Challenging bugs for AST: e.g. *H. influenzae*

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H. influenzae and antimicrobial resistance

- Beta-lactam resistance
  - Beta-lactamase production
    - TEM and ROB
  - PBP3 mutations
    - Groups I, II, III, III-like, III+, III+-like

- Non-beta lactam resistance
  - Fluoroquinolones
  - Tetracyclines
  - Trimethoprim-sulfamethoxazole
Prevalence of PBP3 mutations

Beta-lactam resistance in *H. influenzae* in Kronoberg county, Sweden 2011-2018

<table>
<thead>
<tr>
<th>Country</th>
<th>No of isolates</th>
<th>PCG R (%)</th>
<th>BLA (%)</th>
<th>PBP3 (%)</th>
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<tbody>
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<td>All</td>
<td>304</td>
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<td>Sweden 2</td>
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<td>32</td>
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<td>24</td>
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</table>
**H. influenzae and PBP3 mutations**

Diagram showing different groups and mutations:
- **Group I**: R517H
- **Group II**: N526K
- **Group III (-)**: N526K, S385T
- **Group III (++)**: N526K, S385T, L389F
- **Group III-like (-)**: R517H, S385T
- **Group III-like (+)**: R517H, S385T, L389F

<table>
<thead>
<tr>
<th>KTG</th>
<th>SSN</th>
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<tbody>
<tr>
<td>R517H</td>
<td>S385T</td>
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<tr>
<td>N526K</td>
<td>L389F</td>
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</table>

Compiled by Dagfinn Skaare in thesis "Non-beta-lactamase-mediated beta-lactam resistance in *Haemophilus influenzae*. Mechanisms, epidemiology and susceptibility testing."
PBP3 mutations and beta-lactam resistance

17 *H. influenzae* – all with PBP3 mutations

EUCAST breakpoints are shown as dotted lines

Blue/green bars = PBP3 mutations group II

Red/orange bars = PBP3 mutations group III
Ampicillin 2 µg disk diffusion per beta-lactam resistance mechanism

No β-lactam resistance

- MIC (mg/L): 0.5, 0.25, 0.125, ≤0.06
- No of observations:

β-lactamase positive only

- MIC (mg/L): ≥64, 32, 16, 8, 4, 2
- No of observations:

PBP3 mutations only

- MIC (mg/L): 16, 8, 4, 2, 1, 0.5
- No of observations:

β-lactamase positive + PBP3 mutations

- MIC (mg/L): ≥64, 32, 16, 8
- No of observations:
Cefotaxime 5 µg disk diffusion per beta-lactam resistance mechanism

**No β-lactam resistance**

- **MIC (mg/L):** 0.03, 0.016, 0.008

**β-lactamase positive only**

- **MIC (mg/L):** 0.03, 0.016, 0.008, ≤0.004

**PBP3 mutations only**

- **MIC (mg/L):** 1, 0.5, 0.25, 0.12, 0.06, 0.03, 0.016

**β-lactamase positive + PBP3 mutations**

- **MIC (mg/L):** 0.06, 0.03, 0.016
**H. influenzae and fluoroquinolones**

**Ciprofloxacin 5 µg**

- MIC (mg/L):
  - ≥4
  - 2
  - 1
  - 0.5
  - 0.25
  - 0.125
  - 0.06
  - 0.03
  - 0.016
  - 0.008
  - ≤0.004

**Moxifloxacin 5 µg**

- MIC (mg/L):
  - ≥4
  - 2
  - 1
  - 0.5
  - 0.25
  - 0.125
  - 0.06
  - 0.03
  - 0.016
  - 0.008
  - ≤0.004

**Levofloxacin 5 µg**

- MIC (mg/L):
  - ≥4
  - 2
  - 1
  - 0.5
  - 0.25
  - 0.125
  - 0.06
  - 0.03
  - 0.016
  - 0.008
  - ≤0.004

**Ofloxacin 5 µg**

- MIC (mg/L):
  - 0.5
  - 0.25
  - 0.125
  - 0.06
  - 0.03
  - 0.016
  - 0.008
**H. influenzae** and fluoroquinolones – screening with nalidixic acid

**Nalidixic acid 30 µg vs. Ciprofloxacin MIC**

**Nalidixic acid 30 µg vs. Levofloxacin MIC**

**Nalidixic acid 30 µg vs. Moxifloxacin MIC**

**Nalidixic acid 30 µg vs. Ofloxacin MIC**
**H. influenzae** with tetracyclines and trimethoprim-sulfamethoxazole

### Tetracycline 30 µg

- MIC (mg/L): 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125
- Inhibition zone diameter (mm)

### Trimethoprim-sulfamethoxazole 1.25-23.75 µg

- MIC (mg/L): ≥8, 4, 2, 1, 0.5, 0.25, 0.125, 0.06, 0.03, 0.016, ≤0.004
- Inhibition zone diameter (mm)

### Tetracycline 30 µg vs. Doxycycline MIC

- MIC (mg/L): 4, 2, 1, 0.5, 0.25, 0.125
<table>
<thead>
<tr>
<th></th>
<th>Broth microdilution (BMD)*</th>
<th>Disk diffusion</th>
</tr>
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<tbody>
<tr>
<td><strong>Media</strong></td>
<td>MH-F broth</td>
<td>MH-F agar</td>
</tr>
<tr>
<td><strong>Inoculum</strong></td>
<td>5 x 10^5 CFU/mL</td>
<td>McFarland 0.5</td>
</tr>
<tr>
<td><strong>Incubation</strong></td>
<td>16-20 h</td>
<td>16-20 h</td>
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<tr>
<td></td>
<td>35±1°C</td>
<td>35±1°C</td>
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<tr>
<td></td>
<td>Ambient air (sealed panels)</td>
<td>5% CO₂</td>
</tr>
</tbody>
</table>

* According to ISO 20776-1 but with EUCAST MH-F broth

**MH-F broth** = MH broth with 5% lysed horse blood and 20 mg/L β-NAD
**MH-F agar** = MH agar with 5% defibrinated horse blood and 20 mg/L β-NAD
MH-F broth

- Mueller-Hinton broth with 5% lysed horse blood and 20 mg/L β-NAD

- Mechanically defibrinated horse blood is lysed by repeated freezing and thawing followed by centrifugation.
  - Important with clear broth to facilitate reading MIC endpoints.

- Developed by EUCAST to be used for *S. pneumoniae*, other streptococci, *H. influenzae* and several other fastidious organisms.
  - Both 20 mg/L β-NAD and 5% lysed horse blood are needed to support sufficient growth of *H. influenzae*. 
Reading BMD for *H. influenzae*

Positive control
Reproducibility of BMD for *H. influenzae*

<table>
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<tr>
<th>Isolate</th>
<th>AMC</th>
<th>AMO</th>
<th>AMP</th>
<th>CTX</th>
<th>CRO</th>
<th>CXM</th>
<th>IMI</th>
<th>MER</th>
<th>CIP</th>
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<td>2</td>
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<td>≤0.015</td>
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<td>0.5</td>
<td>≤0.06</td>
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</table>
Gradient tests for *H. influenzae* and beta-lactam agents

Essential agreement for gradient tests vs. BMD for *H. influenzae* with various PBP3 mutations and beta-lactam agents.

17 isolates tested on MH-F from two manufacturers (n=34 per antimicrobial agent).

<table>
<thead>
<tr>
<th>Reading of results</th>
<th>Gradient test brand</th>
<th>Essential agreement (%)</th>
<th>Excluding cefuroxime, imipenem and meropenem</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>All agents</td>
<td></td>
</tr>
<tr>
<td>According to manufacturer¹</td>
<td>Etest</td>
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<td>83</td>
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<td>MICE</td>
<td>70</td>
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<td>First inhibition²</td>
<td>Etest</td>
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<td>MICE</td>
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<tr>
<td></td>
<td>MTS</td>
<td>80</td>
<td>93</td>
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</tbody>
</table>

1. According to manufacturers’ instructions with growth of small colonies within the ellipse and regrowth taken into account
2. First inhibition of growth. Small colonies within the ellipse and regrowth disregarded.

**Agents tested**
- Ampicillin
- Cefepime
- Cefotaxime
- Ceftriaxone
- Cefuroxime
- Imipenem
- Meropenem

Disk diffusion of *H. influenzae*

- **MH-F**: Mueller-Hinton Fastidious agar
  - 5% defibrinated horse blood and 20 mg/L β-NAD

- The MH-F agar was developed to ensure good growth of *S. pneumoniae*, other streptococci and *H. influenzae*
  - Mechanically defibrinated horse blood
  - β-NAD with purity of ≥98%

- Excess humidity in agar plates can cause problems with haze within zones and fuzzy zone edges.
  - No drops of water should be visible on the agar surface or inside the lid
Excess humidity of agar plates - example with MH-F

In-house produced plate stored in ventilated rack
- Clear zone edge

Commercial plate stored in plastic bag
- Fuzzy zone edge
- Regrowth close to disk

If necessary, dry plates either at 20-25°C overnight, or at 35°C, with the lid removed, for 15 min.
Inhibition zone diameter (mm)

**H. influenzae** NCTC 8468 with cefotaxime 5 µg

Storage of MH-F plates in ventilated racks and drying plates in room temperature over night before inoculation has reduced reading problems and resulted in larger zones!
Screening for beta-lactam resistance in *H. influenzae* (EUCAST BP table v 9.0, 2019)

**Disk diffusion test with benzylpenicillin 1 unit disk**

Always perform in parallel with testing of other beta-lactam agents

- **Zone diameter ≥ 12 mm**
  - Excludes all beta-lactam resistance mechanisms
  - Report susceptible (S) to any beta-lactam agents for which clinical breakpoints are available, including those with “Note”.

- **Zone diameter < 12 mm**
  - Beta-lactamase and/or PBP3 mutations
  - **Ampicillin, amoxicillin and piperacillin (without beta-lactamase inhibitor)**
    - **Beta-lactamase positive**
      - Report resistant (R)
    - **Beta-lactamase negative**
      - Report susceptibility according to the clinical breakpoints for the agent in question

- **Other beta-lactam agents**
  - except cefepime, cefpodoxime and imipenem*

*For cefepime, cefpodoxime and imipenem, if resistant by both screen and agent disk diffusion test, report resistant. If resistant by screen test and susceptible by agent disk diffusion test, determine the MIC of the agent and interpret according to breakpoints.
Benzylpenicillin 1 unit vs. \(\beta\)-lactam resistance mechanism

- No \(\beta\)-lactam resistance
- PBP3 mutations only
- \(\beta\)-lactamase only
- \(\beta\)-lactamase + PBP3 mutations
Screening for beta-lactam resistance in *H. influenzae*

**PCG 1 unit vs. Ampicillin MIC**

- No of observations
- Inhibition zone diameter (mm)

**PCG 1 unit vs. Amoxicillin-clav MIC**

- No of observations
- Inhibition zone diameter (mm)

**PCG 1 unit vs. Cefotaxime MIC**

- No of observations
- Inhibition zone diameter (mm)

**PCG 1 unit vs. Meropenem MIC**

- No of observations
- Inhibition zone diameter (mm)
Stability of benzylpenicillin 1 unit disks over more than 5 years

H. influenzae ATCC 49766 with benzylpenicillin 1 unit
Amoxicillin-clavulanic acid 2-1 µg vs. MIC
*H. influenzae*, 167 isolates (360 correlates)
Amoxicillin-clavulanic acid 2-1 µg disk diffusion per beta-lactam resistance mechanism

**No β-lactam resistance**

**β-lactamase positive only**

**PBP3 mutations only**

**β-lactamase positive + PBP3 mutations**

- **MIC (mg/L)**
  - No observations
  - Inhibition zone diameter (mm)
  - MIC (mg/L)
    - 1
    - 0.5
    - 0.25
    - ≤0.125

- **No of observations**

- **Inhibition zone diameter (mm)**

- **ESCMID eLibrary**

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Amoxicillin-clavulanic acid

- Better separation with other combinations of amoxicillin and clavulanic acid?
  - Standard 20-10 µg disk results in poorer separation
  - Additional combinations of amoxicillin and clavulanic acid under investigation

- Better results with specific reading instructions for amoxicillin-clavulanic acid?
  - Look carefully and take all growth into account
  - Disregard thin growth and read the outer zone

- Improved screening test?
  - To differentiate between isolates with β-lactamase only and those with β-lactamase and PBP3 mutations
**H. influenzae** and ATU

- Only for beta-lactam agents
- Only for PCG screen-positive isolates

**Agents with ATU for H. influenzae**
- Ampicillin, amoxicillin-clavulanic acid and piperacillin-tazobactam
- Cefepime, cefotaxime, cefpodoxime, ceftriaxone and cefuroxime
- Imipenem
Ampicillin 2 µg vs. MIC
*H. influenzae*, 166 isolates (305 correlates)

- MIC (mg/L):
  - ≥64
  - 32
  - 16
  - 8
  - 4
  - 2
  - 1
  - 0.5
  - 0.25
  - 0.125
  - ≤0.06

Inhibition zone diameter (mm) vs. No of observations
Piperacillin-tazobactam 30-6 µg vs. MIC

*H. influenzae*, 149 isolates

- ATU

**No of observations**

**MIC (mg/L)**
- 0.5
- 0.25
- 0.125
- 0.06
- 0.03
- ≤0.016
Cefotaxime 5 µg vs. MIC
*H. influenzae*, 198 isolates (309 correlates)
Imipenem 10 µg vs. MIC
*H. influenzae*, 150 isolates (162 correlates)
Standardisation and quality of materials

• All AST must be standardised to get reproducible and reliable results!
  – EUCAST methodology documents and instruction videos
  – Frequent quality control

• Use materials of good quality (good performance according to EUCAST QC criteria)
  – Evaluation of antimicrobial disks, Åhman et al., CMI 25 (2019):345-352
Thanks!

- Jenny Åhman, Amra Basic, Onur Karatuna and Gunnar Kahlmeter, EUCAST Development Laboratory.

- Charlotta Karlsson, Agota Varga and Stina Bengtsson Clinical Microbiology, Växjö and Karlskrona, Sweden.

- EUCAST Network Laboratories contributing with data and/or isolates.
• Check the EUCAST website regularly for updates on methodology, QC ranges and breakpoints.

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• For questions and comments, please contact erika.matuschek@escmid.org or the EUCAST secretariat (see website).