Screening for fluoroquinolone resistance in *Staphylococcus aureus* and *Streptococcus spp.* using norfloxacin and EUCAST disk diffusion criteria

P1572

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**Introduction**

EUCAST recommends the use of the norfloxacin (NOR) 10-µg disk to screen for fluoroquinolone (FQ) resistance in *Staphylococcus* spp., *Streptococcus pneumoniae* and *Streptococcus* groups A, B, C and G. Norfloxacin screen negative isolates (wild type for NOR) can be considered devoid of FQ resistance mechanisms. These can be reported susceptible to all FQs with clinical breakpoints, with the exception of *S. pneumoniae* where wild-type (WT) isolates should be reported intermediate for ofloxacin (not shown) and ciprofloxacin. Norfloxacin screen positive isolates (non-wild type for NOR) should be tested for susceptibility to individual agents. The norfloxacin screen is a useful tool in areas where FQ resistance is still uncommon in *Staphylococcus* and *Streptococcus* spp.

**Objectives**

The objective of this study was to evaluate the norfloxacin 10-µg disk as a screen for fluoroquinolone resistance using EUCAST criteria for *Staphylococcus* spp. (≤17 mm) and *Streptococcus* spp. (≤12 mm).

**Methods**

Clinical isolates of *S. aureus* (98), *S. pneumoniae* (100), *S. pyogenes* (33) and *S. agalactiae* (32), were selected from the SENTRY collection (JMI Laboratories, USA). These included isolates susceptible and resistant to relevant FQs. MIC values were determined by broth microdilution, according to the ISO standard 20776-1, at the EUCAST Laboratory for *S. aureus* and at JMI Laboratories for *streptococci*. For *streptococci*, Mueller-Hinton broth was supplemented with 5% lysed horse blood (by repeated freezing and thawing) and 20 mg/L β-NAD (MH-F broth). All disk diffusion tests were performed at the EUCAST Laboratory according to EUCAST methodology using Mueller-Hinton agar (≤5% defibrinated horse blood and 20 mg/L β-NAD) from two manufacturers (BD and Oxoid/Thermo Fisher Scientific). The NOR disk screen test was evaluated by correlating NOR zone diameters to MICs for ciprofloxacin, levofloxacin and moxifloxacin, respectively.

**Results**

All NOR screen negative isolates were categorised by MIC as wild type for all other FQs investigated (Table 1). Of the NOR screen positive isolates, 8 of 40 *S. aureus*, 11 of 22 *S. pneumoniae* and 1 of 2 *S. pyogenes* were reported wild type according to MIC for one or more FQs. Of these isolates, three *S. aureus*, seven *S. pneumoniae* and one *S. pyogenes* were wild type for all FQs investigated. Inhibition zone diameter distributions for norfloxacin with *S. aureus* and *S. pneumoniae* are shown in Figure 1.

**Table 1. Number of isolates categorised as wild type and non-wild type for norfloxacin and different fluoroquinolones.**

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Ciprofloxacin WT</th>
<th>Ciprofloxacin non-WT</th>
<th>Levofloxacin WT</th>
<th>Levofloxacin non-WT</th>
<th>Moxifloxacin WT</th>
<th>Moxifloxacin non-WT</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em> (n=98)</td>
<td>58 0 58 0 58 0 58 0</td>
<td>3 37 3 30 8 32 8 32</td>
<td>78 0 78 0 78 0</td>
<td>78 0 78 0 78 0</td>
<td>78 0 78 0 78 0</td>
<td>78 0 78 0 78 0</td>
</tr>
<tr>
<td><em>S. pneumoniae</em> (n=100)</td>
<td>58 0 58 0 58 0</td>
<td>3 37 3 30 8 32 8 32</td>
<td>78 0 78 0 78 0</td>
<td>78 0 78 0 78 0</td>
<td>78 0 78 0 78 0</td>
<td>78 0 78 0 78 0</td>
</tr>
<tr>
<td><em>S. pyogenes</em> (n=33)</td>
<td>78 0 78 0 78 0</td>
<td>78 0 78 0 78 0</td>
<td>78 0 78 0 78 0</td>
<td>78 0 78 0 78 0</td>
<td>78 0 78 0 78 0</td>
<td>78 0 78 0 78 0</td>
</tr>
<tr>
<td><em>S. agalactiae</em> (n=32)</td>
<td>78 0 78 0 78 0</td>
<td>78 0 78 0 78 0</td>
<td>78 0 78 0 78 0</td>
<td>78 0 78 0 78 0</td>
<td>78 0 78 0 78 0</td>
<td>78 0 78 0 78 0</td>
</tr>
<tr>
<td><em>S. aureus</em> WT (n=27)</td>
<td>78 0 78 0 78 0</td>
<td>78 0 78 0 78 0</td>
<td>78 0 78 0 78 0</td>
<td>78 0 78 0 78 0</td>
<td>78 0 78 0 78 0</td>
<td>78 0 78 0 78 0</td>
</tr>
<tr>
<td><em>S. pneumoniae</em> WT (n=20)</td>
<td>78 0 78 0 78 0</td>
<td>78 0 78 0 78 0</td>
<td>78 0 78 0 78 0</td>
<td>78 0 78 0 78 0</td>
<td>78 0 78 0 78 0</td>
<td>78 0 78 0 78 0</td>
</tr>
<tr>
<td><em>S. pyogenes</em> WT (n=32)</td>
<td>78 0 78 0 78 0</td>
<td>78 0 78 0 78 0</td>
<td>78 0 78 0 78 0</td>
<td>78 0 78 0 78 0</td>
<td>78 0 78 0 78 0</td>
<td>78 0 78 0 78 0</td>
</tr>
</tbody>
</table>

WT = Wild Type,  = Not analysed due to lack of FQ breakpoints.

**Conclusions**

For *S. aureus* and *Streptococcus spp.*, the EUCAST norfloxacin screen test reliably predicts susceptibility to all fluoroquinolones with clinical breakpoints. However, the test overcalls resistance and norfloxacin screen positive isolates must be tested for susceptibility to individual fluoroquinolones, which is also supported by this study. Due to the high sensitivity and low specificity, the norfloxacin screen test is useful only in areas with low prevalence of fluoroquinolone resistance in relevant species.

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![Figure 1. Inhibition zone diameter distributions for norfloxacin 10 µg with *S. aureus* (n=98) to the left and *S. pneumoniae* (n=100) to the right. MIC values for different FQs are shown as coloured bars. EUCAST screen breakpoints for norfloxacin (≤17 mm for *S. aureus* and ≤12 mm for *S. pneumoniae*) are shown as dotted lines.](image-url)
Improved screen for beta-lactam resistance in *Streptococcus pneumoniae* with the oxacillin 1-µg disk using the EUCAST disk diffusion method

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**Introduction**

EUCAST recommends screening with the oxacillin 1-µg disk to detect beta-lactam non-susceptibility in *Streptococcus pneumoniae*. The disk provides sensitive screening for all degrees of beta-lactam non-susceptibility (oxacillin zone diameter <20 mm), but has hitherto been used only to distinguish between isolates belonging to the wild type (WT) and those with any acquired beta-lactam resistance mechanisms.

**Objective**

The objective of this study was to investigate if the oxacillin screen could be further optimised to reduce additional laboratory testing for non-wild type *S. pneumoniae*.

**Methods**

Clinical isolates of *S. pneumoniae* (n=100) with a variety of beta-lactam resistance mechanisms were selected from the SENTRY collection (JMI Laboratories, USA). MIC values were determined for beta-lactam agents by broth microdilution (BMD) at JMI Laboratories according to the ISO standard 20776-1, 2006. The Mueller-Hinton broth was supplemented with 5% lysed horse blood (by repeated freezing and thawing) and 20 mg/L β-NAD (MH-F broth). Disk diffusion was performed at the EUCAST Laboratory according to EUCAST methodology for fastidious organisms on in-house prepared MH-F plates (Mueller-Hinton agar + 5% defibrinated horse blood and 20 mg/L β-NAD) using agar from two manufacturers (BD and Oxoid/Thermo Fisher Scientific). Data analysis was performed by EUCAST.

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**Results**

Benzylpenicillin MICs for *S. pneumoniae* ranged from ≤0.008 to 4 mg/L (Table 1).

Table 1. Benzylpenicillin susceptibility vs. oxacillin 1-µg disk diameter

<table>
<thead>
<tr>
<th>Benzylpenicillin susceptibility</th>
<th>MIC (mg/L)</th>
<th>No of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible (n=40)</td>
<td>≤0.008</td>
<td>9</td>
</tr>
<tr>
<td>Intermediate (n=55)</td>
<td>0.12-2</td>
<td>44</td>
</tr>
<tr>
<td>Resistant (n=5)</td>
<td>≥4</td>
<td>0</td>
</tr>
</tbody>
</table>

All isolates with oxacillin zones ≥20 mm (n=30) were wild type and categorised as susceptible to all investigated beta-lactam agents. Furthermore, isolates with oxacillin zones ≥8 mm (n=50) were all fully susceptible to ampicillin, amoxicillin, amoxicillin-clavulanate, cefepime, cefotaxime and ceftriaxone (Figure 1). The suggested algorithm for the oxacillin screen test is presented in Table 2.

Table 2. Improved screen for beta-lactam resistance in *S. pneumoniae* with the oxacillin 1-µg disk

<table>
<thead>
<tr>
<th>Oxacillin 1-µg disk zone diameter</th>
<th>Antimicrobial agent</th>
<th>MIC (µg)</th>
<th>Further testing and/or interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤16 mm</td>
<td>&lt;20 µg</td>
<td>Report susceptibility irrespective of clinical breakpoints</td>
<td></td>
</tr>
<tr>
<td>Intermediate zone (16-20 mm)</td>
<td>Ampicillin (without and with beta-lactam inhibitor), cefotaxime, ceftriaxone</td>
<td>Determine MIC and interpret according to the clinical breakpoints</td>
<td></td>
</tr>
<tr>
<td>≤16 mm</td>
<td>Ceftriaxone (without and with beta-lactam inhibitor)</td>
<td>Report susceptible</td>
<td></td>
</tr>
</tbody>
</table>

Conclusions

Based on the oxacillin 1-µg disk screen, we present an improved algorithm to predict susceptibility to clinically important beta-lactam agents in non-wild type *S. pneumoniae*. This reduces laboratory work and allows susceptibility test results to be released earlier. The new screening criteria are available in the EUCAST Clinical Breakpoint Table v 3.1, 2013.

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**Figure 1. Inhibition zone diameter distributions for *S. pneumoniae* with oxacillin 1 µg.** MIC values for different beta-lactam agents are shown as coloured bars, EUCAST improved oxacillin 1-µg screen breakpoints (≤20 mm for benzylpenicillin and ≤8 mm for the other agents) are shown as dotted lines. S=Susceptible, R=Resistant, WT=Wild Type.
Introduction

Ceftaroline, the active metabolite of ceftaroline fosamil, is a novel cephalosporin with activity against many methicillin-resistant Staphylococcus aureus (MRSA) due to enhanced affinity for the penicillin-binding protein 2a (PBP2a). Recently, EUCAST established clinical MIC breakpoints for ceftaroline vs. S. aureus, S≤1 mg/L and R=1 mg/L.

Objectives

The objectives of this study were to evaluate ceftaroline 5-, 10- and 30-µg disks and to establish disk diffusion breakpoints and quality control criteria for S. aureus.

Methods

Antimicrobial susceptibility testing was performed during 2012 on a challenge set of 100 S. aureus, including a large proportion of MRSA. MIC determination was performed at one site with broth microdilution according to the ISO standard 20776-1, 2006. A consensus MIC was calculated from a minimum of three separate tests for each isolate. Disk diffusion was performed on all isolates at two sites with ceftaroline disks (5-100 μg) according to EUCAST methodology. Meca-positive isolates were confirmed with PCR with MICs close to the EUCAST breakpoint (n=17) were tested for reproducibility at both sites. Disk diffusion was also performed on additional isolates of S. aureus (n=180), categorised as MSSA (mecillin susceptible) or MRSA (mecillin resistant) according to EUCAST and CLSI MRSA screening. A tentative quality control (QC) range for S. aureus ATCC 29213 and ceftaroline 5 μg was established by a two-site study. Disk diffusion was performed with ceftaroline disks from two manufacturers (Mast Diagnostics and Oxoid/Thermo Fisher Scientific) and Mueller-Hinton (MH) agar from four manufacturers (BD, bioMérieux, Bio-Rad and Oxoid/Thermo Fisher Scientific). Data from five additional European laboratories were used to validate the tentative QC range.

Results

Zone diameter breakpoints

Ceftaroline MICs ranged from 0.12 to 4 mg/L. The ceftaroline 5-µg disk provided better separation between susceptible (≤1 mg/L) and resistant (>1 mg/L) isolates than the other disk concentrations (data not shown) and supported zone diameter breakpoints of S≤20 mm, R=20 mm (Figure 1a). Discrepant results on 21 and 22 mm belonged to one single isolate. The suggested zone diameter breakpoints were further supported by data on wild-type isolates (MSSA, Figure 1b) and data from repeated testing (MIC and zone diameter) of mecA-positive isolates with MICs close to the breakpoint using disks and MH agar from several manufacturers (Figure 1c). However, the breakpoints bisect the MIC distribution of MRSA (Figure 1d) and we suggest that susceptibility is confirmed with an MIC determination for isolates with zone diameters of 19-21 mm.

Quality control criteria

Data from two sites on S. aureus ATCC 29213 with ceftaroline 5 μg corresponded well, and a tentative range of 24-30 mm (target 27 mm) was established. Differences between disks and MH agar from different manufacturers were small (Figure 2). Readings at the five additional laboratories on local Mueller-Hinton plates were normally distributed with a mean of 27 mm (=EUCAST target), and only 3/50 readings were out of range (all 23 mm).

Conclusions

Following the analysis of MIC-inhibition zone correlates, the 5 µg disk was chosen for EUCAST disk diffusion testing of ceftaroline. Zone diameter breakpoints for S. aureus with ceftaroline were set at S≤20 mm, R=20 mm. The QC range for S. aureus ATCC 29213 was set at 24-30 mm with a target of 27 mm. Clinical breakpoints QC criteria for ceftaroline are now available in the EUCAST Tables on www.eucast.org.
EUCAST objectives
EUCAST was set up to standardize susceptibility testing in Europe so that comparable results and interpretations are produced.

EUCAST structure
EUCAST is a standing ESCMID committee, funded through a contract with ECDC, with additional input, particularly related to the disk diffusion method, from ESCMID. It was formed in 1997 and restructured at the ECDCM in Milan 2002. It consists of a General Committee, with representatives from all European Union and some non-European Union countries, and is led by an ESCMID-appointed Steering Committee, which includes: Chairman, Scientific Secretary, Clinical Data Coordinator, five National Breakpoint committees and three representatives of the EUCAST General Committee. Decisions are made by the Steering Committee after consultation with the General Committee and expert groups.

Committee appointments by ESCMID
- Chairman, Scientific Secretary and Clinical Data Coordinator (three years)
- National breakpoint committee seats on the Steering Committee (three years)
- Three representatives from the EUCAST general committee from countries not otherwise represented on the steering committee (two years).

Committee appointments by countries
- Each country with an active national breakpoint committee appoints one representative to serve for 3 years on the Steering Committee (active national committees only, i.e. committees with >2 meetings per year, are eligible)
- Countries without representatives on the Steering Committee may apply to the ESCMID board for a position on the Steering Committee to serve for 2 years
- Each country appoints one representative to serve on the General Committee (no set term of office).

EUCAST General Committee
- Australia J Turtridge
- Austria P Aptlter
- Belgium J Verhaegen
- Bosnia S Uzunovic-Kamberovic
- Bulgaria K Metodiev
- Croatia A Tambic-Andrasovic
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- Latvia A Balode
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- Luxembourg M Perrin
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- Slovenia I Strumbelj
- Spain L Martinez Martinez
- Sweden B Olsson-Lileqvist
- Switzerland R Zbinden
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- UK A McGowan
- ISC P Tulenko
- FESCI D Livermore

EUCAST steering committee (SC)
Chairman Rafael Canton (to 2014)
Scientific secretary Martin Steinbakn (to 2014)
Clinical Data Co-ordinator Gunnar Kahlmeter (to 2014)
BSAC (UK), Alasdair MacGowan (to 2014)
CA-IFSM (France), Claude-James Saussy / Luc Dubreuil (to 2014)
CRG (Netherlands), Johan Mouton (to 2014)
NIVA (Norway), Martin Steinbakn (to 2014)
SRGA (Sweden), Christian Giske (to 2014)
EUCAST GC representatives
Petra Aptlter, Austria (to 2013)
Luis Martinez Martinez, Spain (to 2014)
Robert Skov, Denmark, (to 2014)
“Visiting” members from General Committees
- Visiting GC member arrangements were implemented for 2013. Any national representative can attend up to two SC meeting per year as a visiting member. A maximum of two visiting members may attend each meeting. In March 2013 ESCMID agreed to provide up to five travel grants per year to support visiting GC members.

EUCAST subcommittees
- Antifungal Susceptibility Testing
- The Antifungal Susceptibility Testing (AFST) subcommittee is a standing EUCAST subcommittee with a mandate of improving breakpoints and susceptibility testing for fungi. The AFST Steering Committee currently consists of Maksen C. Arendrup, (Chairman), Derek Brown (secretary), Cornelia Lass-Floerl, and Manuel Cuenca-Estrella. Voriconazole breakpoints for Aspergillus spp., micafungin breakpoints for Candida spp. and itraconazole breakpoints for Candida spp. and Aspergillus spp. have been released on the EUCAST website.

Antimicrobial resistance mechanisms
- The subcommittee on antimicrobial resistance mechanisms of clinical and/or epidemiological importance (chairman Christian Giske) has produced draft practical guidelines for detection of resistance mechanisms. The draft document will be released for consultation in the near future.

Pharmaceutical industry, susceptibility testing device manufacturers
- These groups are informed of EUCAST activity and consulted via an e-mail network open to all companies with an interest in antimicrobial susceptibility testing.

EUCAST websites
- The EUCAST websites continue to be developed and updated, and all EUCAST breakpoints and documents are freely available from the website (www.eucast.org), including all publications.

EUCAST disk diffusion method
- Version 3.0 of the breakpoints for the EUCAST disk diffusion method calibrated to EUCAST clinical MIC breakpoints is now available on the EUCAST website. Breakpoints for Pasteurella multocida and Campylobacter spp. were added to version 3.0 and the disk diffusion technique is currently being developed for several additional organisms, including Corynebacterium spp.

Screening tests for beta-lactam resistance in S. pneumoniae and H. influenzae have been modified or added to version 3.0 of the breakpoint tables.

Graphs showing MIC-zone diameter correlations also continue to be expanded on the EUCAST website. The distributions highlight wild type populations and give epidemiological cut off values (ECOFTs).

Implementation of breakpoints
- In Europe, the trend from using other breakpoint guidelines to using EUCAST breakpoints and methods continues. In the EARS-Net resistance surveillance exercise quality assessment exercise in May 2012, EUCAST breakpoints were used by 61% of participating laboratories, compared with 47% a year earlier. Several more countries are in the process of changing or plan to change to EUCAST in the near future. There is also increasing interest in EUCAST breakpoints in countries not in Europe.

Implementation of EUCAST breakpoints, January 2013

Automated systems
- The manufacturers of automated susceptibility testing devices continue to implement EUCAST breakpoints in their systems. Up-to-date information on the current EUCAST status of manufacturers is given on the EUCAST website.

EUCAST documents
- Standard Operating Procedures (SOPs) published on the EUCAST website in the last year are:
  - SOP 3.0 Revision of breakpoints
  - SOP 4.0 Organisation of the Steering Committee
  - SOP 5.0 Interaction of EUCAST committees
  - SOP 6.0 Operation and maintenance of EUCAST websites

Several additional rationale documents for EUCAST breakpoints have been released and there are now close to 50 rationale documents on the website...

Guidance documents on Stenotrophomonas maltophilia and direct antimicrobial susceptibility testing are now available.

EUCAST website
- www.eucast.org

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