

# Susceptibility testing of anaerobic bacteria

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Odense, Denmark



# Why?

## From 2000 to 2013

Rising resistance patterns were detected in:

- *Bacteroides* and *Parabacteroides* spp. to cefoxitin/cefotetan,  $\beta$ -lactam/  $\beta$ -lactamase inhibitor combinations and clindamycin,
- in non-difficile *Clostridium* spp. to moxifloxacin,
- in *Prevotella* spp. to penicillins.

Resistance is changing but in general increasing = unpredictable

**Even more serious: reports of carbapenem and metronidazole resistance in the *B. fragilis* group**



Recent evolution of antibiotic resistance in the anaerobes as compared to previous decades



Lyudmila Boyanova <sup>A,\*</sup>, Rossen Kolarov <sup>B</sup>, Ivan Mitov <sup>B</sup>

<sup>A</sup> Department of Medical Microbiology, Medical University of Sofia, Zhene Street 2, 1431 Sofia, Bulgaria  
<sup>B</sup> University Hospital of Maxillofacial Surgery, Sofia, Bulgaria

# How? The phenotypic methods

- **Agar dilution**
- **Broth (micro) dilution**
- **Gradient strips**
- **(Disk diffusion)**

# EUCAST MIC breakpoints

## Gram-positive anaerobes except *Clostridium difficile*

EUCAST Clinical Breakpoint Tables v. 8.0, valid from 2018-01-01

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This group of bacteria includes many genera. The most frequently isolated Gram-positive anaerobes are: *Clostridium*, *Actinomyces*, *Propionibacterium*, *Bifidobacterium*, *Eggerthella*, *Eubacterium*, *Lactobacillus* and anaerobic Gram-positive cocci. Anaerobes are most frequently defined by no growth on culture plates incubated in a CO<sub>2</sub> enriched atmosphere, but many Gram-positive, non-spore forming rods such as *Actinomyces* spp., many *P. acnes* and some *Bifidobacterium* spp. can grow on incubation in CO<sub>2</sub> and may be tolerant enough to grow poorly in air, but are still considered as anaerobic bacteria. Several species of *Clostridium*, including *C. carnis*, *C. histolyticum* and *C. tertium*, can grow but not sporulate in air. For all these species, susceptibility testing should be performed in anaerobic environment.

| Penicillins                                | MIC breakpoint (mg/L) |                 |
|--|-----------------------|-----------------|
|  | S ≤                   | R >             |
| <i>Benzylpenicillin</i> <sup>1</sup>       | 0.25                  | 0.5             |
| <i>Ampicillin</i> <sup>2</sup>             | 4                     | 8               |
| <i>Ampicillin-sulbactam</i>                | 4 <sup>2</sup>        | 8 <sup>2</sup>  |
| <i>Amoxicillin</i> <sup>1</sup>            | 4                     | 8               |
| <i>Amoxicillin-clavulanic acid</i>         | 4 <sup>2</sup>        | 8 <sup>2</sup>  |
| <i>Piperacillin</i> <sup>3</sup>           | 3                     | 18              |
| <i>Piperacillin-tazobactam</i>             | 3 <sup>2</sup>        | 18 <sup>2</sup> |
| <i>Ticarcillin</i> <sup>1</sup>            | 3                     | 18              |
| <i>Ticarcillin-clavulanic acid</i>         | 3 <sup>2</sup>        | 18 <sup>2</sup> |
| <i>Tazobactam</i>                          | --                    | --              |
| <i>Phenoxymethylpenicillin</i>             | 16                    | 8               |
| <i>Oxacillin</i>                           | --                    | --              |
| <i>Cloxacillin</i>                         | --                    | --              |
| <i>Dicloxacillin</i>                       | --                    | --              |
| <i>Fluckloxacillin</i>                     | --                    | --              |
| <i>Mecillinam</i> (uncomplicated UTI only) | --                    | --              |

Notes  
Numbered notes relate to general comments and/or MIC breakpoints.

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## Gram-negative anaerobes

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| <i>Ampicillin-sulbactam</i>                | 0.5 <sup>2</sup>      | --              |
| <i>Amoxicillin</i> <sup>1</sup>            | 0.5                   | --              |
| <i>Amoxicillin-clavulanic acid</i>         | 0.5 <sup>2</sup>      | --              |
| <i>Piperacillin</i> <sup>3</sup>           | 0.7                   | 10 <sup>2</sup> |
| <i>Piperacillin-tazobactam</i>             | 0.7 <sup>2</sup>      | 10 <sup>2</sup> |
| <i>Ticarcillin</i> <sup>1</sup>            | 1.8                   | 18              |
| <i>Ticarcillin-clavulanic acid</i>         | 0.7 <sup>2</sup>      | 10 <sup>2</sup> |
| <i>Tazobactam</i>                          | --                    | --              |
| <i>Phenoxymethylpenicillin</i>             | 8                     | 8               |
| <i>Oxacillin</i>                           | --                    | --              |
| <i>Cloxacillin</i>                         | --                    | --              |
| <i>Dicloxacillin</i>                       | --                    | --              |
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# Agar dilution (gold standard)



CLSI M11-A8

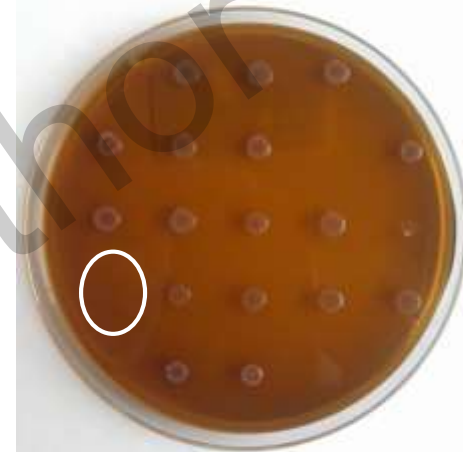
Metronidazole susceptibility testing of *Bacteroides fragilis* group isolates



0.125 mg/L



0.25 mg/L



0.50 mg/L

# Broth (micro) dilution

Commercial assays are available, however ...

... a caveat in the CLSI guideline:

*“until further studies are performed to validate this procedure for testing other organisms, it should be used only for testing members of the B. fragilis group.”*



CLSI M11-A8

# Gradient strips

**Expensive**

**Problems with performance  
and warnings concerning  
specific agents**



*Bacteroides fragilis*  
ATCC 25285

# EUCAST MIC breakpoints

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| <i>Tazobactam</i>                          | --                    | --              |
| <i>Phenoxymethylpenicillin</i>             | 16                    | 32              |
| <i>Oxacillin</i>                           | --                    | --              |
| <i>Cloxacillin</i>                         | --                    | --              |
| <i>Dicloxacillin</i>                       | --                    | --              |
| <i>Fluckloxacillin</i>                     | --                    | --              |
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| <i>Amoxicillin</i> <sup>1</sup>            | 0.5                   | 1               |
| <i>Amoxicillin-clavulanic acid</i>         | 0.5 <sup>2</sup>      | 1 <sup>2</sup>  |
| <i>Piperacillin</i> <sup>1</sup>           | 18                    | 36              |
| <i>Piperacillin-tazobactam</i>             | 18 <sup>2</sup>       | 36 <sup>2</sup> |
| <i>Ticarcillin</i> <sup>1</sup>            | 18                    | 36              |
| <i>Ticarcillin-clavulanic acid</i>         | 18 <sup>2</sup>       | 36 <sup>2</sup> |
| <i>Tazobactam</i>                          | --                    | --              |
| <i>Phenoxymethylpenicillin</i>             | 6                     | 12              |
| <i>Oxacillin</i>                           | --                    | --              |
| <i>Cloxacillin</i>                         | --                    | --              |
| <i>Dicloxacillin</i>                       | --                    | --              |
| <i>Fluckloxacillin</i>                     | --                    | --              |
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“Disk diffusion criteria for antimicrobial susceptibility testing of anaerobes have not yet been defined ...”

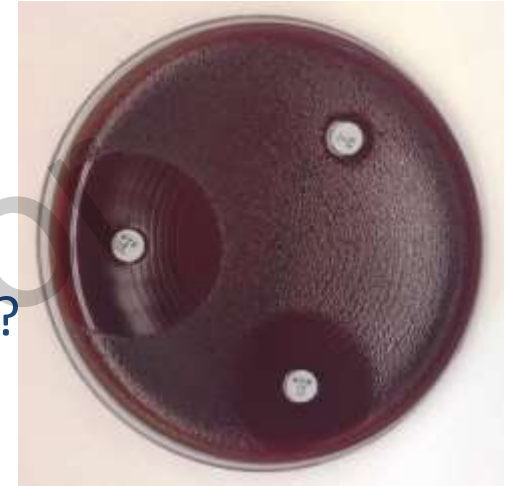


# Disk diffusion

**Why has it not worked before?**

Lack of standardization?

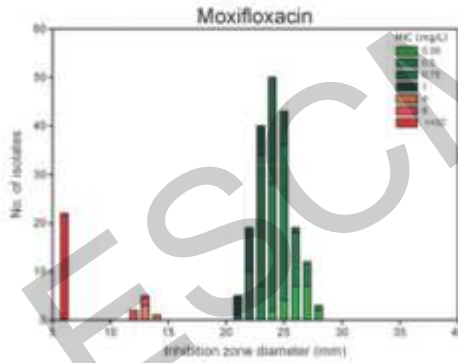
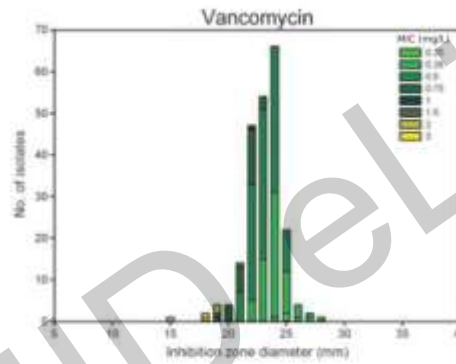
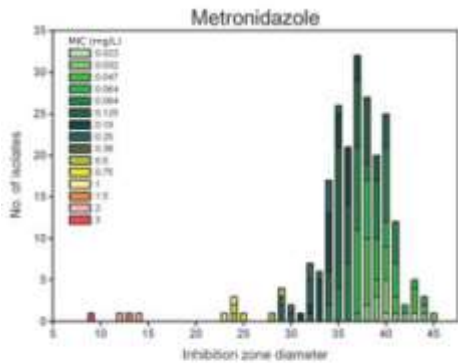
Testing too many different species at the same time?



EUCAST have no genus or species specific breakpoints (except *C. difficile*)

– anaerobic bacteria are a very diverse group of bacteria

# Disk diffusion – *C. difficile*



**TABLE 1.** Categorization of *C. difficile* as wild type or non-wild type using a gradient test and the proposed disk diffusion method. Zone diameter correlates to ECOFFs as determined by EUCAST were used and very major errors (VME) and major errors (ME) calculated

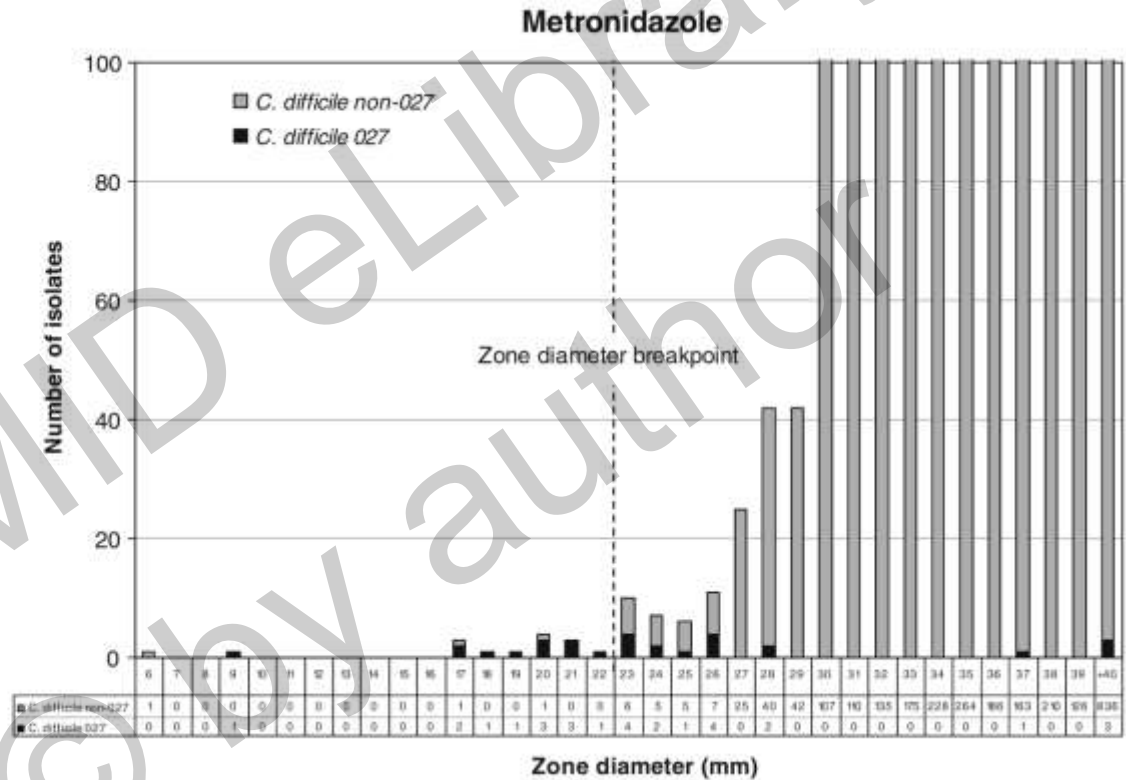
| Antimicrobial agent | Zone diameter* ECOFF (mm) | EUCAST ECOFF (mg/L) | Errors (%) |     |
|---------------------|---------------------------|---------------------|------------|-----|
|                     |                           |                     | VME        | ME  |
| Metronidazole       | WT ≥ 23                   | WT ≤ 2              | 0          | 1.4 |
| Vancomycin          | WT ≥ 19                   | WT ≤ 2              | 0          | 0.5 |
| Moxifloxacin        | WT ≥ 20                   | WT ≤ 4              | 0          | 1.8 |

\*Tentative zone diameter ECOFF for the proposed disk diffusion method.

Very important also to look at essential agreement (and categorical agreement)

# Disk diffusion – *C. difficile*

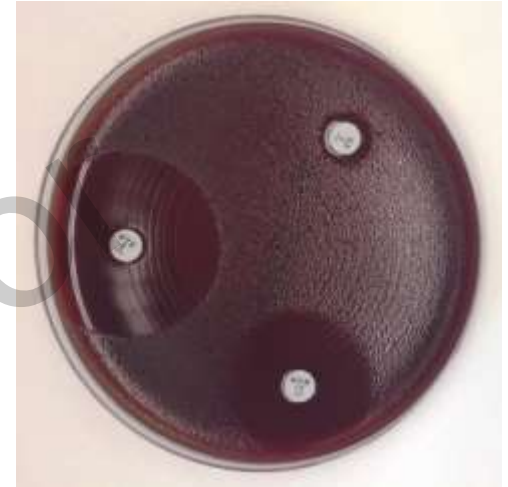
**Fig. 1** Metronidazole and vancomycin zone diameter distribution of 2702 toxin-producing *Clostridium difficile* isolates. Zone diameter breakpoints are indicated by the dotted lines. Polymerase chain reaction (PCR) ribotype 027 is shown in black. The +40 equals zone diameters  $\geq 40$  mm



# Disk diffusion

The Brucella Blood Agar (BBA) was deemed OUT

- only one product worked



# Fastidious Anaerobe Agar (FAA)

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1990, p. 2280-2282  
0066-4804/90/112280-03\$02.00/0  
Copyright © 1990, American Society for Microbiology

Vol. 34, No. 11

## Fastidious Anaerobe Agar Compared with Wilkins-Chalgren Agar, Brain Heart Infusion Agar, and Brucella Agar for Susceptibility Testing of *Fusobacterium* Species

JON S. BRAZIER,<sup>1</sup> ELLIE J. C. GOLDSTEIN,<sup>2\*</sup> DIANE M. CITRON,<sup>2</sup> AND MARGARETA I. OSTOVARI<sup>2</sup>

*Anaerobe Reference Unit, Public Health Laboratory, Luton & Dunstable Hospital, Luton, England,<sup>1</sup> and The R. M. Alden Research Laboratory, Santa Monica Hospital Medical Center, Santa Monica, California 90404<sup>2</sup>*

Received 7 May 1990/Accepted 5 September 1990

**Add 5% fresh mechanically defibrinated horse blood**

| Formula               | g/litre |
|-----------------------|---------|
| Peptone Mix           | 23.0    |
| Sodium chloride       | 5.0     |
| Soluble Starch        | 1.0     |
| Agar No.2             | 12.0    |
| Sodium bicarbonate    | 0.4     |
| Glucose               | 1.0     |
| Sodium pyruvate       | 1.0     |
| L-Cysteine HCl        | 0.5     |
| Haemin                | 0.01    |
| Vitamin K             | 0.001   |
| L - Arginine          | 1.0     |
| Soluble pyrophosphate | 0.25    |
| Sodium succinate      | 0.5     |

pH: 7.2 + 0.2

**Appearance:** Red due to addition of blood. The blood will darken (reduce) with time.

# Could the Fastidious Anaerobe Agar be IN?

**Pilot study simultaneously performed in Cardiff (Wales), Växjö (Sweden) and Odense (Denmark)**

- Same batch of agars and disks
- Inoculum (1 McF)
- Atmosphere (80% N<sub>2</sub>, 10% CO<sub>2</sub> and 10% H<sub>2</sub>)
- Temperature (37°C)
- Incubation time (24 h)

Testing QC strains and measuring zone diameters

# Reading zones is a central issue

From reading:  
the outer-  
most-  
outer zone  
edge



... to reading:  
the most obvious  
zone edge

# Baby steps





# Other variables to test

- Agar depth
  - From fridge or room temperature (for how long)
  - With or without plastic (humidity)
  - Homemade or ready to use agar plates
  - FAA powder from different manufacturers
- The FAA
- Atmosphere: Anaerobic workstation, gas generating envelopes and the Anoxomat
  - Temperature: 35 °C or 37 °C
  - Incubation time: 16 – 20 h or 24h
  - Disk potencies: clindamycin 10 µg and piperacillin-tazobactam 75/10 µg and 100/10 µg

# “The work” by Herjan Bavelaar

Dedicated young Dutch clinical microbiologist in training

Almost 24/7 for 9 months



- Systematically testing the different variables with
- different QC strains representing a wide spectrum of anaerobic bacteria
- at the EUCAST development laboratory in Växjö, Sweden
- under the supervision of Erika Matuschek and Gunnar Kahlmeter

QC strains: *Bacteroides fragilis*, *Clostridium difficile*, *Clostridium perfringens*, *Fusobacterium necrophorum*, *Peptostreptococcus anaerobius*, *Prevotella melaninogenica*

# Poster P0160

**Later today: 15:30 - 16:30**

Session: Antimicrobial susceptibility testing by disk diffusion

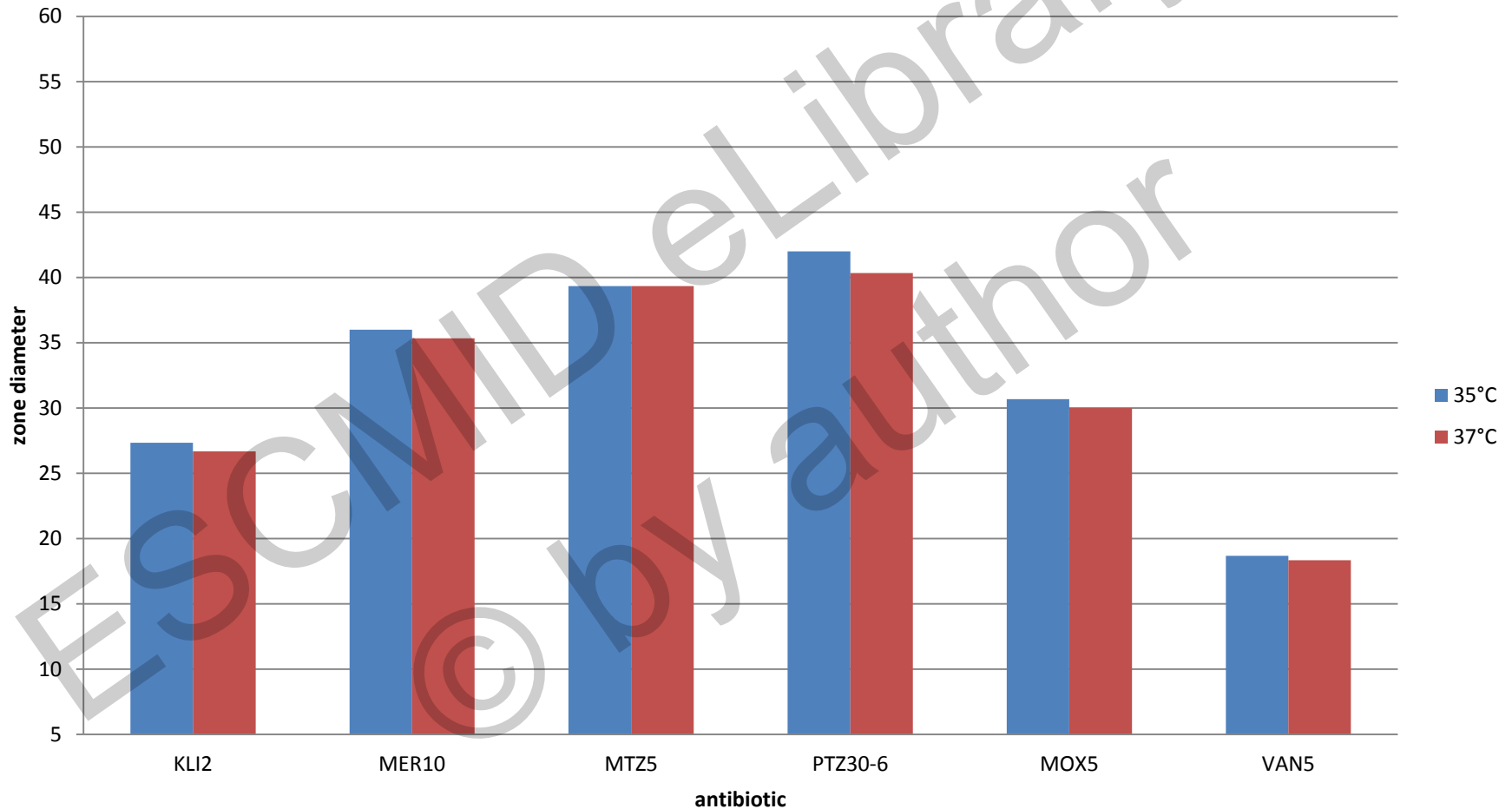
Title: *Development of a EUCAST disk diffusion method for rapidly growing anaerobic bacteria using fastidious anaerobe agar (FAA)*

Herjan Bavelaar et al.





# *Peptostreptococcus anaerobius* ATCC 27337



# Poster P0161

**Later today: 15:30 - 16:30**

Session: Antimicrobial susceptibility testing by disk diffusion

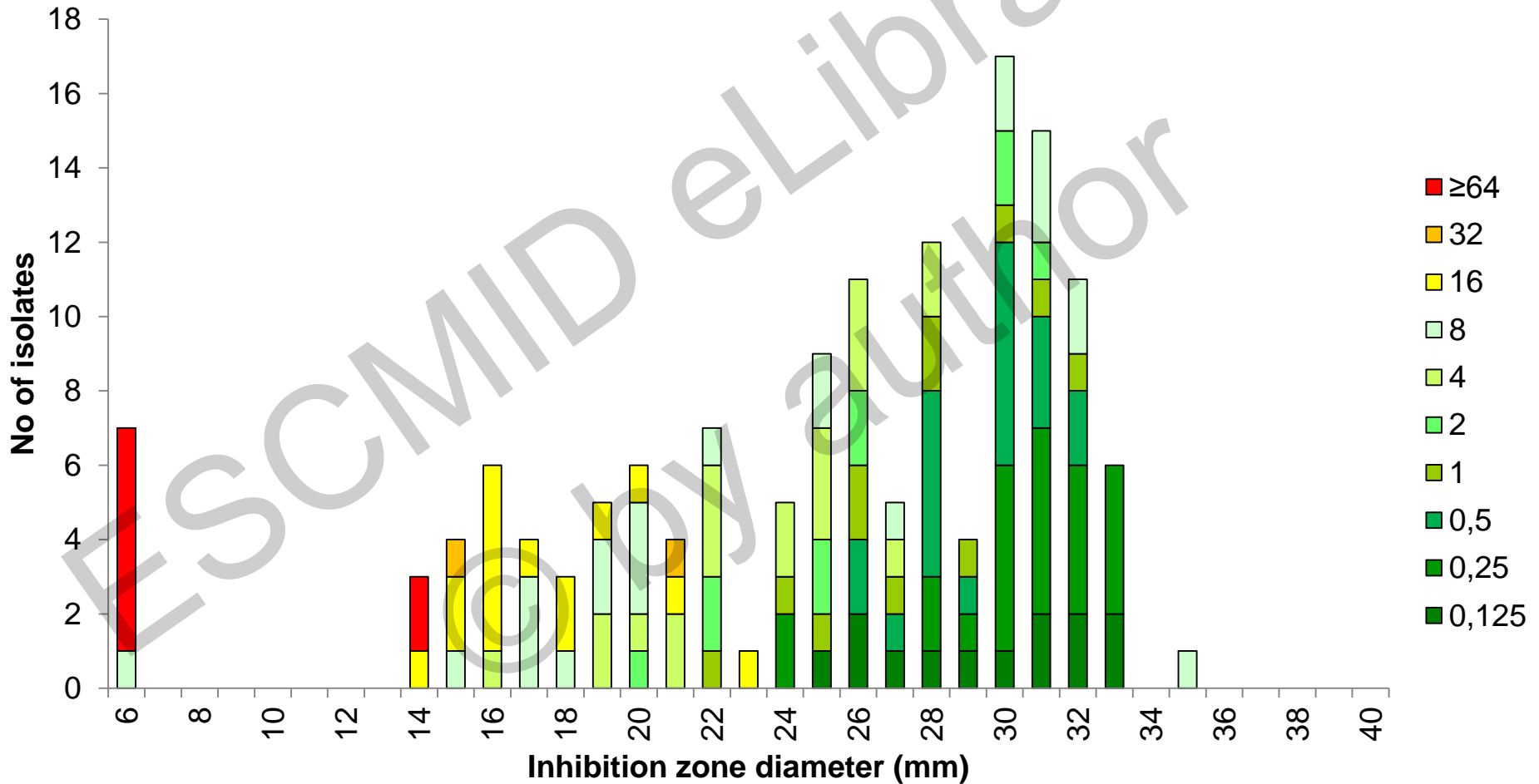
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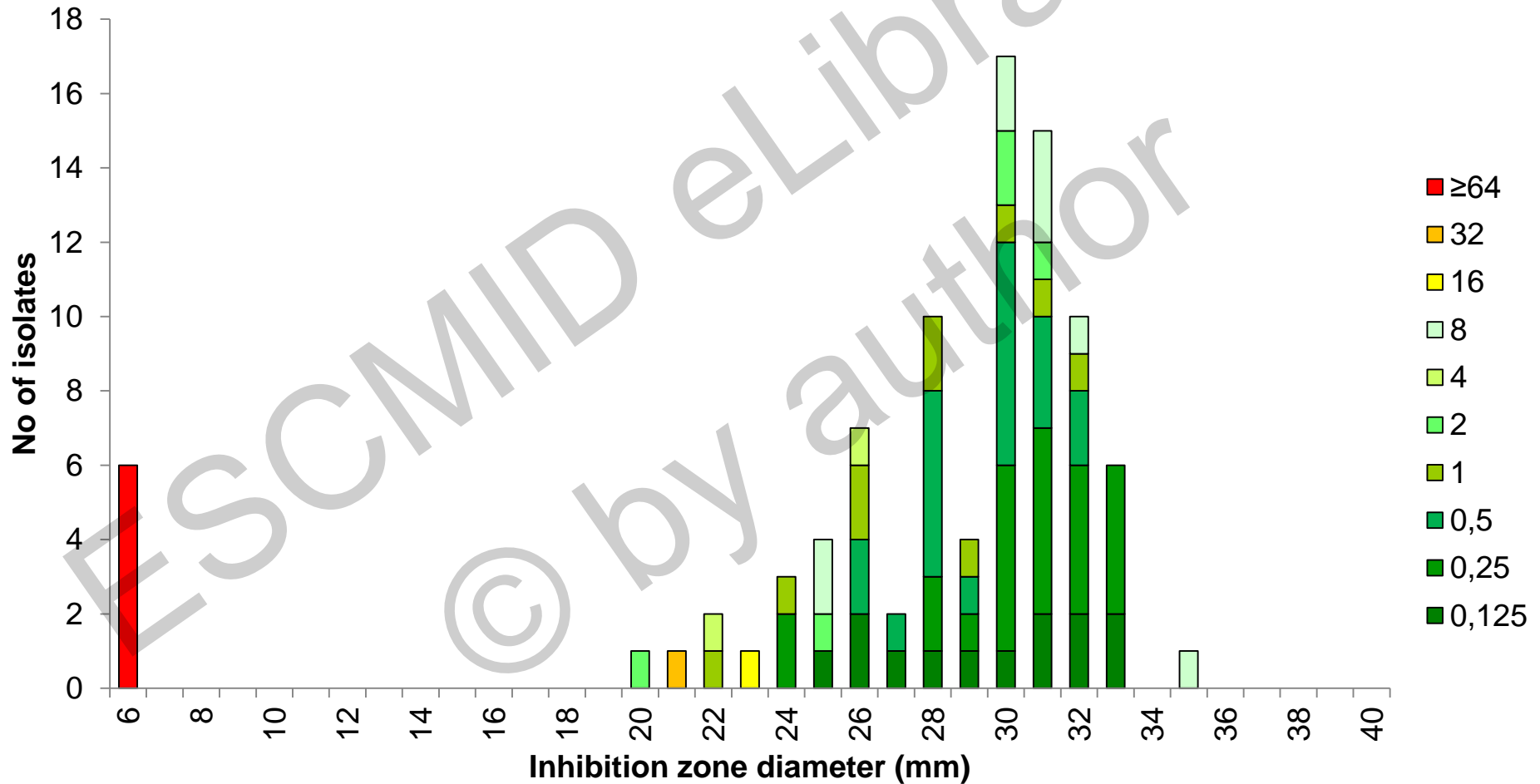


## Piperacillin-tazobactam 30-6 µg vs. MIC *Bacteroides* spp., 146 clinical isolates

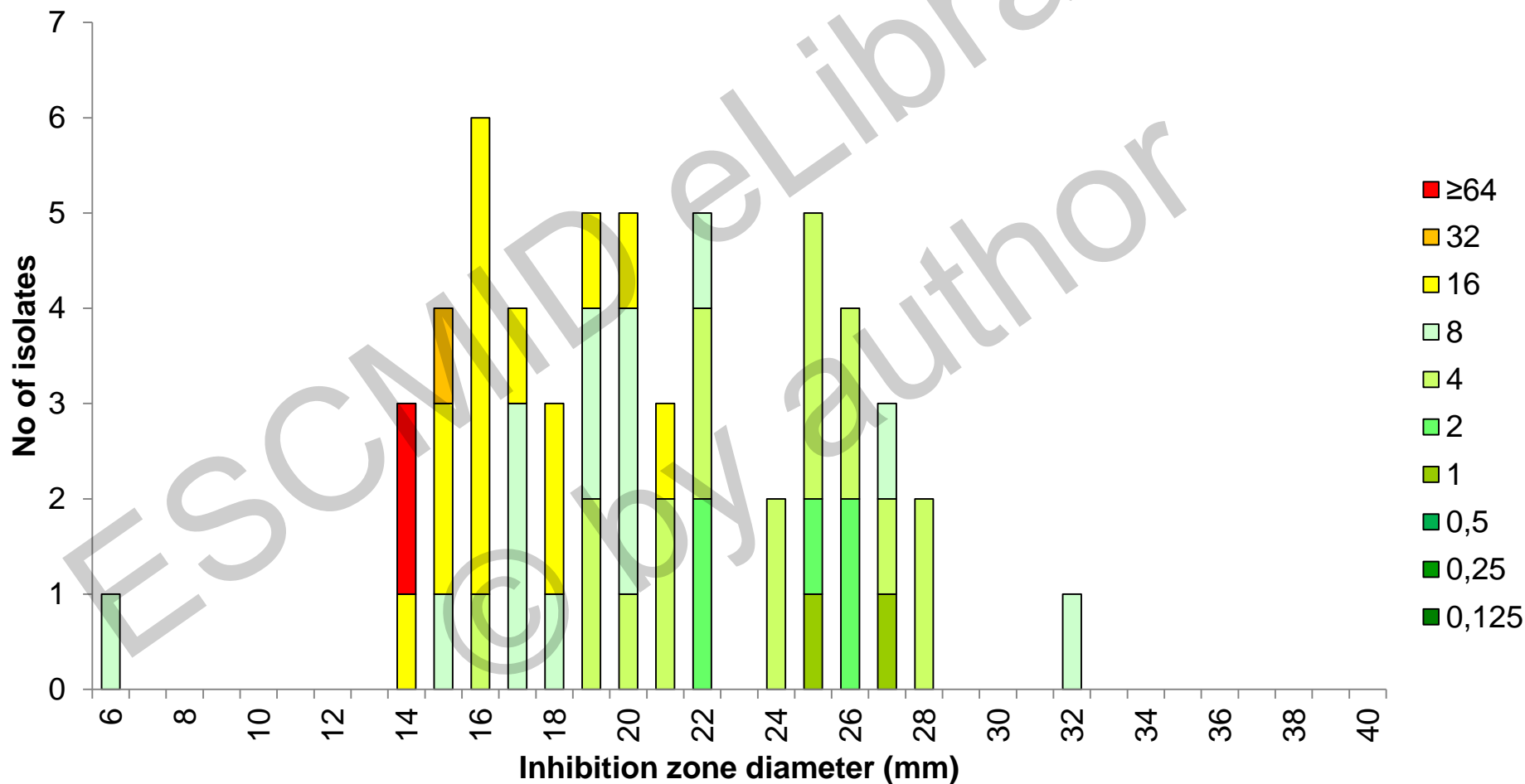




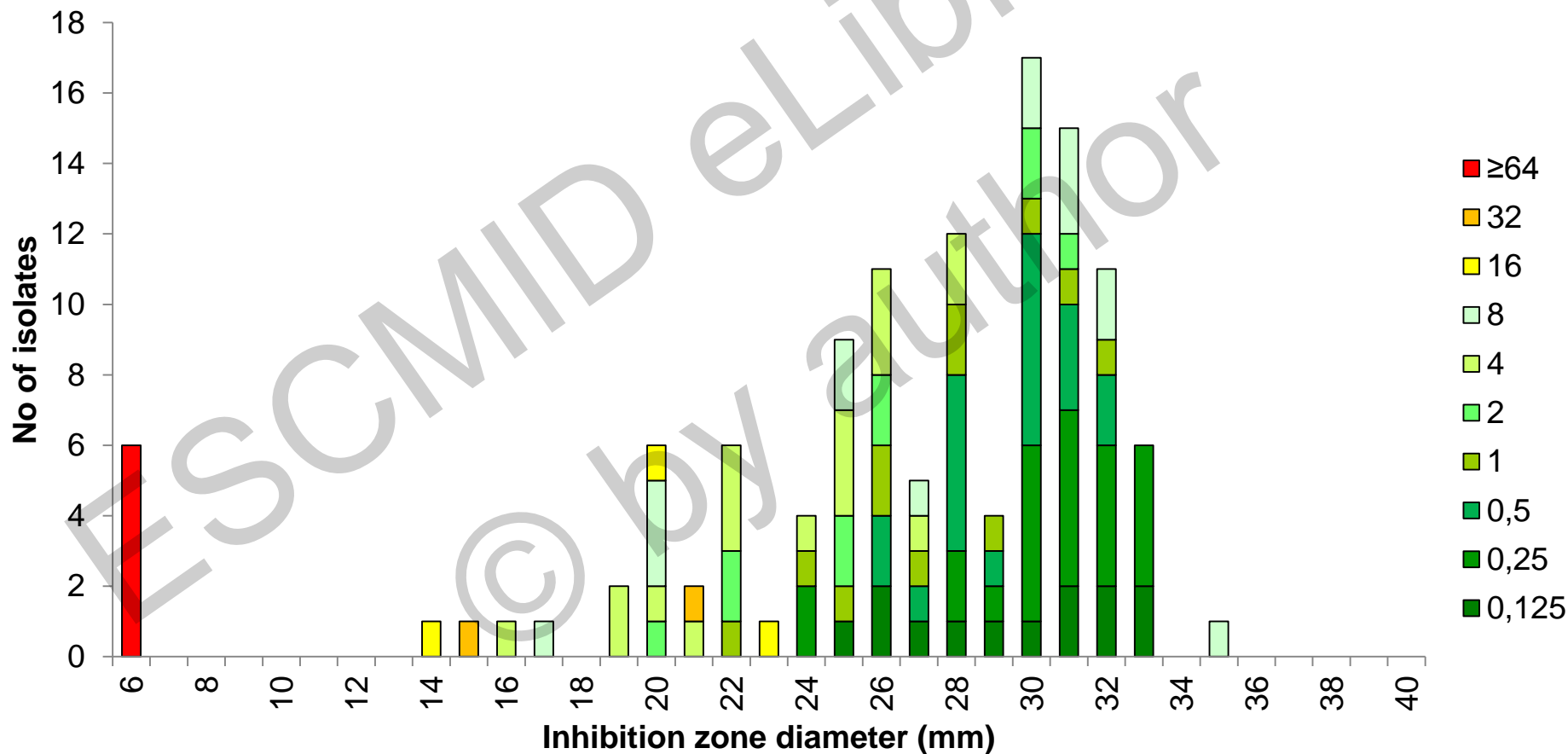
## Piperacillin-tazobactam 30-6 $\mu$ g vs. MIC *Bacteroides fragilis*, 90 clinical isolates



## Piperacillin-tazobactam 30-6 $\mu$ g vs. MIC *Bacteroides non-fragilis*, 56 clinical isolates



**Piperacillin-tazobactam 30-6 µg vs. MIC  
*Bacteroides* spp., except *B. thetaiotaomicron*  
 122 clinical isolates**



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Herjan Bavelaar et al.



# The method so far

- Fastidious Anaerobe Agar (FAA) with 5% horse blood
- Inoculum (1 McF)
- Atmosphere (80% N<sub>2</sub>, 10% CO<sub>2</sub> and 10% H<sub>2</sub>)
- Temperature (36±1 °C)
- Incubation time (16-20 h) – for clindamycin 40-44 h to detect all resistance

Deviation from EUCAST standard disk potencies:

- clindamycin 10 µg for the *B. fragilis* group (standard is 2 µg )



# QC – *B. fragilis* ATCC 25285

Zone diameter targets and ranges are in preparation

# Future plans and perspectives

Multicentre study to evaluate the method with the *B. fragilis* group  
- and defining zone diameter breakpoints

Next species/genera to include:

- *Clostridium perfringens* and other *Clostridium* spp.
- *Fusobacterium necrophorum*
- *Prevotella* spp.
- *Cuti(Propioni)bacterium acnes*
- GPACs (Gram positive anaerobic cocci)



# Acknowledgements

**Herjan Bavelaar, Erika Matuschek and Gunnar Kahlmeter**

- **EUCAST Development Laboratory, Växjö, Sweden**

**Trefor Morris**

- **Anaerobe Reference Unit, Cardiff, Wales**

**Christian Giske and Gunnar Kahlmeter (again)**



**EUCAST**

EUROPEAN COMMITTEE  
ON ANTIMICROBIAL  
SUSCEPTIBILITY TESTING

European Society of Clinical Microbiology and Infectious Diseases

**Elisabeth Nagy and Carl Erik Nord**



**ESGAI**

ESCMID STUDY GROUP  
FOR ANAEROBIC INFECTIONS

European Society of Clinical Microbiology and Infectious Diseases