Disk diffusion for anaerobic bacteria

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Susceptibility testing of Anaerobes

Trefor Morris
Lead Scientist UK Anaerobe Reference Unit
What do we need & why?

Reliable, reproducible, cost effective, timely and relevant susceptibility testing methodology for anaerobes!

Due to increased resistance!

What is currently missing?

1) High profile support from leading authority on susceptibility testing – EUCAST!

2) Technical requirements – media, specific breakpoints, control ranges – currently only CLSI
Overview

- Resistance in anaerobic bacteria, clinical relevance
- Disk diffusion method
- Results from different variables tested
- Results from the first clinical strains
Introduction

- Rising resistance in predominantly *Bacteroides* species to clindamycin, β-lactam / β-lactamase inhibitor combinations, cefoxitin and quinolones
- *Even more worrisome: increasing resistance against carbapenems and metronidazole*

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**Table 6.** Resistance trends of clinical *Bacteroides* isolates in Europe between 1990–2010 (expressed as the percentage of resistant isolates) [94,135,145].

<table>
<thead>
<tr>
<th>Europe</th>
<th>AMP</th>
<th>AMX/CLA</th>
<th>PIP/TAZ</th>
<th>FX</th>
<th>IMP</th>
<th>CLI</th>
<th>TET</th>
<th>MET</th>
<th>CIP</th>
<th>MXF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breakpoints (mg/L)</td>
<td>32/64</td>
<td>8</td>
<td>128</td>
<td>32/64</td>
<td>4/16</td>
<td>4/8</td>
<td>4</td>
<td>8/32</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>1990</td>
<td>16.0%</td>
<td>1.0%</td>
<td>-</td>
<td>3.0%</td>
<td>0.3%</td>
<td>9.0%</td>
<td>64.0%</td>
<td>0.0%</td>
<td>56.0%</td>
<td>-</td>
</tr>
<tr>
<td>2000</td>
<td>99.3%</td>
<td>-</td>
<td>1.0%</td>
<td>6.0%</td>
<td>0.7%</td>
<td>15.0%</td>
<td>-</td>
<td>0.5%</td>
<td>-</td>
<td>9.0%</td>
</tr>
<tr>
<td>2010</td>
<td>98.2%</td>
<td>10.4%</td>
<td>10.3%</td>
<td>17.2%</td>
<td>1.2%</td>
<td>32.4%</td>
<td>-</td>
<td>0.5%</td>
<td>-</td>
<td>13.6%</td>
</tr>
</tbody>
</table>

AMP: ampicillin; AMX/CLA: amoxicillin/clavulanic acid; PIP/TAZ: piperacillin/tazobactam; FX: cefoxitin; IMP: imipenem; CLI: clindamycin; TET: tetracycline; MET: metronidazole; CIP: ciprofloxacin; MXF: moxifloxacin.

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Meropenem

324 clinical isolates of *Bacteroides fragilis* from Växjö 2010 → now

![Graph showing MIC distribution for Meropenem with 4.9% I and 2.5% R criteria.](image-url)
Introduction

→ Moxifloxacin resistance in non-difficile *Clostridium* species.

→ Penicillin resistance in *Prevotella* species.

→ Clindamycin resistance in *Fusobacterium* species.

With increasing resistance and limited options for treatment of anaerobic infections it is becoming more important to test for susceptibility!
Susceptibility testing

Methods

Agar dilution (Gold standard)

Broth microdilution

Gradient strips

→ Disk diffusion
Currently no genus or species-specific breakpoints

Disk diffusion criteria for AST of anaerobes have not yet been defined...
Goal: To develop a disk diffusion method for rapidly growing anaerobic bacteria.

→ Relatively inexpensive compared to the other methods.

→ Easier to perform and interpret.
Introduction

Goal: To develop a disk diffusion method for rapidly growing anaerobic bacteria.

Easy, cheap and reliable susceptibility testing in turn will lead to
→ More surveillance of resistance
→ Better guidance of antibiotic therapy

”Mixed anaerobes susceptible to metronidazole”
”Oral anaerobic flora susceptible to penicillin”
Medium

BBA = Brucella Blood Agar
Medium

BBA = Brucella Blood Agar

FAA = Fastidious Anaerobe Agar

→ Better growth of clinically relevant anaerobes due to addition of peptones (growth stimulation), L-cysteine and L-arginine (better growth of Fusobacteria, Bacteroides, Cutibacterium)

Good results in pilot experiments with disk diffusion.
Results

disk diffusion method has to be usable for (almost) all laboratories!

→ Challenging due to lack of standardization of culture

- Different manufacturers of FAA
- Different incubation methods
- Incubation temperature
- Incubation duration

- Agar depth
- Effect of storage/ refrigeration
- Different disk charges
- Removal of foil before refrigeration

Further detail today
Results

FAA-agar with 5% mechanically defibribrinated horse blood
1,0 McFarland (0,9-1,1) suspension
Incubation 37°C, 24 hours, anaerobic workstation

<table>
<thead>
<tr>
<th>Tested strains</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacteroides fragilis</em></td>
<td>ATCC 25285</td>
</tr>
<tr>
<td><em>Clostridium difficile</em></td>
<td>ATCC 700057</td>
</tr>
<tr>
<td><em>Clostridium perfringens</em></td>
<td>CCUG 1795T</td>
</tr>
<tr>
<td><em>Fusobacterium necrophorum</em></td>
<td>ATCC 25286</td>
</tr>
<tr>
<td><em>Peptostreptococcus anaerobius</em></td>
<td>ATCC 27337</td>
</tr>
<tr>
<td><em>Prevotella melaninogenica</em></td>
<td>CCUG 4944</td>
</tr>
</tbody>
</table>
Results

Different manufacturers
## Results

### 4 manufacturers

LabM, EOlabs, Acumedia, Bioconnections

<table>
<thead>
<tr>
<th></th>
<th>Acumedia</th>
<th>Bioconnections</th>
<th>EandO</th>
<th>LabM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>23</td>
<td>23</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Soluble Starch</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Sodium Bicarbonate</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Glucose</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Sodium Pyruvate</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>L-Cysteine HCl.H2O</td>
<td>0.5</td>
<td>0.4</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Sodium Pyrophosphate</td>
<td>0.25</td>
<td>0.3 (ferric)</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Sodium Succinate</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Hemin</td>
<td>0.01</td>
<td>0.005</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Vitamin K</td>
<td>0.001</td>
<td>0.004</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Agar</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td><strong>total</strong></td>
<td><strong>45,661 g/L</strong></td>
<td><strong>45,609g/L</strong></td>
<td><strong>45,661g/L</strong></td>
<td><strong>45,661g/L</strong></td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td>7.2 +/- 0.2</td>
<td>7.3 +/- 0.2</td>
<td>7.2 +/- 0.2</td>
<td>7.2 +/- 0.2</td>
</tr>
</tbody>
</table>
Results

→ 4 different agars tested with all QC strains.
  - 9 tests per agar/antibiotic/strain combination
  - Different batches of FAA and horse blood every week

For example: *Bacteroides fragilis* and clindamycin is tested 36 times:
9x LabM, 9x EOlabs, 9x Acumedia, 9x Bioconnections
**Bacteroides fragilis ATCC 25285**

**Clindamycin 10**

<table>
<thead>
<tr>
<th></th>
<th>LabM</th>
<th>EOlabs</th>
<th>Bioconnections</th>
<th>Acumedia</th>
</tr>
</thead>
<tbody>
<tr>
<td>mean LM</td>
<td>29,8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean EO</td>
<td>30,2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean BC</td>
<td>29,3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean AC</td>
<td>30,0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Zone diameter vs. number of observations**

- LabM
- EOlabs
- Bioconnections
- Acumedia
### Bacteroides fragilis ATCC 25285

**Meropenem 10**

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Zone Diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean LM</strong></td>
<td>36.1</td>
<td></td>
</tr>
<tr>
<td><strong>Mean EO</strong></td>
<td>37.4</td>
<td></td>
</tr>
<tr>
<td><strong>Mean BC</strong></td>
<td>37.4</td>
<td></td>
</tr>
<tr>
<td><strong>Mean AC</strong></td>
<td>38.6</td>
<td></td>
</tr>
</tbody>
</table>

**Number of Observations**

- **LabM**
- **EOlabs**
- **Bioconnections**
- **Acumedia**

**Diagram:**

- X-axis: Zone Diameter
- Y-axis: Number of Observations

**Institute:**

- Radboud UMC
- EUCAST
### Bacteroides fragilis ATCC 25285

**Piperacillin-tazobactam 30-6**

<table>
<thead>
<tr>
<th></th>
<th>LM</th>
<th>EO</th>
<th>BC</th>
<th>AC</th>
</tr>
</thead>
<tbody>
<tr>
<td>mean</td>
<td>32.6</td>
<td>33.1</td>
<td>33.1</td>
<td>35.4</td>
</tr>
</tbody>
</table>

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**Graph Description:**
- **Y-axis:** Number of observations
- **X-axis:** Zone diameter
- **Legend:**
  - LabM
  - EOlabs
  - Bioconnections
  - Acumedia
**Bacteroides fragilis ATCC 25285**

**Metronidazole 5**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
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</tr>
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<tbody>
<tr>
<td>mean LM</td>
<td>31,4</td>
</tr>
<tr>
<td>mean EO</td>
<td>29,3</td>
</tr>
<tr>
<td>mean BC</td>
<td>30,4</td>
</tr>
<tr>
<td>mean AC</td>
<td>26,7</td>
</tr>
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</table>

**number of observations**

<table>
<thead>
<tr>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
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</tr>
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</table>

<table>
<thead>
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<th>18</th>
<th>20</th>
<th>22</th>
<th>24</th>
<th>26</th>
<th>28</th>
<th>30</th>
<th>32</th>
<th>34</th>
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</table>

<table>
<thead>
<tr>
<th>36</th>
<th>38</th>
<th>40</th>
<th>42</th>
<th>44</th>
<th>46</th>
<th>48</th>
<th>50</th>
<th>52</th>
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<tbody>
<tr>
<td></td>
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<td></td>
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<td></td>
<td></td>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>54</th>
<th>56</th>
<th>58</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**zone diameter**

- **LabM**
- **EOlabs**
- **Bioconnections**
- **Acumedia**
**Clostridium difficile ATCC 700057**

### Metronidazole 5

- **mean LM**: 34.9
- **mean EO**: 37.2
- **mean BC**: 35.3
- **mean AC**: 41.0

### Chart

- **LabM**
- **EOlabs**
- **Bioconnections**
- **Acumedia**
Fusobacterium necrophorum ATCC 25286

Clindamycin 10

<table>
<thead>
<tr>
<th></th>
<th>Mean LM</th>
<th>Mean EO</th>
<th>Mean BC</th>
<th>Mean AC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>44,6</td>
<td>45,6</td>
<td>45,4</td>
<td>45,4</td>
</tr>
</tbody>
</table>
Fusobacterium necrophorum ATCC 25286

Metronidazole 5

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>mean LM</td>
<td>30,7</td>
</tr>
<tr>
<td>mean EO</td>
<td>31,1</td>
</tr>
<tr>
<td>mean BC</td>
<td>31,1</td>
</tr>
<tr>
<td>mean AC</td>
<td>31,3</td>
</tr>
</tbody>
</table>

zone diameter

number of observations

LabM
EOlabs
Bioconnections
Acumedia
Conclusion

→ LabM and Bioconnections usually show better growth since zone diameters are smaller.

→ The quality of growth is not very different (mostly confluent growth), LabM shows best contrast.

→ Acumedia usually has largest zones with important outliers (major errors):
  - Metronidazole for *Bacteroides fragilis*
  - Metronidazole for *Clostridium difficile*
Results

Different anaerobic incubation methods
Results

→ Comparison of gas-generating envelopes

• Anaerobic workstation
• VS GasPak EZ (BD)
• VS AnaeroGen (Thermo Fisher Scientific)

Incubation: 37°C, 24h

Values are averages of 3 independent runs with all strain/antibiotic combinations
Bacteroides fragilis ATCC 25285

![Bacteroides fragilis](image)

- KLI10
- MER10
- IMI10
- MTZ5
- PTZ30-6
- MOX5
- AMC20-10
- CHLO30

**Antibiotics:***
- Anaerogen
- Gazpak
- Anaerobic box

---

**Zone Diameter:**
- 5
- 10
- 15
- 20
- 25
- 30
- 35
- 40
- 45
- 50
- 55
- 60

---

29
**Fusobacterium necrophorum**

![Bar chart showing zone diameter for different antibiotics](image)

**Antibiotics Tested:**
- KLI10
- MTZ5
- PTZ30-6
- MOX5
- PEN 1

**Antibiotic Groups:**
- **Anaerogen**
- **Gazpak**
- **Anaerobic box**

**Results:**
- The bar chart illustrates the zone diameter for each antibiotic group.
- The chart shows how different antibiotics perform against the bacterium **Fusobacterium necrophorum**.
Results

→ Anaerobic workstation vs Anoxomat

• Anaerobic workstation
• VS Anoxomat system

Incubation: 37°C, 16h

Values are averages of 3 independent runs with all strain/ antibiotic combinations
Bacteroides fragilis ATCC 25285

Bacteroides fragilis

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Workstation</th>
<th>Anoxomat</th>
</tr>
</thead>
<tbody>
<tr>
<td>KLI10</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>MER10</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>IMI10</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>MTZ5</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>PTZ30-6</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>MOX5</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>AMC20-10</td>
<td>45</td>
<td>45</td>
</tr>
<tr>
<td>CHLO30</td>
<td>25</td>
<td>25</td>
</tr>
</tbody>
</table>

zone diameter

antibiotic

Workstation
Anoxomat
Fusobacterium necrophorum ATCC 25286

Fusobacterium necrophorum

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MOX5</th>
<th>PEN1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Workstation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anoxomat</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Zone diameter
Conclusion

No significant differences for any of the antibiotic/strain combinations regarding
- quality of growth
- legibility of zones
- zone diameters
Varying incubation temperature
Results

The Brucella blood agar for disk diffusion antimicrobial susceptibility testing of the Bacteroides fragilis group?

U.S. Justesen*, T. K. Danielsen, E. Matuschek, G. Kahlmeter (Odense C, DK; Växjö, SE)

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Bacteroides fragilis Zone diameter (mm)</th>
<th>Bacteroides thetaiotaomicron Zone diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>37°C</td>
<td>35°C</td>
</tr>
<tr>
<td>Piperacillin/tazobactam</td>
<td>32.4 (30.6-33.5)</td>
<td>37.7 (36.5-38.9)</td>
</tr>
<tr>
<td>(30/6 mikrog)</td>
<td>32.2 (31.3-33.7)</td>
<td></td>
</tr>
<tr>
<td>Meropenem</td>
<td>37.6 (35.7-39.0)</td>
<td>41.2 (39.3-42.7)</td>
</tr>
<tr>
<td>(10 mikrog)</td>
<td>36.7 (35.9-38.1)</td>
<td></td>
</tr>
<tr>
<td>Metronidazole</td>
<td>30.2 (29.3-31.7)</td>
<td>35.5 (34.2-37.1)</td>
</tr>
<tr>
<td>(5 mikrog)</td>
<td>30.3 (28.5-32.9)</td>
<td></td>
</tr>
<tr>
<td>Clindamycin</td>
<td>18.7 (17.0-20.3)</td>
<td>30.9 (29.7-32.4)</td>
</tr>
<tr>
<td>(2 mikrog)</td>
<td>21.6 (20.9-22.8)</td>
<td></td>
</tr>
</tbody>
</table>
Results

Strains incubated in 35°C and 37°C using anaerobic jars with Anaerogen sachets.

Values are averages of 3 independent runs with all strain/ antibiotic combinations
We also tested 10 random *Bacteroides fragilis*-group strains:

Maximum difference in zone diameter + 1,5mm for 35°C
**Fusobacterium necrophorum ATCC 25286**

**Fusobacterium necrophorum**

![Bar chart showing zone diameter for F. necrophorum at 35°C and 37°C across different antibiotics](chart.png)

- **KLI2**: 35°C and 37°C show similar zone diameters.
- **MER10**: 37°C has a higher zone diameter compared to 35°C.
- **MTZ5**: 35°C shows a higher zone diameter compared to 37°C.
- **PTZ30-6**: 37°C has a higher zone diameter compared to 35°C.
- **MOX5**: 37°C shows a higher zone diameter compared to 35°C.

**Antibiotics**

- KLI2
- MER10
- MTZ5
- PTZ30-6
- MOX5

**Temperatures**

- 35°C
- 37°C
Incubation at 37°C usually produces slightly smaller zones.

Seems unlikely that this difference is significant.

Advising 36°C ± 1°C is a clear benefit for clinical laboratories.
Results

Clinical *Bacteroides* strains
Results

170 clinical strains from Odense (Denmark), Tønsberg (Norway) and Växjö (Sweden) with known agar dilution MIC’s (from Cardiff) for
- Metronidazole
- Clindamycin
- Meropenem
- Piperacillin-tazobactam

All Bacteroides (and Parabacteroides) species.

Zones read after 16-20 hours incubation in 37°C.
Results

**Clinical Strains**

Metronidazole 5 µg vs. MIC
*Bacteroides spp.*, 170 isolates

MTZ: S ≤ 4, R > 4
Results

Meropenem 10 \( \mu \text{g vs. MIC} \)

*Bacteroides spp.*, 170 isolates

MEM: \( S \leq 2, R > 8 \)
Results

Piperacillin-tazobactam 30-6 µg vs. MIC
*Bacteroides spp.*, 170 isolates

PTZ: S ≤ 8, R > 16
Results

Piperacillin-tazobactam 30-6 µg vs. MIC
*Bacteroides spp* without *thetaiotaomicron*, 147 isolates

PTZ: S ≤ 8, R > 16
Results

Clindamycin 10 µg (16-20 h) vs. MIC

*Bacteroides spp.*, 170 isolates

CLI: S ≤ 4, R > 4
Results

Clindamycin 10 µg (40-44 h) vs. MIC

*Bacteroides spp.*, 170 isolates

CLI: S ≤ 4, R > 4
Method so far...

- FAA with 5% mechanically defibrinated horse blood
- 1 McFarland inoculum
- Temperature 36 ± 1°C
- Incubation time 16-20 hours (with exception of clindamycin)
- Without having to change anaerobic incubation system

→ Clindamycin disk potency deviation (10µg) from standard EUCAST recommendation (2µg) for *Bacteroides fragilis* group
To do

• Multicenter study for evaluation *Bacteroides* in preparation

• Ongoing evaluation of the method for clinical strains of
  - *Clostridium perfringens* and *species*.
  - *Fusobacterium sp.*
  - *Prevotella sp.*
  - *Cutibacterium sp.*
  - *Anaerobic gram-positive cocci.*
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