Update on EUCAST susceptibility testing

Dr Mandy Wootton
What antibiotics do for us!
The Problem with Infections

• 35,000 or 7% of all deaths in the UK are caused by infectious diseases.

• 66 different antibiotics prescribed - top 15 account for 98% in general practice and 88% in hospitals.

• 35 million courses of antibiotics are prescribed by GPs in England each year.

• Without antimicrobials, the rate of post-operative infection for clean surgery could be 0-50% and that about 30% of those with a serious infection will die.
So we have some resistant bacteria?

- **25,000** people per year in Europe die of sepsis caused by resistant bacteria

- **23,000** deaths per year from sepsis caused by resistant bacteria in United States (conservative estimate)

- **1 child every 5 minutes** dies of infection caused by resistant bacteria in South East Asia
Increasing cost of AMR

Estimates of Burden of Antibacterial Resistance

**European Union**
- population 500m
- 25,000 deaths per year
- 2.5m extra hospital days

**Overall societal costs**
- € 900 million, hosp. days
- Approx. €1.5 billion per year

*Source: ECDG 2007*

**Thailand**
- population 70m
- >38,000 deaths
- >3.2m hospital days

**Overall societal costs**
- US$ 84.6–202.8 mill. direct
- >US$1.3 billion indirect

*Source: Pumart et al 2012*

**United States**
- population 300m
- >23,000 deaths
- >2.0m illnesses

**Overall societal costs**
- Up to $20 billion direct
- Up to $35 billion indirect

*Source: US CDC 2013*

Surveillance needs good quality, comparable susceptibility testing results
Harmonisation of breakpoints – we can now agree if something is resistant

Enterobactericeae 1975 - 2001

<table>
<thead>
<tr>
<th>Committee</th>
<th>Amoxicillin</th>
<th>Cefotaxime</th>
<th>Piperacillin/ tazobactam</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSAC (UK)</td>
<td>8 / 16</td>
<td>2 / 2</td>
<td>16 / 16</td>
</tr>
<tr>
<td>CA-SFM (Fr)</td>
<td>4 / 16</td>
<td>4 / 32</td>
<td>8 / 64</td>
</tr>
<tr>
<td>CRG (NL)</td>
<td>2 / 16</td>
<td>4 / 8</td>
<td>0.25 / 4</td>
</tr>
<tr>
<td>DIN (Ger)</td>
<td>2 / 8</td>
<td>2 / 8</td>
<td>0.12 / 1</td>
</tr>
<tr>
<td>NWGA (Nor)</td>
<td>0.5 / 8</td>
<td>1 / 2</td>
<td>8 / 16</td>
</tr>
<tr>
<td>SRGA (Sw)</td>
<td>1 / 8</td>
<td>0.5 / 1</td>
<td>16 / 16</td>
</tr>
<tr>
<td>NCCLS (USA)</td>
<td>8 / 16</td>
<td>8 / 32</td>
<td>16 / 64</td>
</tr>
</tbody>
</table>
How to determine susceptibility/resistance

Minimum Inhibitory Concentration

- Macro broth dilution
- Micro broth dilution
- Agar dilution
- Gradient Strips
- Automated systems

Category (S/I/R)

- Disc
- Breakpoint method
Standardisation

- Critical population of bacterial cells
  - Known inoculum
  - Defined media
- Defined media
  - Batch variation
  - pH
  - Gaseous environment

<table>
<thead>
<tr>
<th>Drug</th>
<th>5.5</th>
<th>6.5</th>
<th>7.5</th>
<th>8.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptomycin</td>
<td>+64</td>
<td>+16</td>
<td>1</td>
<td>-8</td>
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<tr>
<td>Gentamicin</td>
<td>+16</td>
<td>+4</td>
<td>1</td>
<td>-2</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>+16</td>
<td>+8</td>
<td>1</td>
<td>-2</td>
</tr>
<tr>
<td>Amikacin</td>
<td>+32</td>
<td>+4</td>
<td>1</td>
<td>-2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Drug</th>
<th>Increased - Acidic</th>
<th>Increased - Alkaline</th>
<th>Decreased - Acidic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amikacin</td>
<td></td>
<td></td>
<td>Azithromycin</td>
</tr>
<tr>
<td>Ampicillin</td>
<td></td>
<td></td>
<td>Clindamycin</td>
</tr>
<tr>
<td>Cloxacillin</td>
<td></td>
<td>Metronidazole</td>
<td>Metronidazole</td>
</tr>
<tr>
<td>Doxycycline</td>
<td></td>
<td>Clarithromycin</td>
<td></td>
</tr>
<tr>
<td>Minocycline</td>
<td></td>
<td>Erythromycin</td>
<td></td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td></td>
<td>Nalidixic acid</td>
<td></td>
</tr>
<tr>
<td>Piperacillin</td>
<td></td>
<td>Quinolones</td>
<td></td>
</tr>
<tr>
<td>Tetracycline</td>
<td></td>
<td>Tobramycin</td>
<td></td>
</tr>
</tbody>
</table>
Standardisation

- Defined media cont.
  - Cation concentration (Mg$^{2+}$ & Ca$^{2+}$)
  - Osmolarity (NaCl)
  - Thymine/thymidine

- Antimicrobial
  - Solvents with no antimicrobial activity

- Incubation temperature / time
  - Enterococci & glycopeptides
  - Staphylococci & cefoxitin
ON THE ANTIBACTERIAL ACTION OF CULTURES OF A PENICILLIUM, WITH SPECIAL REFERENCE TO THEIR USE IN THE ISOLATION OF B. INFLUENZÆ.

ALEXANDER FLEMING, F.R.C.S.

From the Laboratories of the Inoculation Department, St Mary’s Hospital, London.

Received for publication May 10th, 1929.

While working with staphylococcus variants a number of culture-plates were set aside on the laboratory bench and examined from time to time. In the examinations these plates were necessarily exposed to the air and they became contaminated with various micro-organisms. It was noticed that around a large colony of a contaminating mould the staphylococcus colonies became transparent and were obviously undergoing lysis (see Fig. 1).
Macrobroth dilution (tube dilution)

- Broth media (1-2mL)
- Antimicrobial dilution: log2
  - Control tube
- Inoculum; direct or growth
  - touching 2 to 5 morphologically similar colonies
  - 0.5 McFarland (10^8); dilute to achieve 10^5
  - Control organisms
- Incubation
- Reading

Minimum Inhibitory Concentration

British Infection Association – Trainees Day
Microbroth dilution

“Gold Standard”
Gradient strips

- Validated against MBD
- Easy to use
- Variety of manufacturers & antimicrobials
- QC
- Reading

British Infection Association – Trainees Day
Automated AST systems

- More reliable/consistent results
  - Closer to standard
  - Reduced scope for error
  - Reproducible
- Easy to use
- Speed of results
  - 2-2.5 hrs for ID, 4-7hrs for AST
- Gram neg, Gram pos, Yeast panels
- Large range of antimicrobials
Disc diffusion method

- Most used method
- Cheap & easy to perform
- No MIC
- Correlated against MIC method
- Governed by three dynamics

**Zone of inhibition formation:**
- Critical concentration: conc just capable of inhibiting growth & conc at zone edge at critical time
- Critical time: time it takes for critical conc to be reached
- Critical population: Number of bacterial cells found at the critical time at the ultimate zone edge
Disc diffusion method - Dynamics

Critical concentration
- Antimicrobial diffuses in a decreasing gradient
  - Diffusion rate
    - Depth of & osmolarity of agar
    - Initial concentration
    - Molecular size
    - Shape of drug
    - Charge of drug

Zone of inhibition formation:
Critical concentration: conc^n just capable of inhibiting growth & conc^n at zone edge at critical time
Critical time: time it takes for critical conc^n to be reached
Critical population: Number of bacterial cells found at the critical time at the ultimate zone edge
Disc diffusion method - Dynamics

Critical time
- 3 to 4 hours under standard conditions
- Standard: observable growth at 18hrs
- Longer for some bug:drug combinations
- Apply discs within 15-30mins after inoculation
- Incubate 15-30mins after application of discs

Zone of inhibition formation:
Critical concentration: conc^n just capable of inhibiting growth & conc^n at zone edge at critical time
Critical time: time it takes for critical conc^n to be reached
Critical population: Number of bacterial cells found at the critical time at the ultimate zone edge
Disc diffusion method - Dynamics

Critical population

- Known inoculum
  - Too high: quicker to critical population, overwhelms antimicrobial so smaller zone (FALSE RESISTANCE)
  - Too low: slower to critical population, antimicrobial overwhelms bacteria (FALSE SUSCEPTIBILITY)
- Known lag phase & generation times
- Most antimicrobial work on dividing cells

Zone of inhibition formation:
Critical concentration: conc^n just capable of inhibiting growth & conc^n at zone edge at critical time
Critical time: time it takes for critical conc^n to be reached
Critical population: Number of bacterial cells found at the critical time at the ultimate zone edge
Disc diffusion method – correlation with MIC
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British Infection Association – Trainees Day
Disc diffusion method – correlation with MIC

False sensitive rate 8.4% (14/166) [target ≤ 1%]

False resistance rate 0% [target ≤ 5%]

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Disc diffusion method – correlation with MIC
Disc diffusion method (BSAC)

- Antimicrobial in paper disc
- IsoSensitest agar (ISA) or ISA + 5% sheep blood + NAD
- Semi confluent inoculum by direct method
  - Touching 2-5 morphologically similar colonies
  - Dilute 0.5 McFarland

<table>
<thead>
<tr>
<th>Dilute 1:100</th>
<th>Dilute 1:10</th>
<th>No dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Haemolytic streptococci</td>
<td>Staphylococci</td>
<td>Neisseria gonorrhoeae</td>
</tr>
<tr>
<td>Enterococci</td>
<td>Serratia spp.</td>
<td>Campylobacter spp.</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>Streptococcus pneumoniae</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas spp.</td>
<td>Neisseria meningitidis</td>
<td></td>
</tr>
<tr>
<td>Stenotrophomonas maltophilia</td>
<td>Moraxella catarrhalis</td>
<td></td>
</tr>
<tr>
<td>Acinetobacter spp.</td>
<td>α-haemolytic streptococci</td>
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<tr>
<td>Haemophilus spp.</td>
<td>Clostridium perfringens</td>
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</tr>
<tr>
<td>Pasteurella multocida</td>
<td>Coryneform organisms</td>
<td></td>
</tr>
<tr>
<td>Bacteroides fragilis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteroides thetaiotaomicron</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Application of discs
- 15 minute rule
Disc diffusion method (BSAC)

- 18-20hr at 35-37°C in air
- VAN/TEIC resistance in enterococci: 24hr
- FOX resistance in Staphylococci: 35°C
- Campylobacter: 42°C microaerophilic for 24hr
- Fastidious orgs: 4-6% CO₂
- Anaerobes: 10% hydrogen/10%CO₂/80% Nitrogen

Reading
BSAC or EUCAST disc diffusion method

<table>
<thead>
<tr>
<th>Committee</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSAC</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>CA-SFM</td>
<td>France</td>
</tr>
<tr>
<td>CRG</td>
<td>The Netherlands</td>
</tr>
<tr>
<td>DIN</td>
<td>Germany</td>
</tr>
<tr>
<td>NWGA</td>
<td>Norway</td>
</tr>
<tr>
<td>SRGA</td>
<td>Sweden</td>
</tr>
<tr>
<td>NCCLS (CLSI)</td>
<td>USA</td>
</tr>
</tbody>
</table>

BSAC Susceptibility Testing Method

From January 2016, the British Society for Antimicrobial Chemotherapy (BSAC) will be supporting the EUCAST disc diffusion method for antimicrobial susceptibility testing. This is to help with the transition from the BSAC disc diffusion method to the EUCAST method.

- BSAC will continue to offer support and development of the BSAC disc diffusion method.
- EUCAST will support laboratories that continue to use the BSAC disc diffusion method during the transition period.
- EUCAST will launch a series of workshops to support the transition to the EUCAST disc diffusion method.
- BSAC will offer training and guidance on the EUCAST method.
- EUCAST will also continue to offer training and guidance on the BSAC disc diffusion method.

British Infection Association – Trainees Day
EUCAST disk diffusion test

- Based on a well-known technique (Kirby-Bauer)
- Calibrated to EUCAST MIC breakpoints
- Methodology, breakpoints and QC criteria are freely available on the EUCAST website
EUCAST susceptibility testing media

- **Mueller-Hinton agar (MH)**
  Enterobacteriaceae,
  Pseudomonas, staphylococci
  and enterococci

- **Mueller-Hinton agar with 5% defibrinated horse blood and 20 mg/L β-NAD (MH-F)**
  for fastidious organisms: *S. pneumoniae* and other streptococci, Haemophilus, Moraxella, Pasteurella,
  Listeria, Campylobacter, and Corynebacterium
Inoculum and incubation

- The method requires an inoculum suspension equivalent to a 0.5 McFarland standard (0.85% saline)

- Inoculation should result in confluent growth without being too heavy

- Incubation for 16-20 h at 35±1°C
  - MH plates in air
  - MH-F plates in 5% CO₂
The 15-15-15 minute rule

- Use the inoculum within **15 minutes** of preparation – and always within 60 minutes
- Apply disks within **15 minutes** of inoculating plates
- Start incubation within **15 minutes** of application of disks

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The growth should be confluent and evenly spread over the plate

Plates should look like this..  ..and NOT like this!

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Reading of zones

- MH plates
  Read zones from the back of the plate against a dark background and illuminated with reflected light

- MH-F plates
  Read zones from the front of the plate with the lid removed and illuminated with reflected light
Reading of zones

Read zone edges at the point where no obvious growth is detected by the unaided eye with the plate held about 30 cm from the eye.

Examples:

- *E. coli*
  - Ciprofloxacin
- *S. aureus*
  - Erythromycin
- CoNS
  - Trimethoprim
- *S. pneumoniae*
  - Rifampicin

Reading guide available at [www.eucast.org](http://www.eucast.org)
Stenotrophomonas maltophilia and trimethoprim-sulfamethoxazole

- Ignore growth within the inhibition zone, which is common for Stenotrophomonas maltophilia and trimethoprim-sulfamethoxazole. The density of growth in the zone may vary from a fine haze to substantial growth.

Ignore growth and read an inhibition zone if any zone edge can be seen.

= Susceptible if zone diameter ≥ 16 mm

Growth up to the disk and no sign of inhibition zone = Resistant
Enterococci and vancomycin

- Examine with transmitted light (plate held up to light).
  - Fuzzy zone edges and colonies within zone indicate vancomycin resistance. If the zone diameter is ≥ 12 mm and the zone edge is fuzzy, investigate further.

E. faecalis
non-VRE

E. faecium
VRE
S. aureus and benzylpenicillin

- Examine with transmitted light (plate held up to light).
  - Disk diffusion is more reliable than MIC for detection of penicillinase producers, provided the zone diameter is measured AND the zone edge closely inspected.

- **S. aureus** with **sharp zone edge and zone diameter ≥ 26 mm** = Resistant
- **S. aureus** with **fuzzy zone edge and zone diameter ≥ 26 mm** = Susceptible
Developing breakpoints for new agents (with EMA)

- New glycopeptides
  - Dalbavancin (approved)
  - Oritavancin (approved)
- New oxazolidinone agent
  - Tedizolid (approved)
- β-lactam-inhibitor agents
  - Ceftolozane-tazobactam (in process)
  - Ceftazidime-avibactam (in process)
  - Carbapenem-inhibitor (in development)
Existing breakpoints under review

- Carbapenems
  - Imipenem, meropenem, ertapenem
  - Doripenem withdrawn in Europe
- Colistin
- Tigecycline for Enterobacteriaceae
- Fluoroquinolones

- Change in dosing or administration
- Change in indications
- Change in target organisms
- New clinical Pk/Pd data
- New resistance mechanisms
- New agents in class
Agents without EUCAST breakpoints and under consideration

- Temocillin
- Nitroxoline

Expected 2016
Organisms with no breakpoints and EUCAST test

- Aerococcus spp.
- Kingella kingae
- Nocardia spp.
- Aeromonas spp.
- Vibrio spp.
- Streptomyces spp.
- Leuconostoc spp.
- Lactobacillus spp.
- Pediococcus spp.
- Other HACEK organisms

On-going

No disc method
- Neisseria species
- Anaerobes

From 2016

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Aerococcus spp. and Kingella kingae
Disc diffusion

- EUCAST disk diffusion method for fastidious organisms
  - MH-F agar
  - McFarland 0.5
  - 5% CO₂
  - 16-20 h incubation
    - with possibility to prolong incubation to 40-44 h if growth is not-sufficient

- Calibration vs. broth microdilution & agar dilution
  - MH-F broth / Mueller Hinton agar
Difficult tests: Fosfomycin

- Fosfomycin is difficult to test with all methods
  - Glucose-6-phosphate is added to improve results

- Agar dilution as reference

- Gradient tests
  - Etest: Disregard colonies if < 5
  - No specific reading instruction for MIC Test Strip
  - M.I.C.E not available

- No EUCAST disk test
AST results vs. WGS

"Resistance genes NOT likely, but results are uncertain"

"Resistance genes likely, but results are uncertain"
Early reading of disc diffusion tests

Examples with *E. coli* and cefotaxime 5 µg

- **6 h incubation**
- **8 h incubation**
- **16-20 h incubation**

- Poorer separation between wild type and non-wild type with short incubation time
- Testing of additional isolates with known resistance mechanisms is on-going

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Other recent recommendations

- Improved screen for beta-lactam resistance in *S. pneumoniae*

- Screen for fluoroquinolone resistance in *Salmonella* spp. with the pefloxacin 5 µg disk
### β-lactam resistance in *S. pneumoniae*

<table>
<thead>
<tr>
<th><strong>Oxacillin 1 µg</strong></th>
<th><strong>Agent</strong></th>
<th><strong>Further testing and/or interpretation</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>≥ 20 mm</td>
<td>All β-lactams</td>
<td>Report S to all agents with clinical breakpoints</td>
</tr>
<tr>
<td></td>
<td>Benzylpenicillin</td>
<td>Meningitis: Report resistant Other infections: Determine the MIC</td>
</tr>
<tr>
<td>&lt; 20 mm</td>
<td>Ampicillin, amoxicillin and piperacillin (± inhibitors)</td>
<td>OXA ≥ 8 mm: Report S. In meningitis, consider determining the MIC.</td>
</tr>
<tr>
<td></td>
<td>Cefepime, cefotaxime, ceftaroline, ceftobiprole and ceftriaxone</td>
<td>OXA &lt; 8 mm: Determine the MIC</td>
</tr>
<tr>
<td></td>
<td>Other β-lactams</td>
<td>Determine the MIC</td>
</tr>
</tbody>
</table>

**EUCAST Breakpoint Table v 5.0**
**β-lactam resistance in S. pneumoniae**

Oxacillin 1 µg vs. Ampicillin MIC  
*S. pneumoniae*, 153 clinical isolates

All isolates with oxacillin 1 µg ≥ 8 mm can be reported susceptible without further testing

**Breakpoints**

- **Ampicillin MIC**  
  S≤0.5, R>2 mg/L  
  ECOFF  
  WT≤0.06 mg/L

- **Oxacillin zone diameter (screen)**  
  S≥8 mm

British Infection Association – Trainees Day
Fluoroquinolone resistance in *Salmonella* spp.

Enterobacteriaceae

<table>
<thead>
<tr>
<th>Fluoroquinolones</th>
<th>MIC breakpoint (mg/L)</th>
<th>Disk content (µg)</th>
<th>Zone diameter breakpoint (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S ≤</td>
<td>R &gt;</td>
<td>S ≥</td>
</tr>
<tr>
<td><strong>Ciprofloxacin, <em>Salmonella</em> spp.</strong></td>
<td>0.06</td>
<td>0.06</td>
<td>Note(^A)</td>
</tr>
<tr>
<td><strong>Pefloxacin (screen), <em>Salmonella</em> spp.</strong></td>
<td>NA</td>
<td>NA</td>
<td>5</td>
</tr>
</tbody>
</table>

A. Susceptibility of *Salmonella* spp. to ciprofloxacin can be inferred from the pefloxacin disk diffusion susceptibility test result.

EUCAST Breakpoint Table v 5.0
Fluoroquinolone resistance in *Salmonella* spp.

Pefloxacin 5 µg vs. FQ resistance mechanism

*Salmonella* spp., 126 isolates (1044 readings)

3 test sites
Media from 4 manufacturers
Disks from 3 manufacturers
6 readers

FQ resistance mechanism

- aac6
- QRDR
- qnr
- None

British Infection Association – Trainees Day
Fluoroquinolone resistance in *Salmonella* spp.

Pefloxacin 5 µg vs. Ciprofloxacin MIC
*Salmonella* spp., 126 isolates (1044 readings)

- 3 test sites
- Media from 4 manufacturers
- Disks from 3 manufacturers
- 6 readers