

# Disk diffusion method for anaerobes

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# Why?

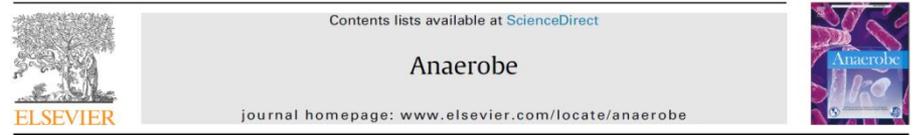
## From 2000 to 2013

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.

**Reports of carbapenem and metronidazole resistance in the *B. fragilis* group**

**Conclusion: Resistance is changing but in general increasing = unpredictable**



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



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# How? The phenotypic methods

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

# Agar dilution (gold standard)

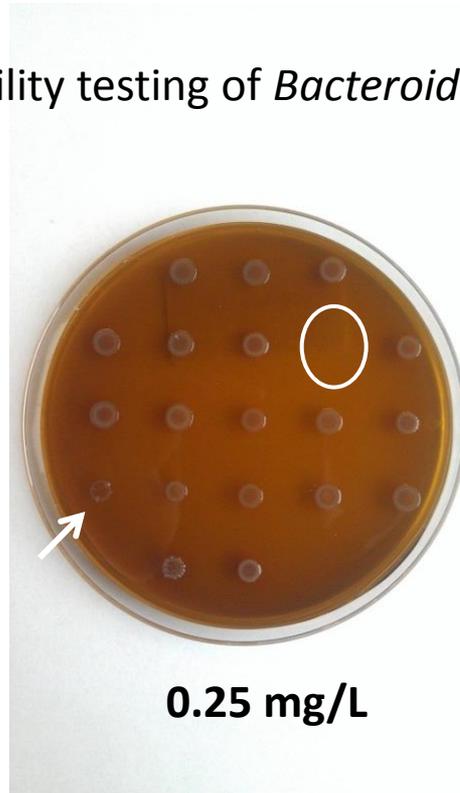


CLSI M11-A8

Metronidazole susceptibility testing of *Bacteroides fragilis* group isolates



0.125 mg/L



0.25 mg/L



0.50 mg/L

# Broth (micro) dilution



CLSI M11-A8

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.”*

# Gradient strips

**Expensive**

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



*Bacteroides fragilis*  
ATCC 25285

# EUCAST MIC breakpoints

## Gram-positive anaerobes except *Clostridium difficile*

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

Disk diffusion criteria for antimicrobial susceptibility testing of anaerobes have not yet been defined and an MIC method should be used. If a commercial MIC method is used, follow the manufacturer's instructions.

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.

## Gram-negative anaerobes

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

Disk diffusion criteria for antimicrobial susceptibility testing of anaerobes have not yet been defined and an MIC method should be used. If a commercial MIC method is used, follow the manufacturer's instructions.

This group of bacteria includes many genera. The most frequently isolated Gram-negative anaerobes are *Bacteroides*, *Prevotella*, *Porphyromonas*, *Fusobacterium*, *Bifidobacterium* and *Mobiluncus*. Anaerobes are most frequently defined by no growth on culture plates incubated in a CO<sub>2</sub> enriched atmosphere. For all these species, susceptibility testing should be performed in anaerobic environment.

Penicillins	MIC breakpoint (mg/L)		Notes
	S ≤	R >	
Benzympenicillin <sup>1</sup>	0.25	0.5	1. Susceptibility to ampicillin, amoxicillin, piperacillin and ticarcillin can be inferred from si 2. For susceptibility testing purposes, the concentration of sulbactam is fixed at 4 mg/L. 3. For susceptibility testing purposes, the concentration of clavulanic acid is fixed at 2 mg/L. 4. For susceptibility testing purposes, the concentration of tazobactam is fixed at 4 mg/L.
Ampicillin <sup>1</sup>	4	8	
Ampicillin-sulbactam	4 <sup>1</sup>	8 <sup>2</sup>	
Amoxicillin <sup>1</sup>	4	8	
Amoxicillin-clavulanic acid	4 <sup>1</sup>	8 <sup>2</sup>	
Piperacillin <sup>1</sup>	8	16	
Piperacillin-tazobactam	8 <sup>1</sup>	16 <sup>2</sup>	
Ticarcillin <sup>1</sup>	8	16	
Ticarcillin-clavulanic acid	8 <sup>1</sup>	16 <sup>2</sup>	
Temocillin	-	-	
Phenoxymethylpenicillin	IE	IE	
Oxacillin	-	-	
Cloxacillin	-	-	
Dicloxacillin	-	-	
Flucloxacillin	-	-	
Mecillinam (uncomplicated UTI only)	-	-	

Penicillins	MIC breakpoint (mg/L)		Notes
	S ≤	R >	
Benzympenicillin <sup>1</sup>	0.25	0.5	1. Susceptibility to ampicillin, amoxicillin, piperacillin and ticarcillin can be inferred from susceptibility to benzympenicillin. 2. For susceptibility testing purposes, the concentration of sulbactam is fixed at 4 mg/L. 3. For susceptibility testing purposes, the concentration of clavulanic acid is fixed at 2 mg/L. 4. For susceptibility testing purposes, the concentration of tazobactam is fixed at 4 mg/L.
Ampicillin <sup>1</sup>	0.5	2	
Ampicillin-sulbactam	4 <sup>1</sup>	8 <sup>2</sup>	
Amoxicillin <sup>1</sup>	0.5	2	
Amoxicillin-clavulanic acid	4 <sup>1</sup>	8 <sup>2</sup>	
Piperacillin <sup>1</sup>	16	16	
Piperacillin-tazobactam	8 <sup>1</sup>	16 <sup>2</sup>	
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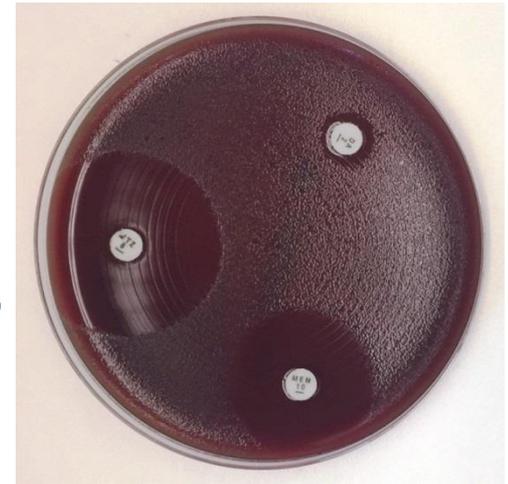
“Disk diffusion criteria for antimicrobial susceptibility testing of anaerobes have not yet been defined ...”

# Disk diffusion

**Why has it not worked before?**

Lack of standardization?

Testing too many different species at the same time?

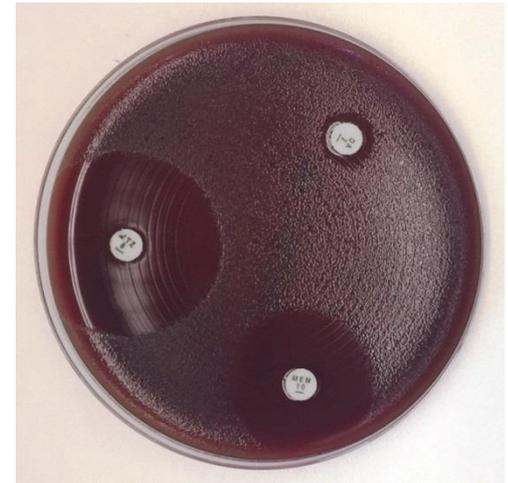


Anaerobic bacteria are a very diverse group of bacteria

# Disk diffusion

We have tried the Brucella Blood Agar (BBA),  
but 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

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*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**

**- guest appearance by Erika just after this presentation**

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?

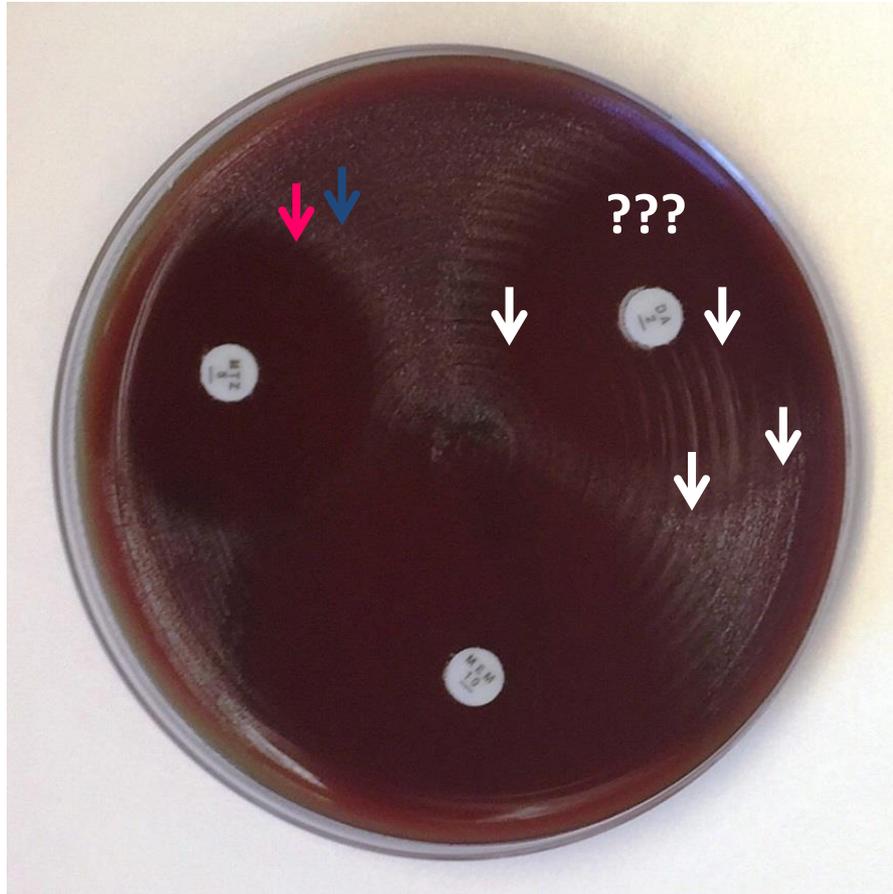
**Pilot study simultaneously performed in Cardiff (Wales), Växjö (Sweden) and Odense (Denmark)**

- 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



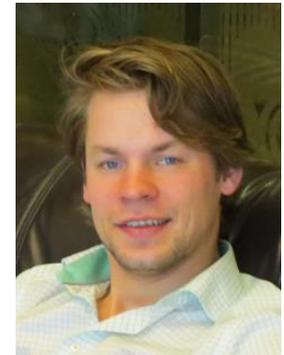
# Other variables to test

- 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

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

# Poster P0160

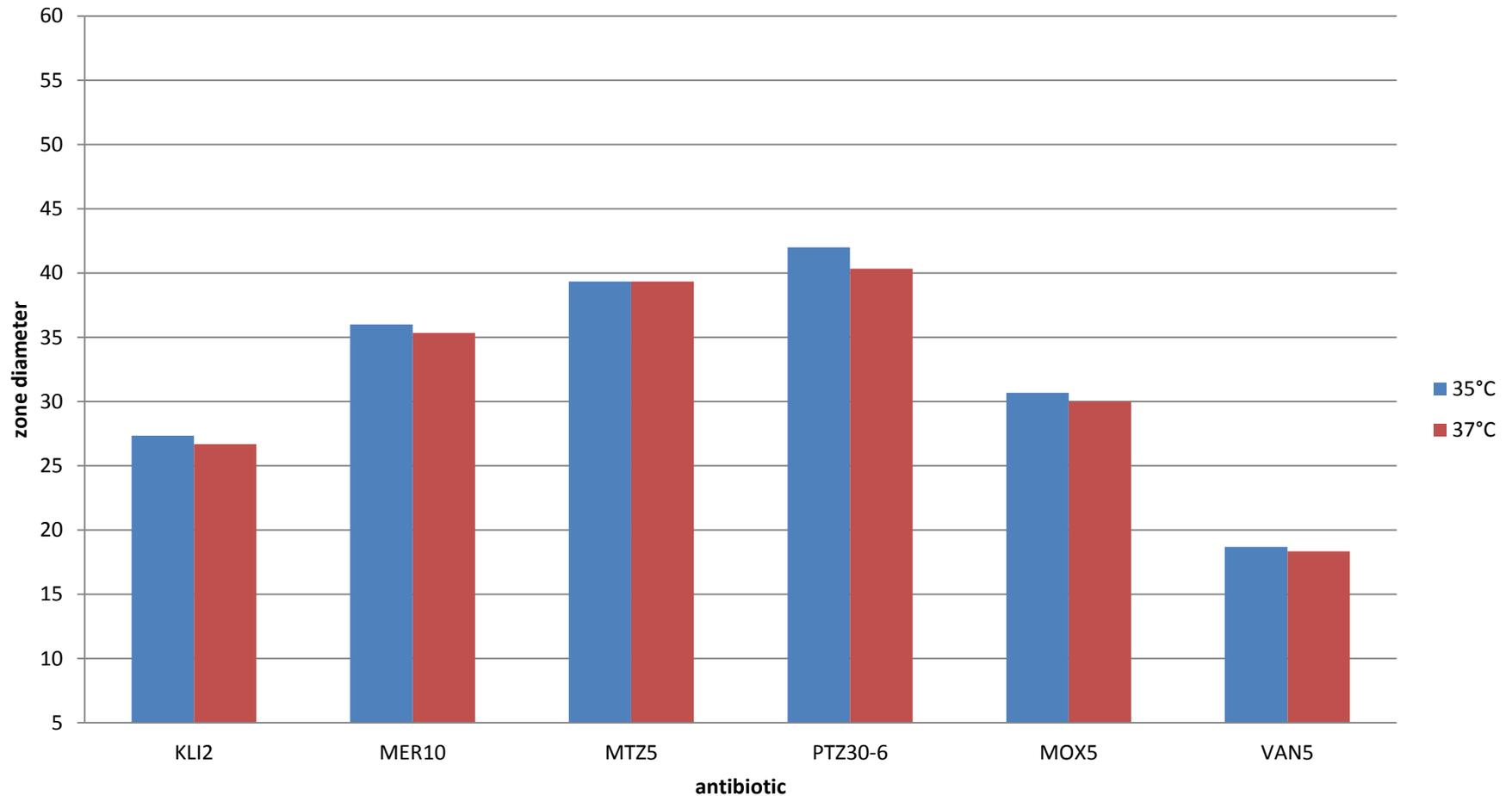
Session: Antimicrobial susceptibility testing by disk diffusion

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

Session: Antimicrobial susceptibility testing by disk diffusion

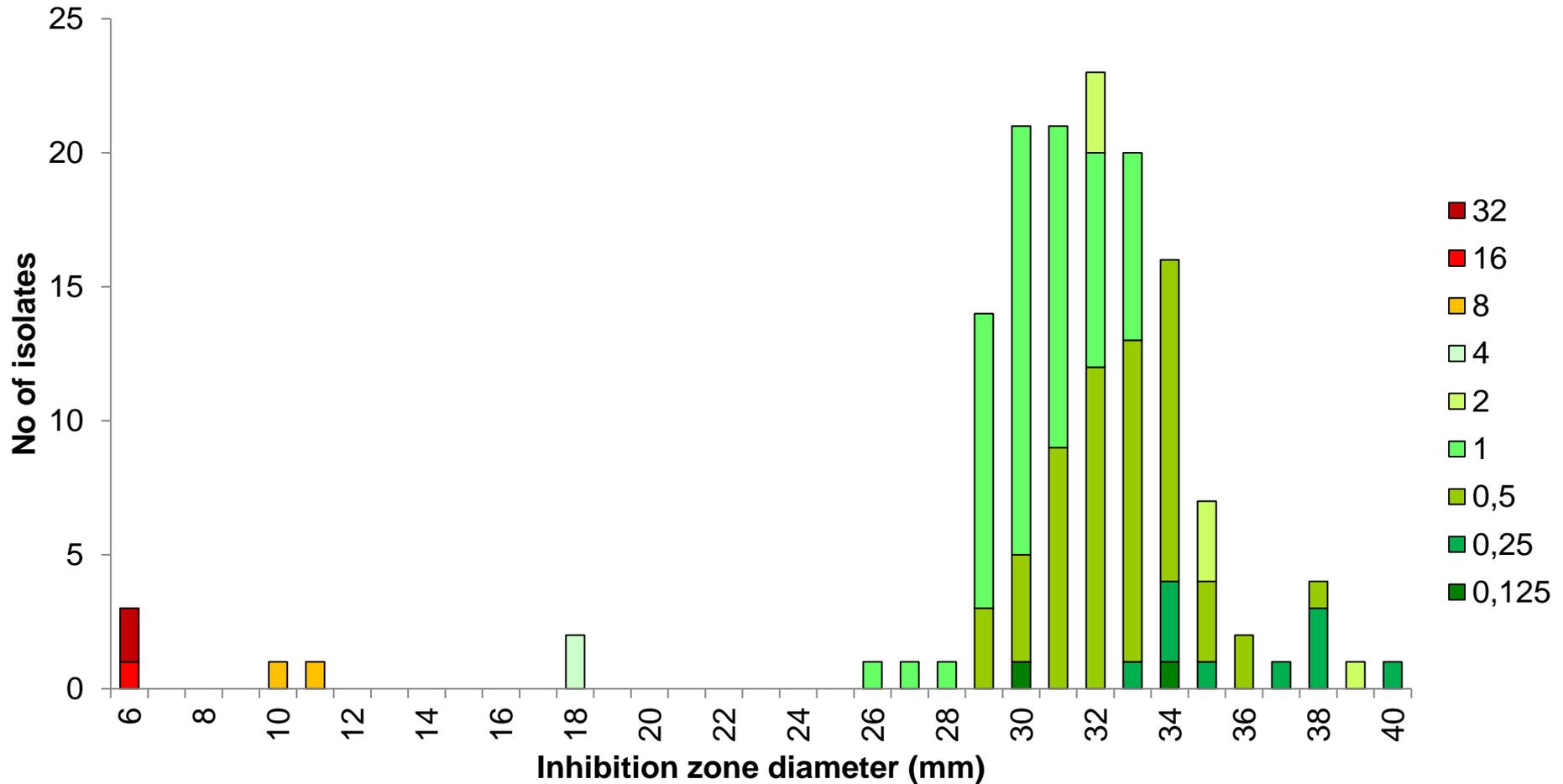
Title: *Performance of a new EUCAST disk diffusion method using fastidious anaerobe agar (FAA) for clinical Bacteroides fragilis group isolates*

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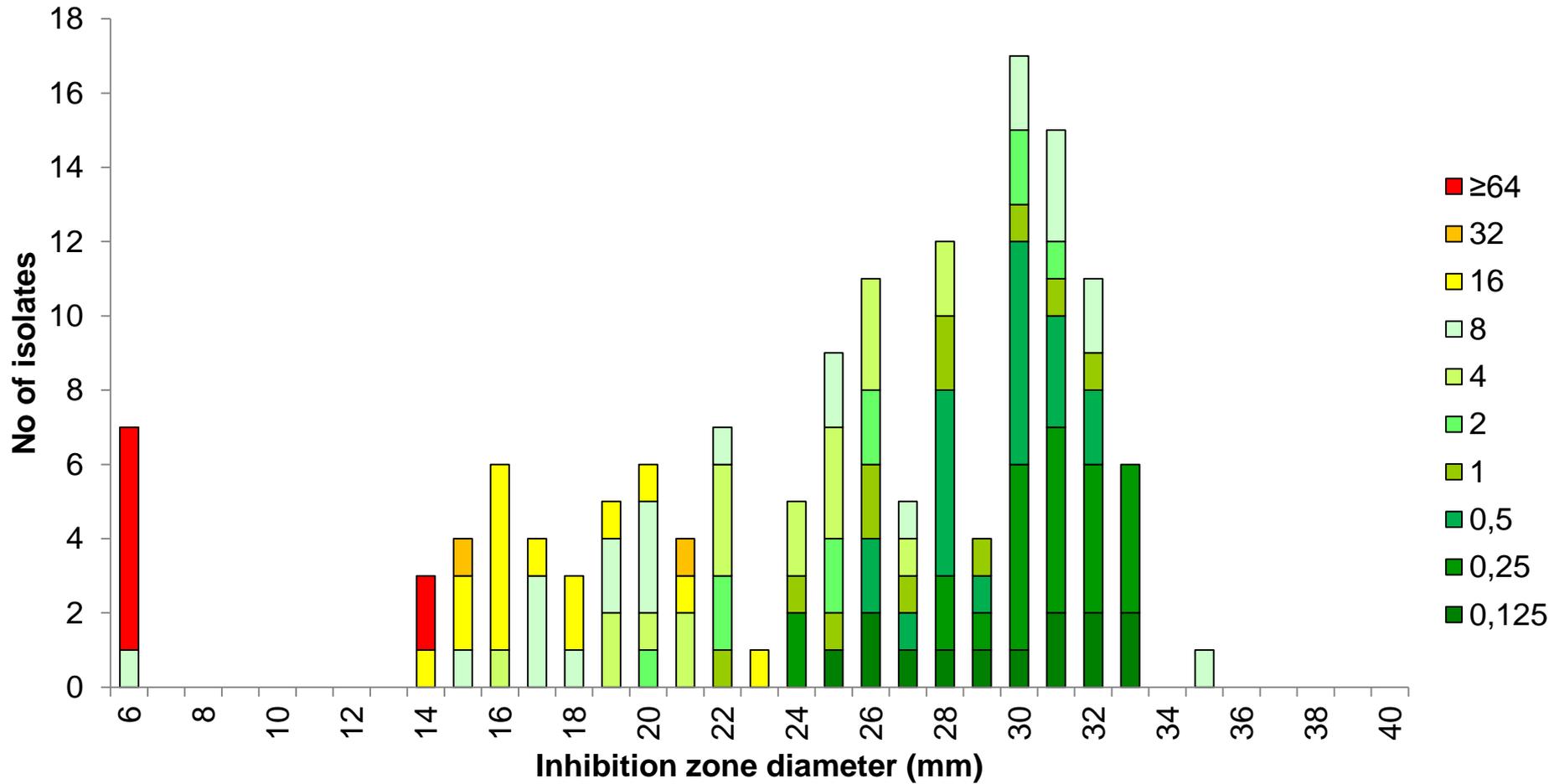


# Metronidazole 5 µg vs. MIC

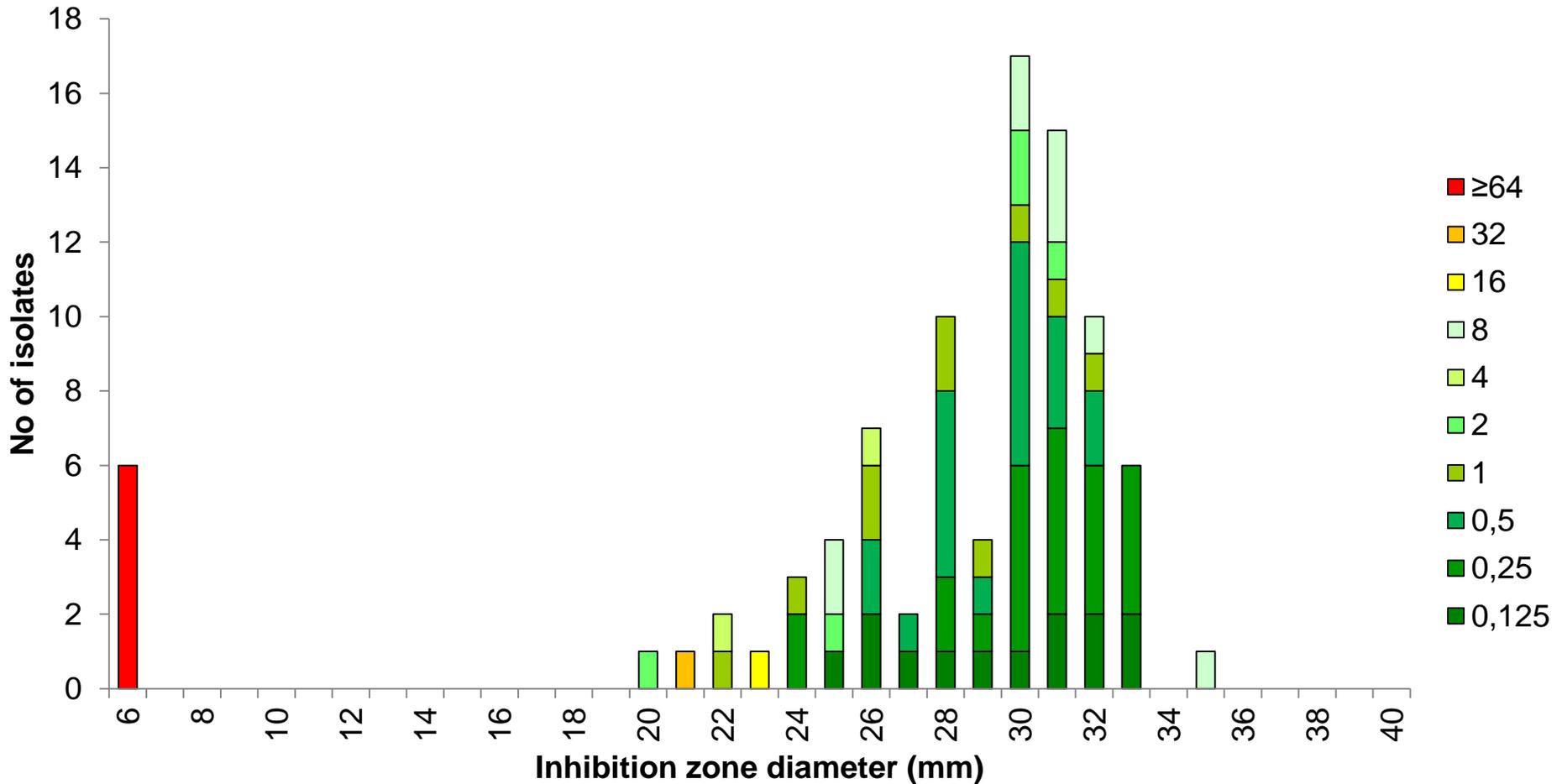
## *Bacteroides* spp., 141 clinical isolates



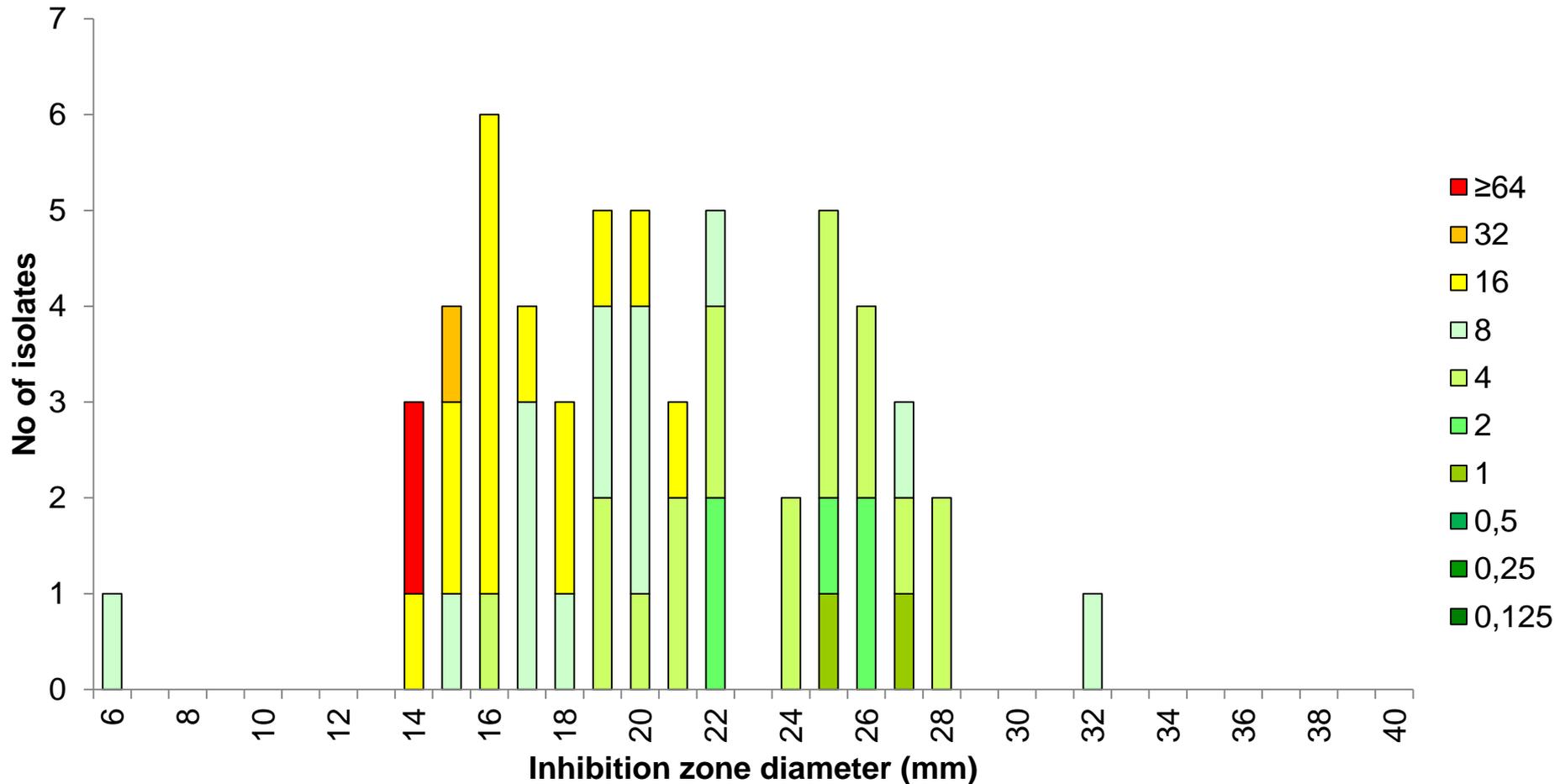
## Piperacillin-tazobactam 30-6 $\mu$ g vs. MIC *Bacteroides* spp., 146 clinical isolates



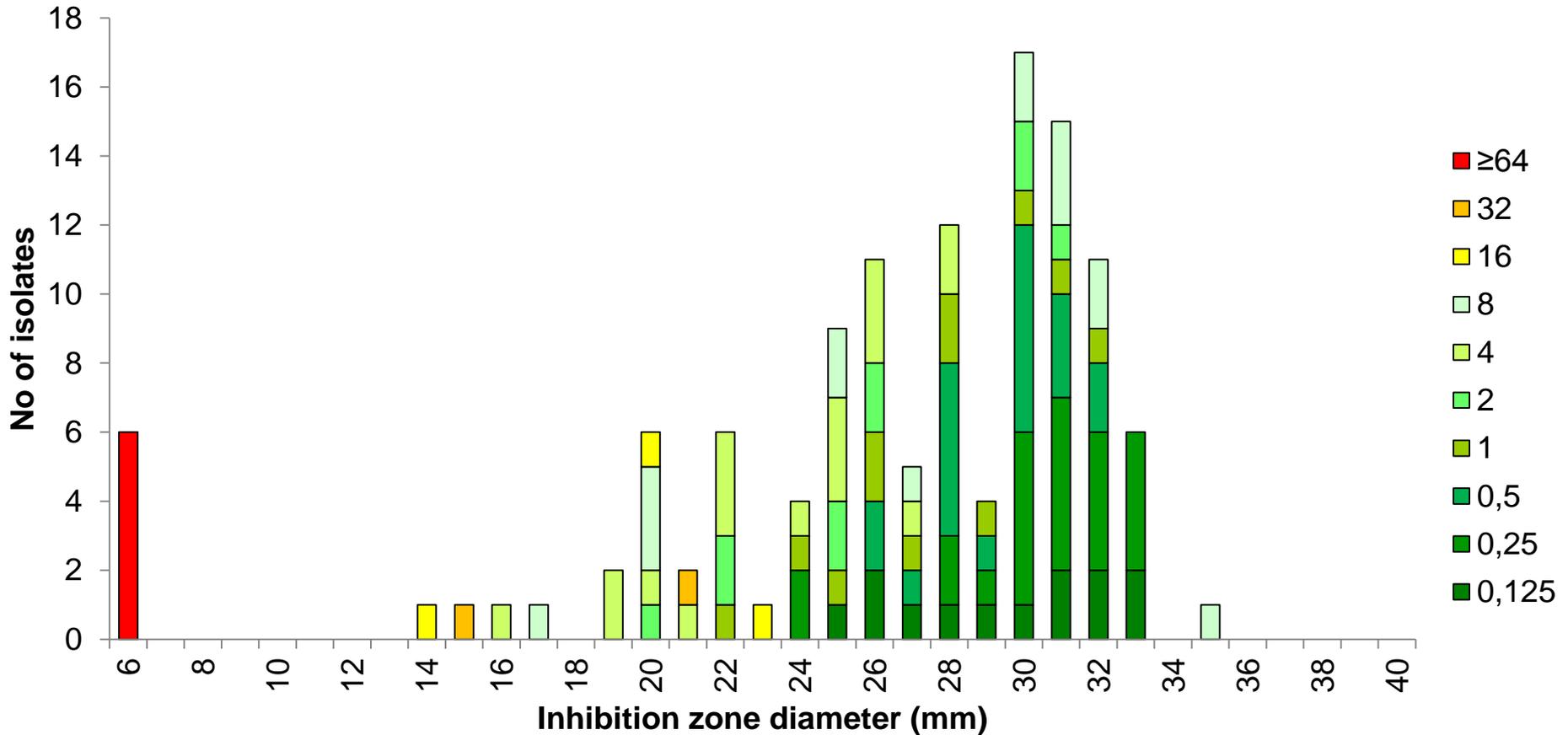
## Piperacillin-tazobactam 30-6 µg vs. MIC *Bacteroides fragilis*, 90 clinical isolates



## Piperacillin-tazobactam 30-6 µg vs. MIC *Bacteroides non-fragilis*, 56 clinical isolates



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



# 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**

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FOR ANAEROBIC INFECTIONS

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