



EUCAST disk diffusion method for anaerobic bacteria

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The development of a disk diffusion method for anaerobes

1. Investigation of parameters affecting the result*
2. MIC-zone diameter correlations for five organisms /organism groups (agar dilution MICs as reference)
 - Media from different manufacturers (in house and commercial), different systems for generating an anaerobic environment
3. Field trial at 16 laboratories
 - Phase 1: Disk diffusion of a common collection of 35 isolates with known MICs
 - Phase 2: Disk diffusion of local isolates

* Bavelaar et al. Clin Microbiol Infect. 2021 Nov;27(11):1695.e1-1695.e6.

Species and antimicrobial agents

- 5 commonly isolated anaerobic and rapidly growing bacteria
 - *Bacteroides* spp. (including *Parabacteroides*)
 - *Prevotella* spp.
 - *Fusobacterium necrophorum*
 - *Clostridium perfringens*
 - *Cutibacterium acnes*
- Clinically relevant antimicrobial agents
 - Benzylpenicillin 1 unit
 - Piperacillin-tazobactam 30-6 μg
 - Meropenem 10 μg
 - Vancomycin 5 μg
 - Clindamycin 2 μg
 - Metronidazole 5 μg

Disk diffusion methodology

- **Medium:** Fastidious Anaerobe Agar with 5% mechanically defibrinated horse blood (FAA-HB)
 - Agar depth 4.0 ± 0.5 mm
- **Inoculum:** McF 1.0 (overnight culture from non-selective media)
- **Incubation:** Anaerobic environment
35-37°C for 16-20 h

FAA-HB media

- Dry FAA-HB plates prior to inoculation.
 - Excess humidity may result in fuzzy zone edges, swarming and/or haze within zones.
- Commercial FAA-HB plates should be dried in two steps:
 - Room temperature overnight, followed by 15 minutes at 35°C without lids
- In-house prepared plates are usually less humid and zones are easier to read.

Streaking of plates and application of disks

- For *Bacteroides* spp., remove excess fluid by turning the swab against the inside of the tube to avoid over-inoculation.
- Spread the inoculum evenly over the entire agar surface, ensuring that there are no gaps between streaks.
 - This is particularly important for *Cutibacterium acnes*, which grows with small colonies and poor contrast to the FAA-HB media.
- Limit the number of disks on each plate to allow good growth and to avoid overlapping of zones.

Incubation atmosphere and time

- The anaerobic atmosphere (irrespective of how it is created) should be checked regularly.
 - Note that the quality of the anaerobic atmosphere might change over time (especially workstations)
- Breakpoints and QC criteria are only valid for 16-20 h incubation.
 - Prolonged incubation is not allowed since this affect zone sizes significantly.

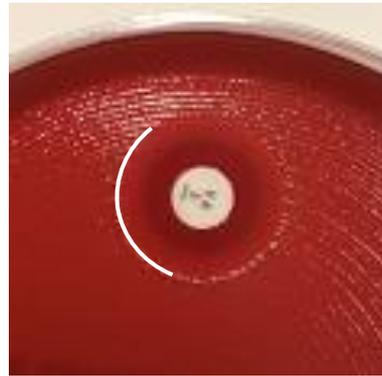
Reading of zones

General instructions

- Read FAA-HB plates from the front with the lid removed illuminated with reflected light.
- Hold the plate about 30 cm from the eye at a 45-degree angle to the work bench when reading.
- Measure zone diameters with a calliper or ruler at the point of complete inhibition as judged by the naked eye.

Reading of zones

Specific instructions



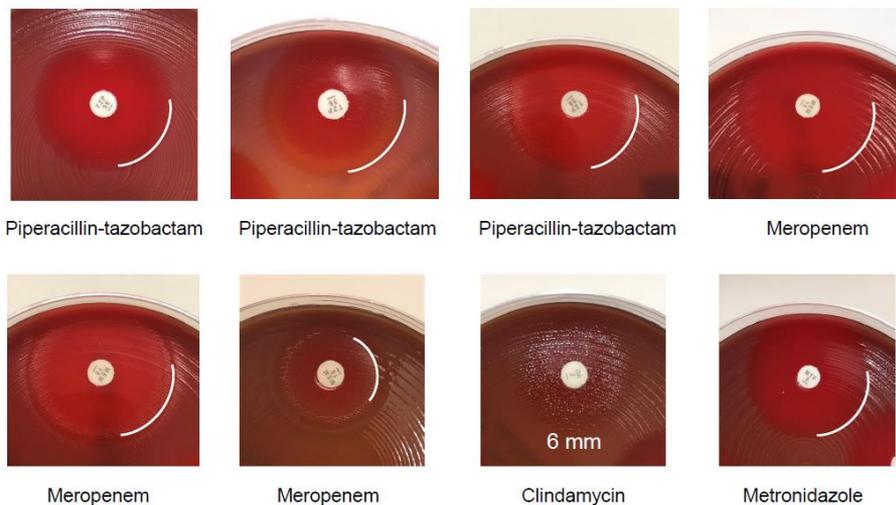
- Ignore any faint haze within the inhibition zone and read the most obvious zone. **Tilt the plate towards you to better define the obvious zone edge.**
- Ignore haemolysis and swarming and read inhibition of growth.
- Isolated colonies within the inhibition zone should be taken into account. **For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

Reading guide

EUCAST disk diffusion for selected rapidly growing anaerobic bacteria on Fastidious Anaerobe Agar (FAA)

Version 1.0
September 2021

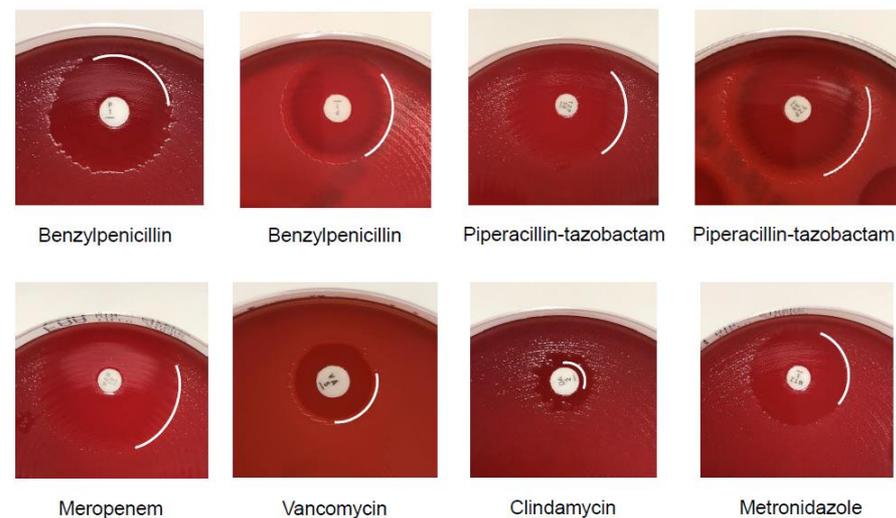
Bacteroides spp.



Fusobacterium necrophorum



Clostridium perfringens



AST of bacteria

- Organization
- Consultations
- EUCAST News
- New definitions of S, I and R
- Clinical breakpoints and dosing
- Rapid AST in blood cultures
- Expert rules and intrinsic resistance
- Resistance mechanisms
- Guidance documents
- SOP
- MIC and zone distributions and ECOFFs
- AST of bacteria**
 - 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



... Disk diffusion methodology 

EUCAST Disk Diffusion Test Methodology

The EUCAST disk diffusion test is based on MH media and disks of a good quality. It is calibrated to EUCAST clinical breakpoints using broth microdilution for MIC determination. Updates are published regularly.

See also [EUCAST instruction videos](#).

[Disk diffusion - Manual v 9.0](#) (1 January, 2021)

[Disk diffusion - Slide show v 9.0](#) (1 January, 2021)

[Disk diffusion - Reading guide v 8.0](#) (1 January, 2021)

EUCAST disk diffusion of anaerobic bacteria is under development 2021. Reviewed clinical breakpoints and disk diffusion correlates will be published with breakpoint table v 12.0 (1 January, 2022). The method will be valid for 5 species (*Bacteroides* spp, *Prevotella* spp, *Fusobacterium necrophorum*, *Clostridium perfringens* and *Cutibacterium acnes*) and for anaerobic incubation for 16 - 20h (extended incubation not allowed). For anyone who wants to prepare and practice, EUCAST already now publish the methodology, reading guide and QC criteria.

- [Disk diffusion and QC criteria for anaerobic bacteria - Manual v 1.0](#) (20 September, 2021)
- [Disk diffusion of anaerobic bacteria - Reading guide v 1.0](#) (20 September, 2021)

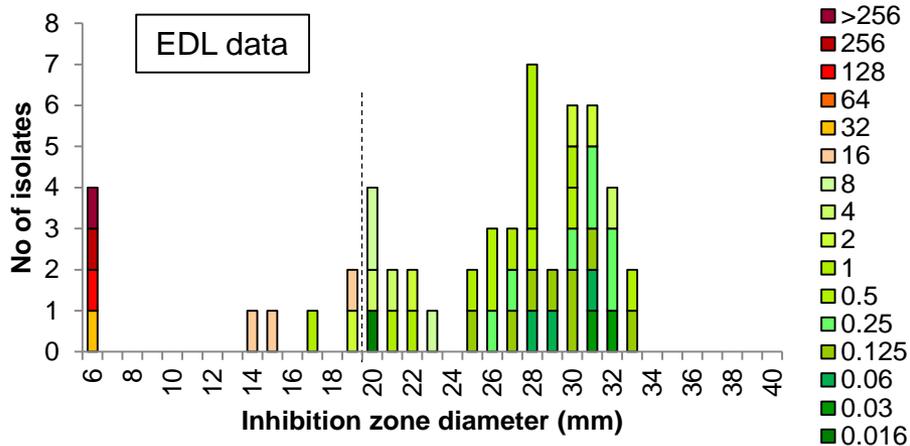
For translations to other languages - see [Translations](#).

For previous versions of documents - see [Previous versions](#).

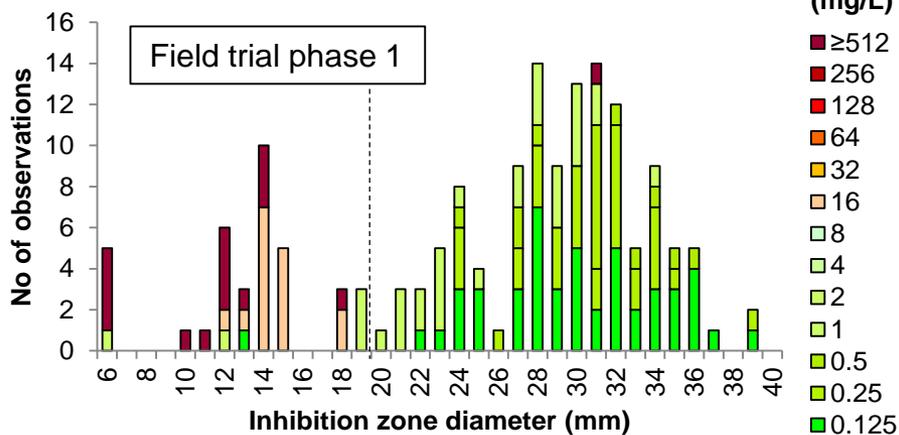
***Bacteroides* spp. (except *B. thetaiotaomicron*)**

Piperacillin-tazobactam 30-6 µg

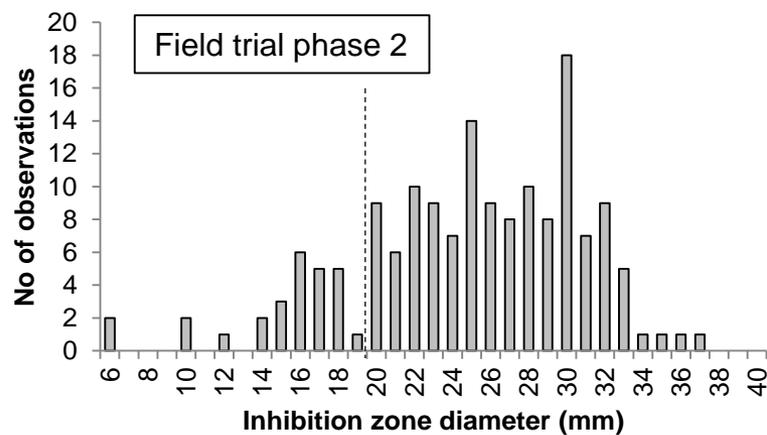
Piperacillin-tazobactam 30-6 µg vs. FAA-HB MIC
***Bacteroides* spp., 53 clinical isolates**



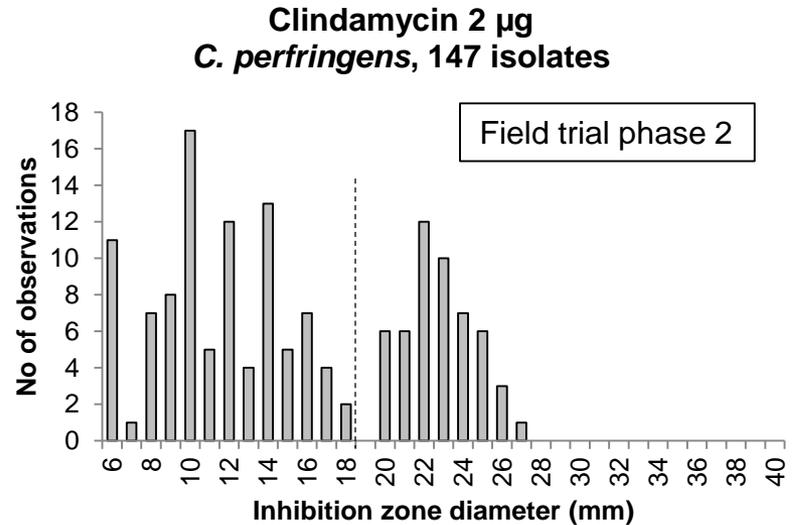
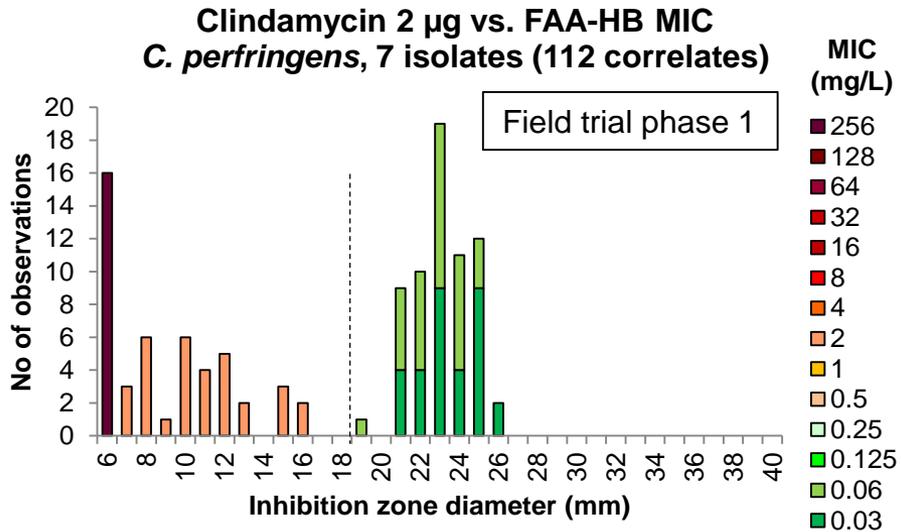
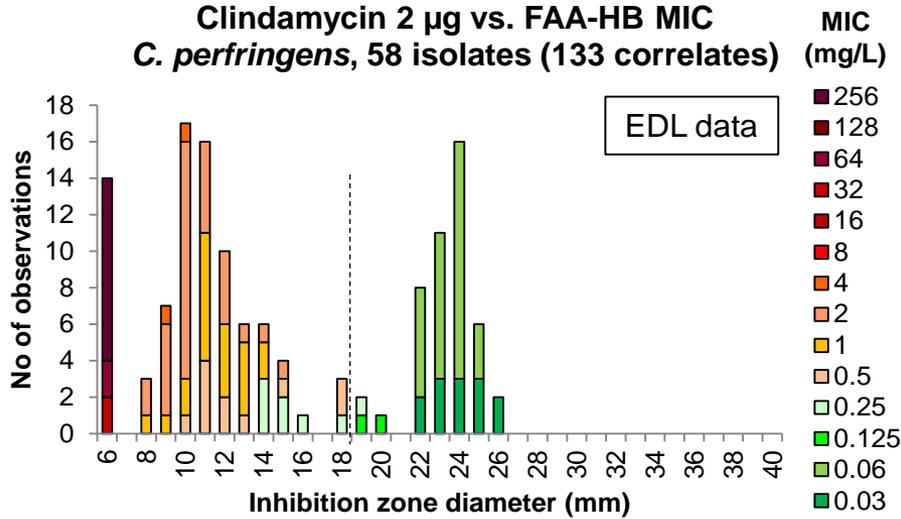
Piperacillin-tazobactam 30-6 µg vs. FAA-HB MIC
***Bacteroides* spp., 10 isolates (160 correlates)**



Piperacillin-tazobactam 30-6 µg
***Bacteroides* spp., 160 isolates**

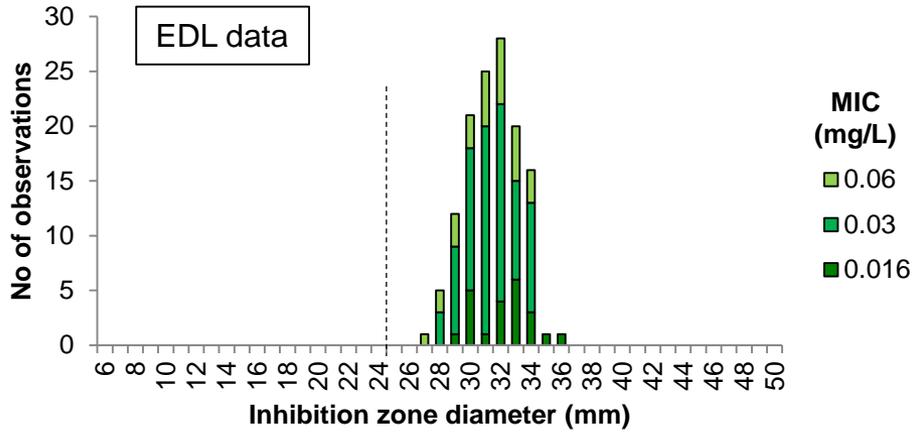


C. perfringens Clindamycin 2 µg

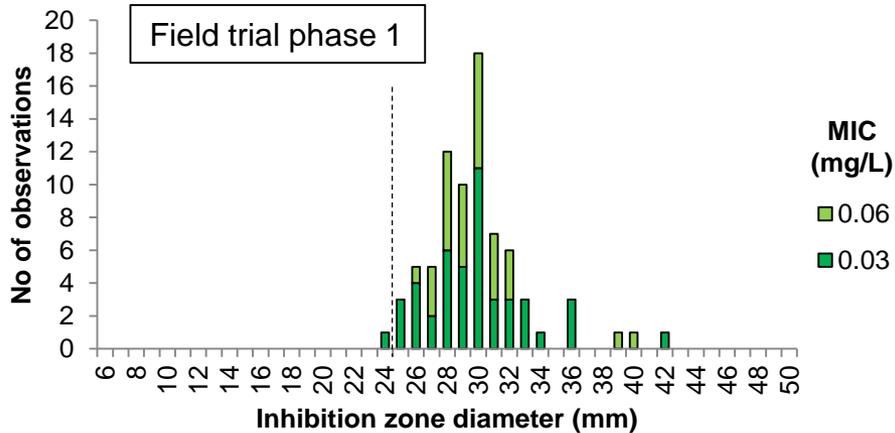


F. necrophorum Benzylpenicillin 1 unit

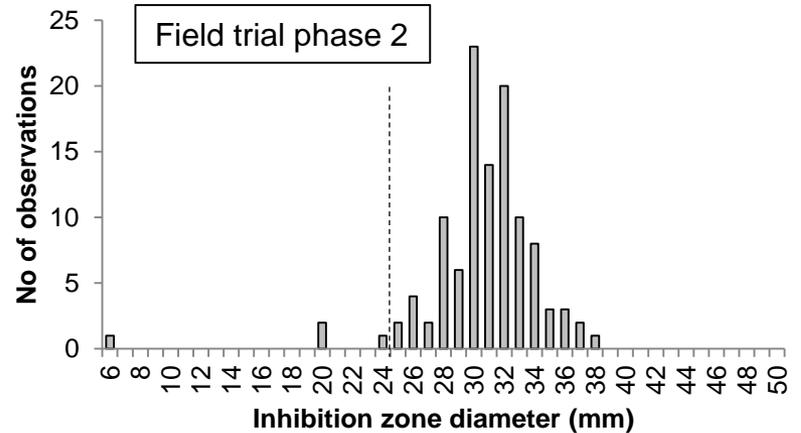
Benzylpenicillin 1 unit vs. FAA-HB MIC
F. necrophorum, 51 isolates (130 correlates)



Benzylpenicillin 1 unit vs. FAA-HB MIC
F. necrophorum, 5 isolates (77 correlates)



Benzylpenicillin 1 unit
F. necrophorum, 112 isolates

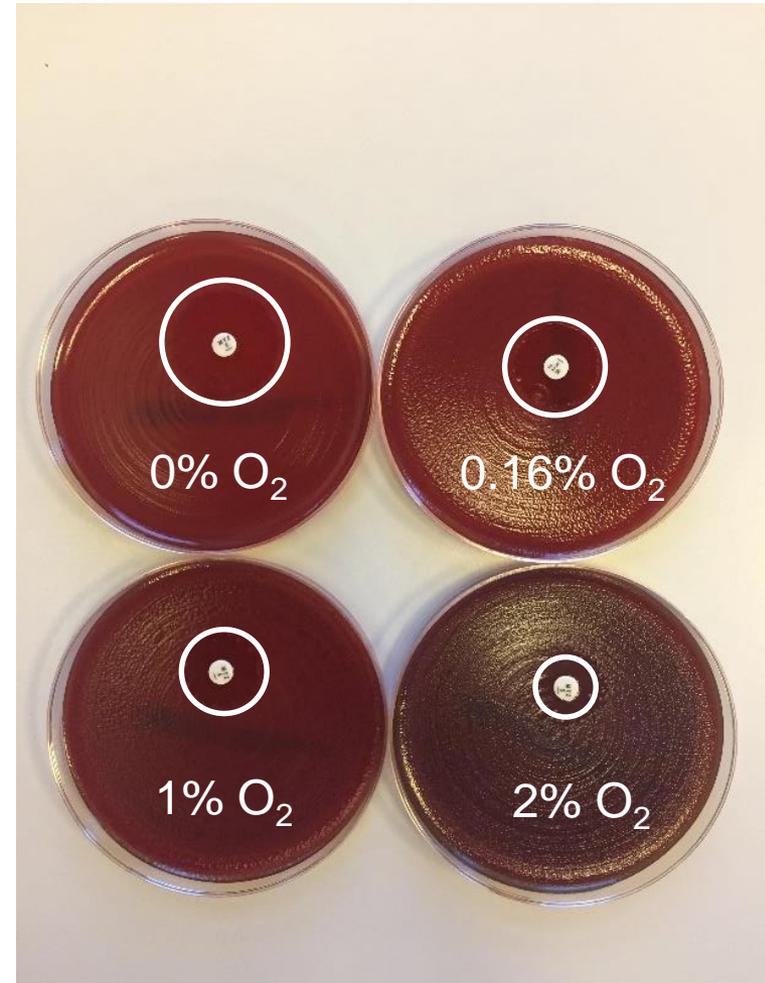


Quality control

- To monitor the anaerobic atmosphere:
 - *C. perfringens* DSM 25589 with metronidazole 5 µg

Monitoring the anaerobic atmosphere

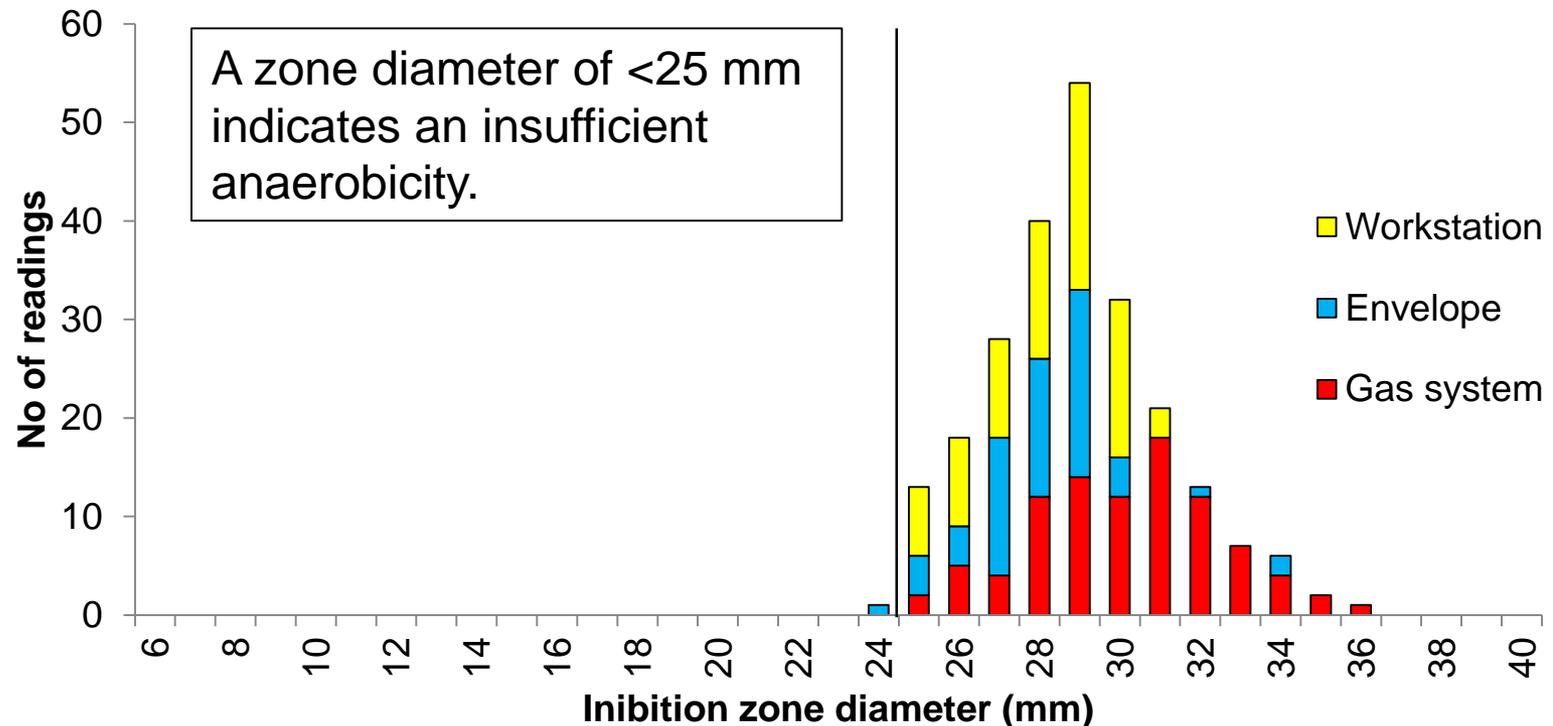
- DSM 25589 / CCUG 75076
- Aerotolerant *Clostridium perfringens*
- Will grow at 4% oxygen and even higher
- Decrease in the metronidazole 5 μg zone diameter from 30 to 22 mm at 0.16% of oxygen (extremely sensitive).



Courtesy of Ulrik Stenz Justesen
Odense University Hospital, Denmark

Monitoring the anaerobic atmosphere: Reproducibility

Metronidazole 5 μ g vs. incubation method
C. perfringens DSM 25589 (n=236)



Quality control

- To monitor the anaerobic atmosphere:
 - *C. perfringens* DSM 25589 with metronidazole 5 µg
- To monitor test performance:
 - *B. fragilis* ATCC 25285
 - *C. perfringens* ATCC 13124
 - Test frequency:
 - At each test occasion or at least 4 times per week.

Tentative QC criteria

Bacteroides fragilis ATCC 25285

(NCTC 9343, DSM 2151, CCUG 4856T)

Antimicrobial agent	Disk content (µg)	Inhibition zone diameter (mm)	
		Target ¹	Range ¹
Piperacillin-tazobactam	30-6	32	29-35
Meropenem	10	35-36	32-39
Clindamycin	2	26	23-29
Metronidazole	5	32-33	29-36

Clostridium perfringens ATCC 13124

(NCTC 8237, CIP 103409, DSM 756, CCUG 1795T, CECT 376 T)

Antimicrobial agent	Disk content (µg)	Inhibition zone diameter (mm)	
		Target ¹	Range ¹
Benzylpenicillin	1 unit	26	23-29
Piperacillin-tazobactam	30-6	33	30-36
Meropenem	10	37	34-40
Vancomycin	5	17	14-20
Clindamycin	2	23	20-26
Metronidazole	5	23	20-26

Range

Set to allow for random variation

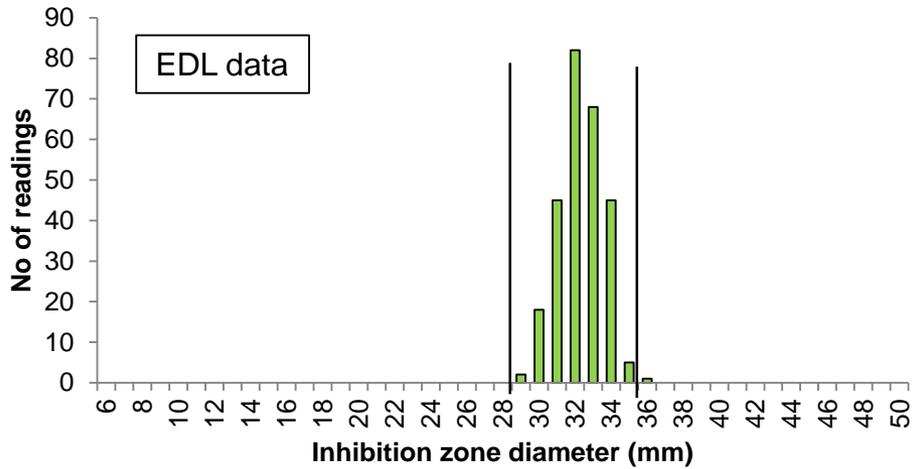
Target

Mean values from repeated measurements should optimally be on target \pm 1 mm

B. fragilis ATCC 25285

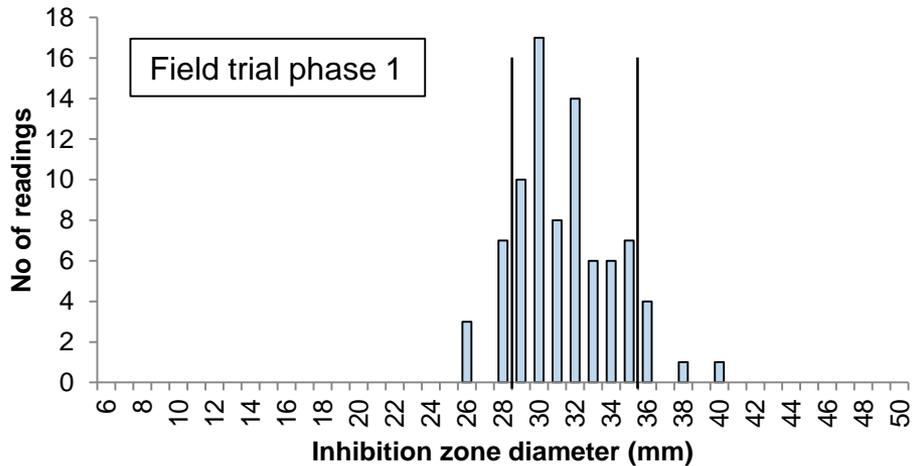
Piperacillin-tazobactam 30-6 μg

B. fragilis ATCC 25285 (n=266)

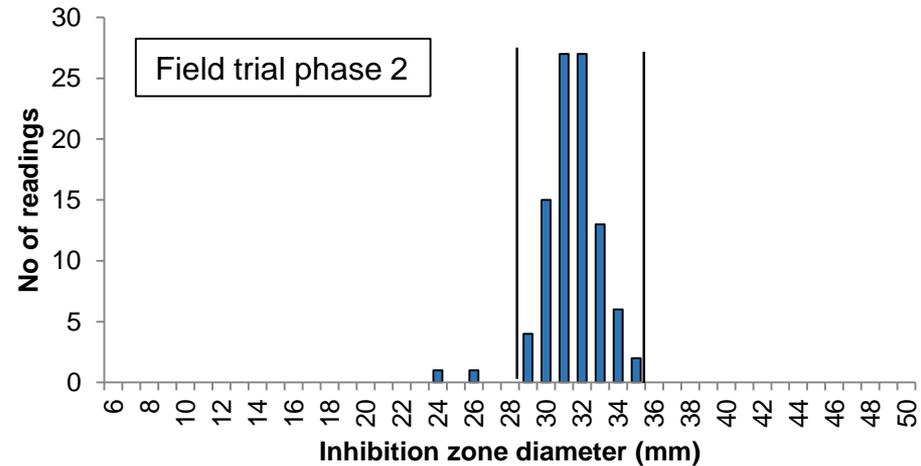


QC criteria:
Range 29-35 mm
Target 32 mm

B. fragilis ATCC 25285 (n=84)



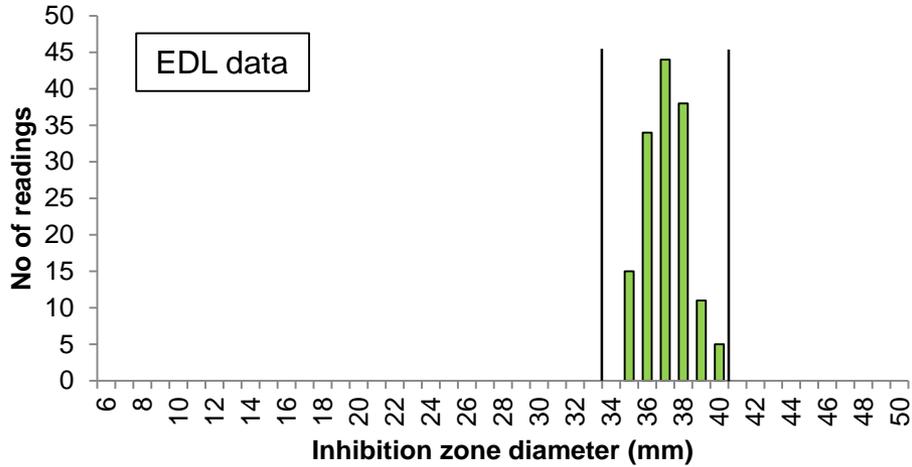
B. fragilis ATCC 25285 (n=96)



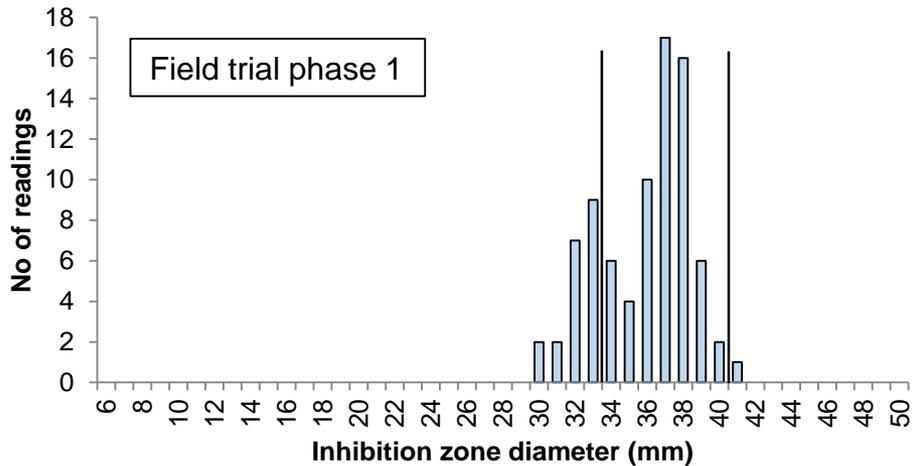
C. perfringens ATCC 13124

Meropenem 10 µg

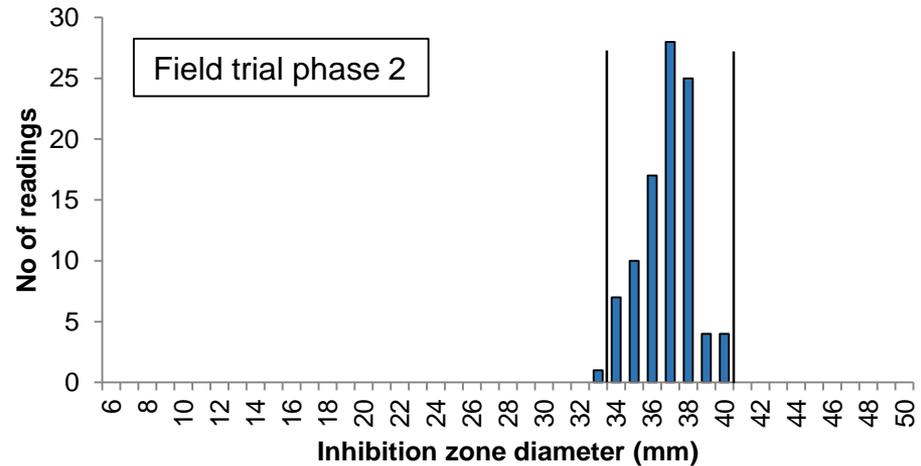
C. perfringens ATCC 13124 (n=147)



C. perfringens ATCC 13124 (n=82)



C. perfringens ATCC 13124 (n=96)



Implementation of disk diffusion of anaerobic bacteria

- Education of “champion”
 - EUCAST documents and webinar
 - Training with QC strains
 - Consult EDL if needed
- Education of staff
 - Preparation, storage and handling of media
 - Inoculation
 - Reading of zones
 - EUCAST Reading Guide
 - Reading exercises with feedback

https://www.eucast.org/videos_and_online_seminars/online_seminars

Implementation of disk diffusion of anaerobic bacteria

- Implementation
 - QC strains and clinical isolates
 - Changed routines in laboratory
 - Storage and drying of media
 - Application of disks (dispensers?)
 - Routine QC
 - New routines for other species of anaerobic bacteria
- Follow up (4-6 months after implementation)
 - Review routine QC data and distributions for clinical isolates
 - Consult EDL if needed

Questions and/or comments?

Please contact erika.matuschek@eucast.org



EUCAST

EUROPEAN COMMITTEE
ON ANTIMICROBIAL
SUSCEPTIBILITY TESTING

European Society of Clinical Microbiology and Infectious Diseases