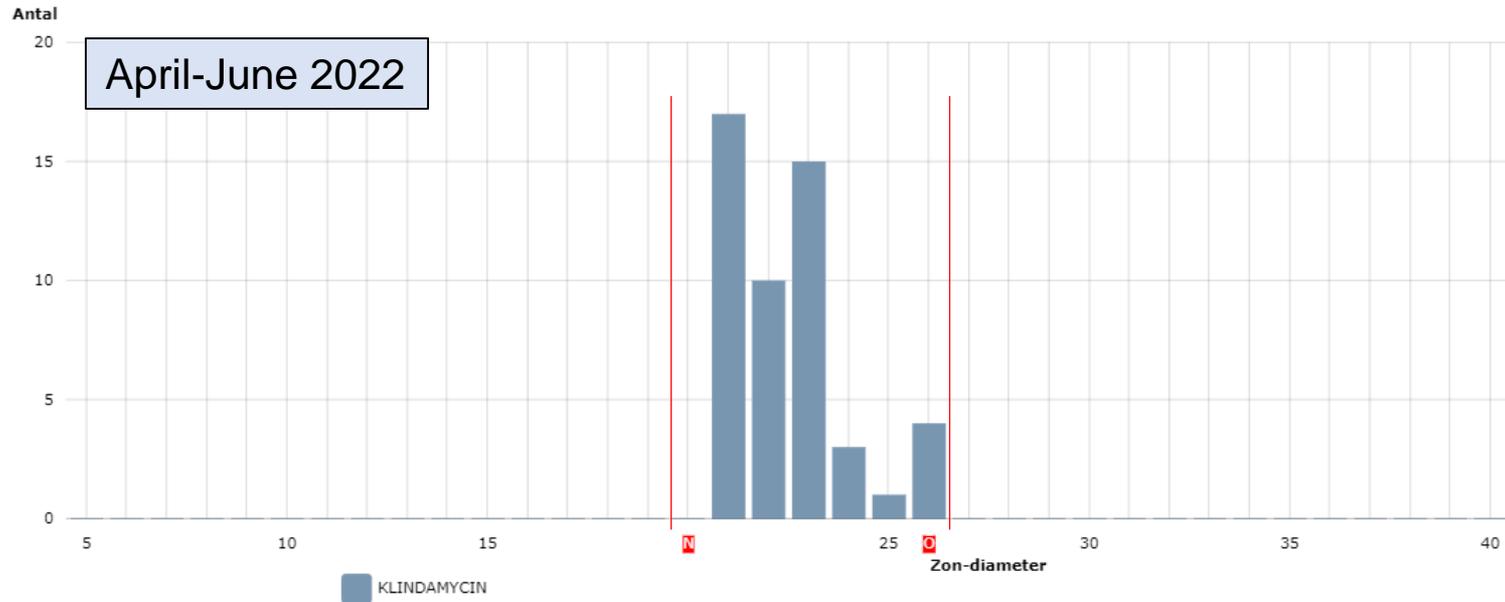
METRONIDAZOL



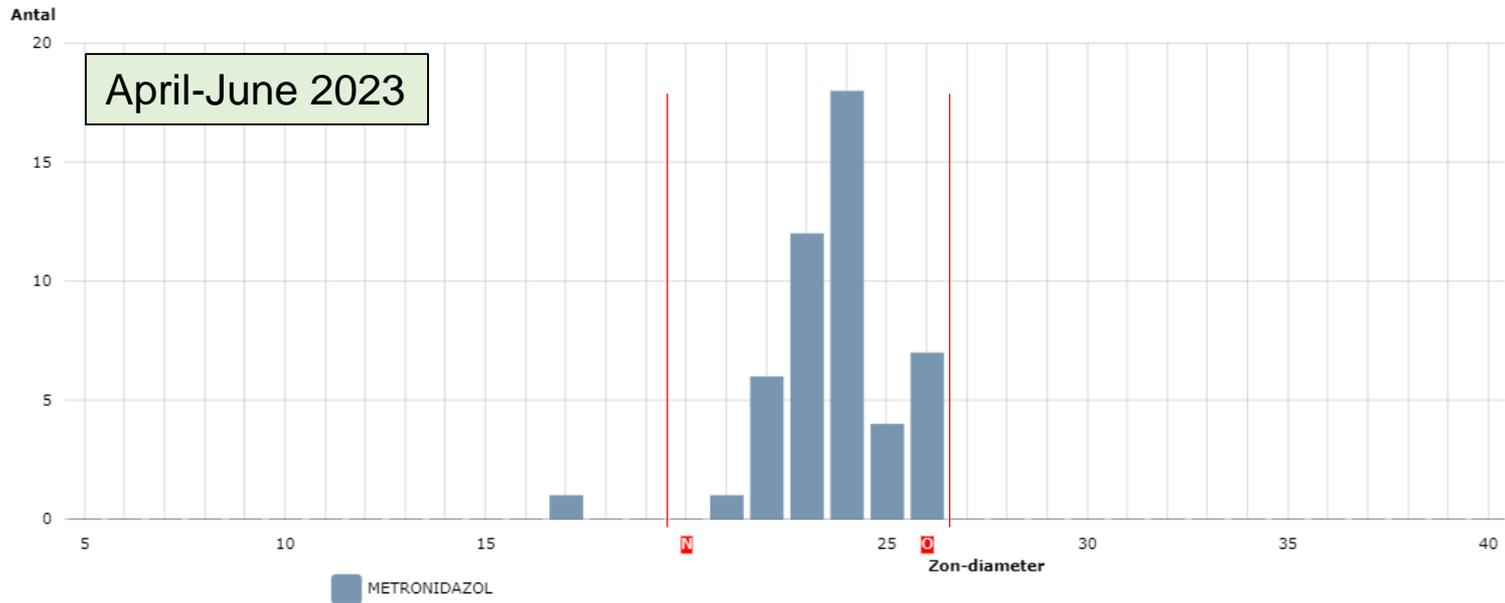
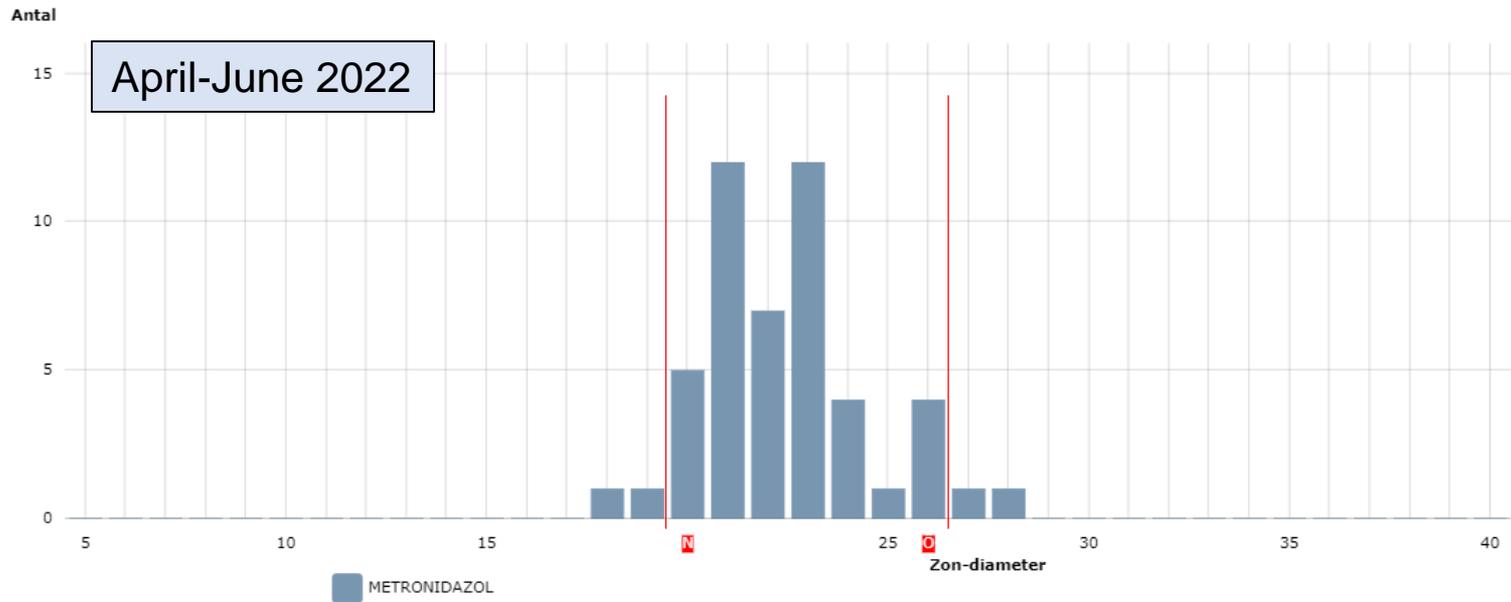
C. perfringens ATCC 13124 and piperacillin-tazobactam 30-6 µg



C. perfringens ATCC 13124 and clindamycin 2 µg



C. perfringens ATCC 13124 and metronidazole 5 µg

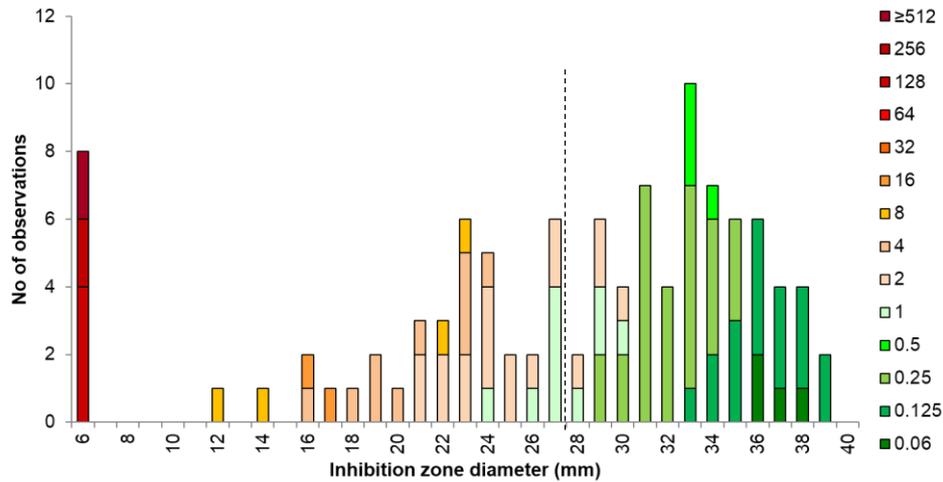
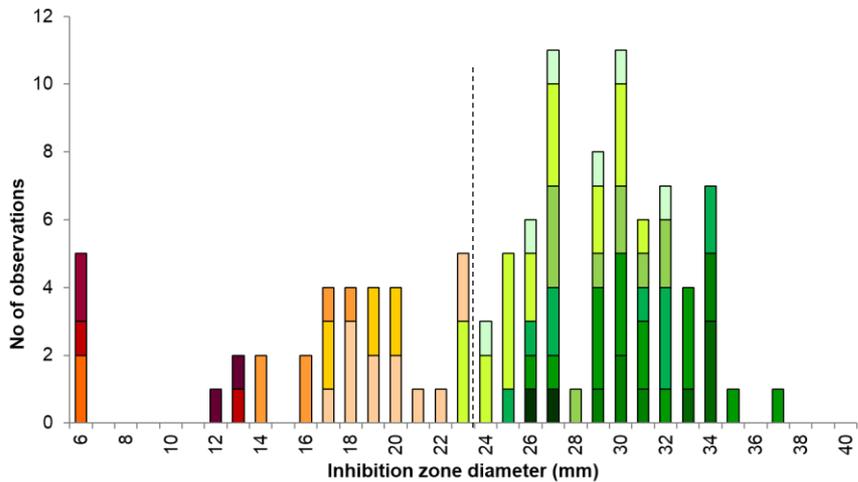


Bacteroides spp.

Piperacillin-tazobactam

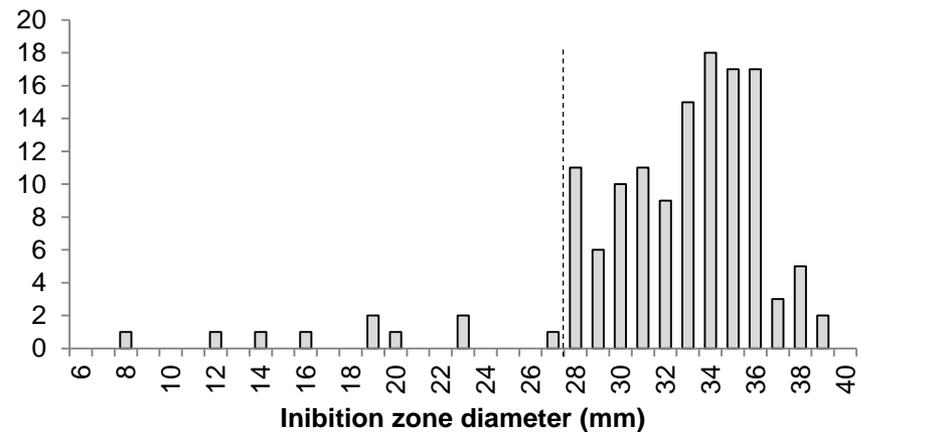
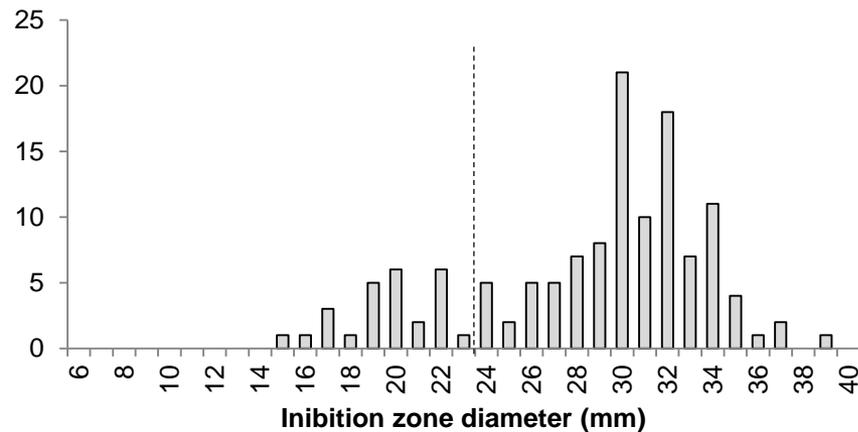


Meropenem



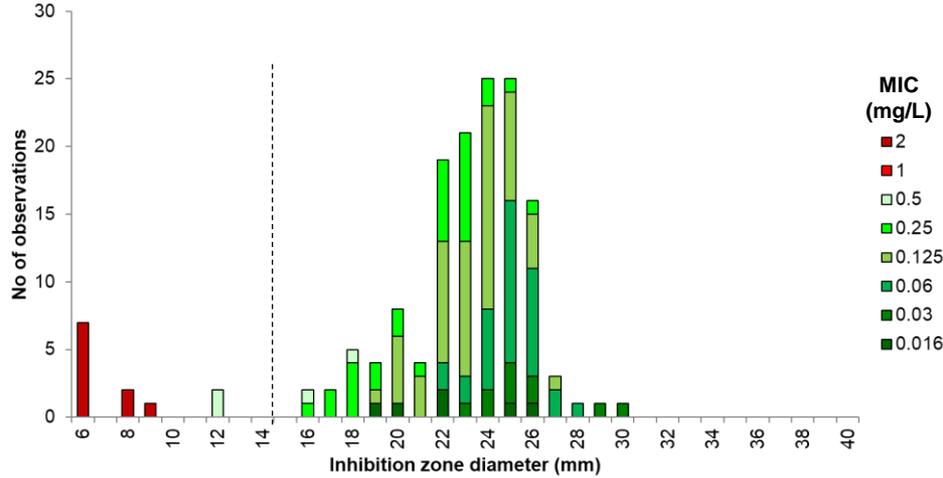
Piperacillin-tazobactam

Meropenem

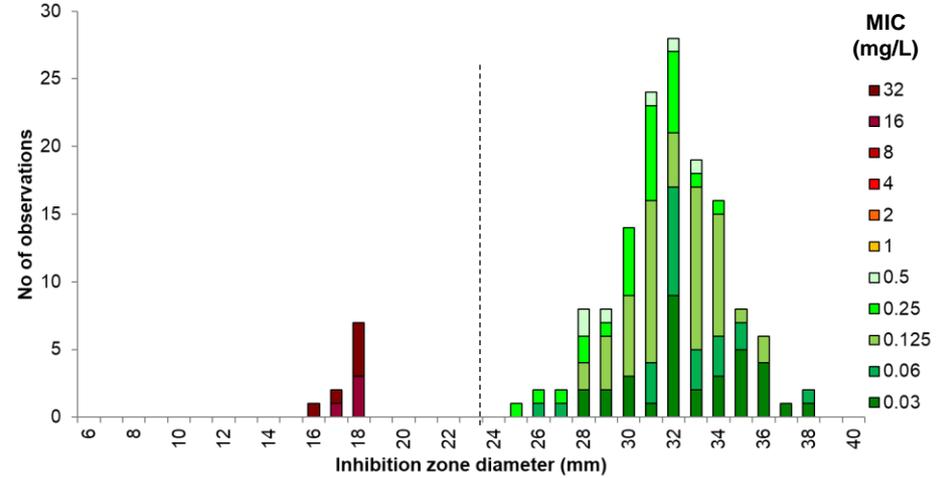


Clostridium perfringens

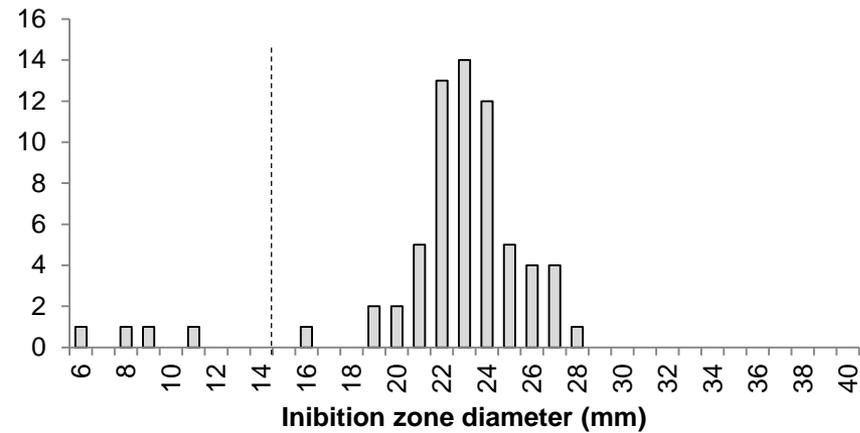
Benzylpenicillin



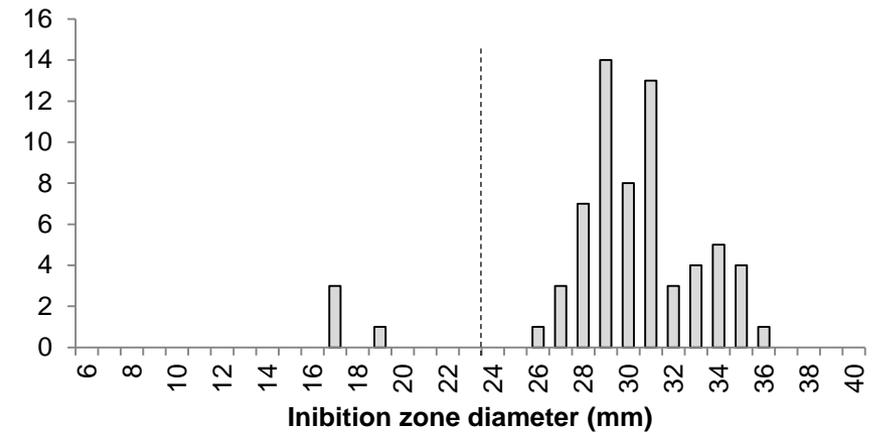
Piperacillin-tazobactam



Benzylpenicillin

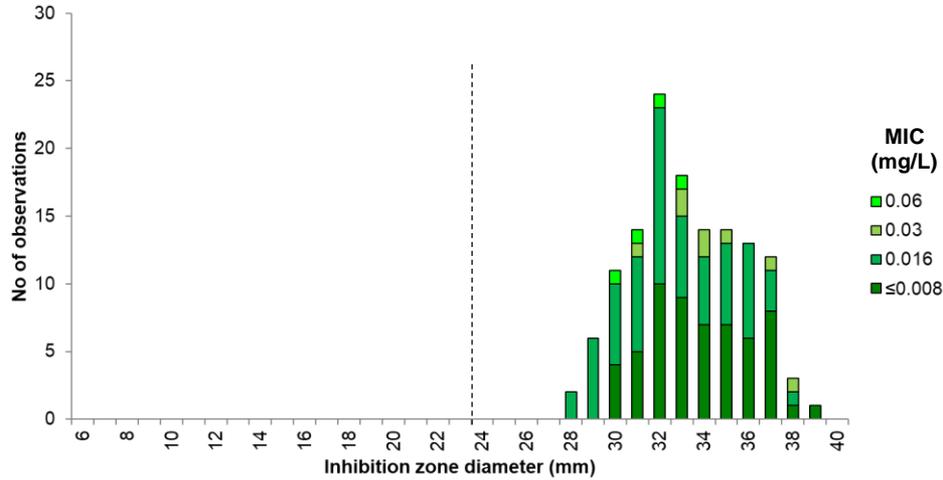


Piperacillin-tazobactam

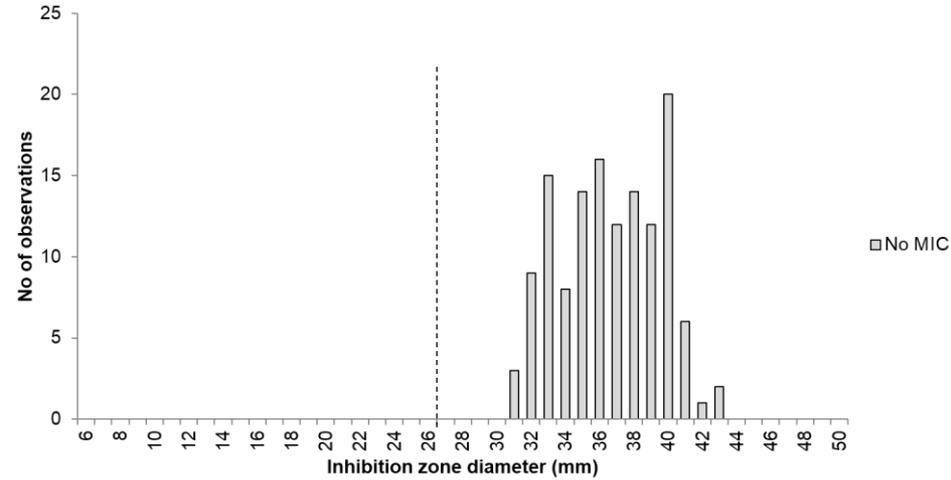


Cutibacterium acnes

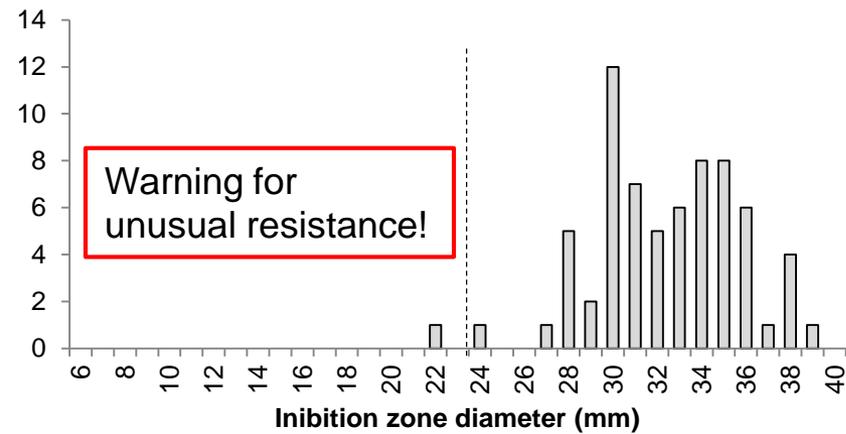
Benzylpenicillin



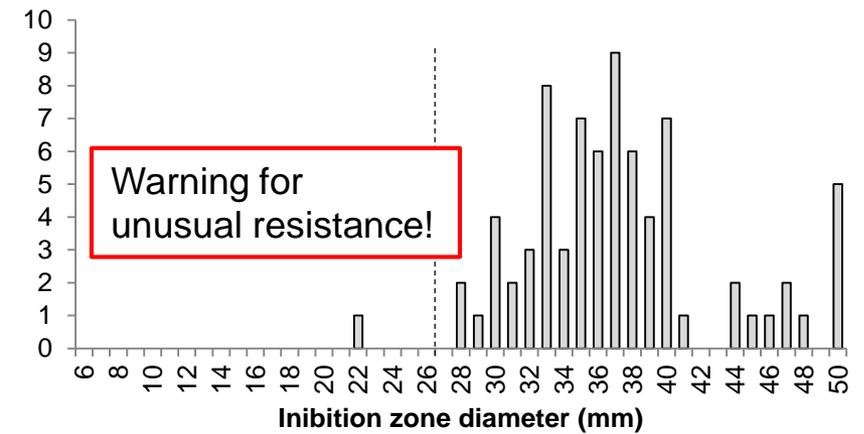
Piperacillin-tazobactam



Benzylpenicillin



Piperacillin-tazobactam



Implementation of disk diffusion of anaerobes - Lessons learned

- Problems with the anaerobic workstation lead to QC results out of range (especially for metronidazole)
 - Important to control the anaerobic environment!
- Important to dry plates before inoculation
- Important to follow the reading instructions
 - Repeated reading exercises lead to harmonisation in reading between staff and less QC results out of range
- Review QC data and distributions for clinical isolates after implementation
- Local warnings (e.g. ATUs) can be used to warn for unusual resistance if there is a tendency to reading smaller zones

Ongoing review of QC criteria

- Validation study ongoing
 - EDL
 - FAA-HB from 3 manufacturers
 - Anaerobic work station, Anoxomat, gas-generating envelopes
 - Validation at external laboratories
- Tendency towards zones in the lower part of the range or below the lower limit for some strain-agent combinations.
- All QC ranges for the anaerobic QC strains will be reviewed for the next version of the EUCAST QC Tables (January 2025).

Species without breakpoints

- Is AST needed?

Species without breakpoints

- Determine the MIC (agar dilution or according to manufacturer's instructions for commercial tests)
 - Check the product insert for gradient tests!
 - Which species are the product validated for?
 - Which medium is the product validated for?
- Consult the EUCAST Guidance document on when there are no breakpoints (updated 29 February 2024):
<https://www.eucast.org/eucastguidancedocuments>

Table 2. Antimicrobial agents relevant for the treatment of anaerobic bacteria with guidance for bacteria lacking breakpoints in standard EUCAST breakpoint tables.

Agents and notes for anaerobic bacteria	MIC-values above which therapy with the agent should be discouraged	
Benzyloenicillin	0.5	Breakpoints for anaerobic bacteria in the breakpoint table are 0.06 – 0.5 mg/L. If a beta-lactamase is detected, report resistant without further testing.
Amoxicillin	0.5	Breakpoints for anaerobic bacteria in the breakpoint table are 0.25 – 0.5 mg/L. If a beta-lactamase is detected, report resistant without further testing.
Amoxicillin-clavulanic acid	0.5	Breakpoints for anaerobic bacteria in the breakpoint table are 0.25 – 0.5 mg/L.
Ampicillin-sulbactam	0.5	Breakpoints for anaerobic bacteria in the breakpoint table are 0.25 – 0.5 mg/L.
Piperacillin-tazobactam	2	Breakpoints for anaerobic bacteria in the breakpoint table are 0.5 – 2 mg/L.
Meropenem	1	Breakpoints for anaerobic bacteria in the breakpoint table are 0.03 – 1 mg/L.
Imipenem	1	Breakpoints for anaerobic bacteria in the breakpoint table are 0.03 – 1 mg/L.
Ertapenem	0.25	Breakpoints for anaerobic bacteria in the breakpoint table are 0.06 – 0.5 mg/L.
Clindamycin	0.5	Breakpoints for anaerobic bacteria in the breakpoint table are 0.25 mg/L.
Metronidazole	4	Breakpoints for anaerobic bacteria in the breakpoint table are 0.5 - 4 mg/L.
Vancomycin (Gram-positive)	2	Only relevant for a few gram-positive anaerobic bacteria. A breakpoint of 2 mg/L is common for targeted species.
Rifampicin (Gram-positive)	0.125	Breakpoints for species already in the EUCAST breakpoint tables are 0.06 – 0.125 mg/L.
Linezolid (mixed infections)	Pending	Linezolid has been used in the treatment of mixed infections where anaerobic bacteria were considered causative, but rarely for targeted therapy of anaerobic infections.
Moxifloxacin (mixed infections)	Pending	Moxifloxacin has been used in the treatment of mixed infections where anaerobic bacteria were considered causative, but rarely for targeted therapy of anaerobic infections.

Species without breakpoints

- The proposed values are based on:
 - A compromise between current EUCAST susceptible (S or I) breakpoints for anaerobic species
 - Wild-type distributions for microorganisms when available
 - PK/PD cut-off values
- If the MIC is above the value, advise against the use of the agent for treatment (alternatively “report as resistant”).
- If the MIC is equal to or below the value, interpret with caution and avoid categorical reporting.

Species without breakpoints

- Comment used in our routine laboratory
 - Anaerobic bacteria can, if resistance is not present, be treated with benzylpenicillin, piperacillin-tazobactam, clindamycin, meropenem and metronidazole.
 - Breakpoints are lacking for some species and full susceptibility testing results can therefore not be given.
 - Isolates will be reported as resistant to a given agent when the laboratory has identified resistance to the agent in question.

Development of breakpoints and disk diffusion criteria for additional species

- *Clostridium ramosum*
- *Clostridium innocuum*
- *Clostridium tertium*
- *Clostridium septicum*
- *Cutibacterium avidum*
- *Fusobacterium nucleatum*
- *Finegoldia magna*
- *Parvimonas micra*
- *Peptostreptococcus anaerobius*
- *Peptoniphilus* spp.

Problems or questions?

Please contact us!

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ON ANTIMICROBIAL
SUSCEPTIBILITY TESTING

European Society of Clinical Microbiology and Infectious Diseases