Cefoxitin resistance as a surrogate marker for the detection of methicillin-resistant *Staphylococcus aureus*

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on behalf of the Australian Group on Antimicrobial Resistance (AGAR)

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**Objectives**: To evaluate the usefulness of cefoxitin when used as a surrogate marker for the detection of methicillin resistance.

**Patients and methods**: Eight hundred and seventy-one strains of *Staphylococcus aureus*, collected from eight tertiary referral centres serving diverse socio-economic populations, were included in the study using NCCLS disc diffusion and the agar dilution methods.

**Results**: Using cefoxitin and NCCLS criteria for disc diffusion, the sensitivity and specificity for recognizing methicillin resistance were both 100%. Similar results were obtained when the strains were tested by the agar dilution method. The cefoxitin MICs for methicillin-susceptible strains were \(< 4 \text{mg/L.} \)

**Conclusions**: Testing with cefoxitin as a surrogate marker for the detection of methicillin resistance was very accurate with both disc diffusion and agar dilution methods. Such testing clearly distinguished methicillin-resistant strains of *S. aureus* from methicillin-susceptible strains.

Keywords: community-onset MRSA infections, hospital-acquired infections, non-multiresistant *Staphylococcus aureus*, oxacillin resistance

**Introduction**

Detection of methicillin resistance using routine susceptibility test methods is known to be problematic.¹⁻⁵ There are several reports in the literature suggesting modifications to normal susceptibility testing procedures.⁶⁻⁸ These include appropriate media, use of higher inoculum densities, incubation for prolonged periods or lower temperatures and the addition of 2–5% NaCl to the test medium. Generally, they are aimed at augmenting the expression of methicillin resistance, thus increasing the sensitivity of a test procedure, particularly in those strains that are heterogeneously resistant,¹ but many modifications result in slower growth and thus potentially a delay in diagnosis.

Until recently, most strains of methicillin-resistant *Staphylococcus aureus* (MRSA) were isolated from hospitalized patients and were multidrug resistant. This feature was helpful in their recognition in the clinical laboratory. However, MRSA have now appeared in the community worldwide.⁹⁻¹⁷ These strains are not multidrug resistant (nmMRSA). In addition, our experience (unpublished data) and other reports¹⁸ have noted strains of *S. aureus* that are not methicillin resistant, but which are still multiresistant to non-β-lactam antibiotics. The *mecA* gene (which encodes methicillin resistance) does not appear to be present in these strains.

The gold standard for identifying MRSA is to detect the *mecA* gene,³ or its product, PBP2a, by latex agglutination.¹⁹,²⁰ However, these tests are not within the scope of many clinical laboratories and are relatively expensive. Cefoxitin and moxalactam have been reported as surrogate markers for the detection of methicillin resistance.²¹,²² The NCCLS has recently reported cefoxitin zone diameter interpretive criteria for the prediction of *mecA*-mediated resistance.²³ The objectives of this study were: (i) to compare the MIC distributions of cefoxitin and oxacillin in *S. aureus* strains with different resistance antibiograms; (ii) to establish a suitable concentration of cefoxitin that could be used in agar media which would distinguish methicillin-resistant...
strains from methicillin-susceptible strains; (iii) to evaluate the usefulness of the cefoxitin disc test in identifying MRSA in our local strains; and (iv) to determine the usefulness of cefoxitin in correctly identifying a collection of methicillin-susceptible strains of \( S. aureus \) that were multiresistant to non-\( \beta \)-lactam antibiotics (RSA).

Materials and methods

**Bacterial strains**

Bacterial strains were collected at eight major tertiary care centres. Each centre collected 100 consecutive isolates from individual patients. Cultures were identified using a set of conventional tests specified by AGAR\textsuperscript{24} and maintained at \(-70^\circ\)C. Additional strains of nmrMRSA and also multiresistant but methicillin-susceptible strains of \( S. aureus \) (RSA), obtained from a collection maintained at one laboratory (RNSH), were also included in the study.

**Susceptibility tests**

**General.** Susceptibility tests were performed by the agar dilution method (NCCLS)\textsuperscript{25} or by the disc diffusion method (NCCLS).\textsuperscript{26} In these studies, Mueller–Hinton II Agar (BBL 211438) was used, and NCCLS methodology for testing and interpretation was followed.\textsuperscript{23,25,26} Methicillin-susceptible \( S. aureus \) (MSSA) and MRSA strains were categorized by phenotypic criteria: their susceptibility to oxacillin at 2 mg/L and, additionally, susceptibility to other \( \beta \)-lactam antibiotics.

**Resistance surveillance.** Testing was performed by the individual laboratories. All cultures were tested against 16 antibacterials by agar dilution at concentrations recommended by the NCCLS\textsuperscript{25} to define susceptibility.

**Cefoxitin testing.** Cefoxitin MIC by agar dilution at 35°C using a range of 0.25–256 mg/L, was also performed by each laboratory. In addition, five laboratories performed disc testing using a 30 \( \mu \)g cefoxitin disc on Mueller–Hinton agar (NCCLS).\textsuperscript{23,26} All media were prepared at one laboratory.

**Further tests.** Selected cultures, including all nmrMRSA strains, were forwarded to one laboratory (RNSH) for further testing. MICs of oxacillin and cefoxitin were determined using Mueller–Hinton II Agar (BBL 211438) with and without added 2% NaCl. Cefoxitin MICs were also determined using Oxoid Mueller–Hinton Agar (CM337) and in Oxoid Columbia Agar (CM 331). Oxacillin and methicillin MICs were determined only for nmrMRSA and RSA strains, and not for MSSA and MRSA strains.

### Detection of mecA by PCR

The multiplex PCR procedure for the detection of mecA and nuc genes was carried out according to Brakstad et al.\textsuperscript{27,28} DNA was extracted by suspending portions of four to five colonies in 50 \( \mu \)L of lysostaphin (100 mg/L). After incubation for 10 min at 37°C, 50 \( \mu \)L of proteinase K (100 mg/L) and 150 \( \mu \)L of TE buffer (1 mM EDTA/10 mM Tris; pH 7.5) were added and incubated for a further 10 min at 37°C. The specimens were then incubated at 95°C for 10 min and centrifuged at 12000 rpm for 3 min; 5 \( \mu \)L of the supernatant was used in each PCR mixture.

The PCR mixture of 25 \( \mu \)L consisted of 0.2 U of AmpliTaq Gold, 2.5 \( \mu \)L of 10X PCR buffer, 2.5 \( \mu \)L of 25 mM MgCl\( _2 \), 0.35 \( \mu \)L of 20 mM primers for mecA and 0.25 \( \mu \)L of 20 mM primers for nuc. The PCR was carried out in an OmniGene thermocycler using the following program: 10 min at 94°C, 35 cycles of 1 min at 94.5°C, 30 s at 53°C, 1 min at 72°C, followed by 5 min at 72°C. PCR products were analysed by gel electrophoresis; mecA product banded at 533 bp and the nuc at 267 bp. All nmrMRSA and RSA strains were confirmed for the presence or absence of the mecA gene by PCR.

### Results

Cefoxitin MICs were determined on 871 isolates of \( S. aureus \). Cefoxitin disc diffusion susceptibility tests were performed on 598 isolates. This was done in eight laboratories as part of the annual AGAR surveillance programme.\textsuperscript{24} The surveillance studies included 16 antibacterials, tested at concentrations recommended by the NCCLS.\textsuperscript{23,25} Based on the antibiotic resistance profiles, the collection of cultures was classified into four categories: 575 MSSA, 177 MRSA, 95 strains of nmrMRSA and the fourth category comprised the collection of 24 RSA maintained at one of the laboratories.

| Table 1. Cefoxitin MICs for \( S. aureus \), nmrMRSA, MRSA and RSA\textsuperscript{a} |
|---------------------------------|---------------------------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| **Organism** | **Number of strains** | **MIC (mg/L)** | **0.25** | **0.5** | **1** | **2** | **4** | **8** | **16** | **32** | **64** | **128** | **256** | > **256** |
| MSSA | NT | 575 | 4 | 4 | 3 | 44 | 520 | | | | | | | |
| nmrMRSA | 2 | 6 | | | | | | | | | | | | |
| | 16 | 3 | | | | | | | | | | | | |
| | 32 | 23 | | | | | | | | | | | | |
| | 64 | 19 | | | | | | | | | | | | |
| | 128 | 41 | | | | | | | | | | | | |
| | 256 | 5 | | | | | | | | | | | | |
| MRSA | NT | 177 | | | | | | | | | | | | |
| RSA | 0.25–2 | 15 | | | | | | | | | | | | |
| | 4 | 7 | | | | | | | | | | | | |
| | 8 | 2 | | | | | | | | | | | | |

\( NT \), not tested.

\( \textsuperscript{a} \)Methicillin-susceptible strains of \( S. aureus \) that were multiresistant to non-\( \beta \)-lactam classes of antibiotics.
There was little difference between Mueller–Hinton agars sourced from BBL and from Oxoid. However, cefoxitin MICs when tested on Oxoid Columbia agar were two- to eight-fold lower than MICs obtained on BBL Mueller–Hinton agar for 85 of the 95 strains.

**Discussion**

The present study has addressed several issues relating to the use of cefoxitin in the disc diffusion test and in agar dilution susceptibility testing as a surrogate marker for detecting methicillin resistance in *S. aureus*. Both cefoxitin disc testing and 4 mg/L cefoxitin in agar appear to be very accurate and easy to perform methods for detecting methicillin-resistant isolates of *S. aureus*. The large number of strains included in this study were sourced from eight tertiary referral centres, servicing patient populations of different socio-economic status, and so cover most isolates likely to be encountered in our clinical laboratories.

There was a clear difference in the MICs of cefoxitin between the methicillin-susceptible strains (MSSA and RSA) and those that were methicillin resistant (nmrMRSA and MRSA). Although all strains of MSSA and RSA were inhibited by cefoxitin at ≤ 4 mg/L, 90% of strains had an MIC of 4 mg/L. MRSA strains showed high-level resistance to cefoxitin. MICs ranged from 8 to > 256 mg/L, with 170 of the 177 strains having an MIC of 128 – > 256 mg/L. Against the nmrMRSA strains, cefoxitin MICs were distributed over a range of 8 – 256 mg/L; for 88 of the 95 strains the cefoxitin MIC was between 32 – 128 mg/L. For one strain, the cefoxitin MIC was 8 mg/L; this strain was mecA-positive but tested susceptible to oxacillin and to methicillin. Such strains are probably very heterogeneous in their expression of methicillin resistance and have been reported to be rare.\(^4\) Nine of the 24 RSA strains appeared resistant to oxacillin by standard testing with oxacillin agar dilution. Oxacillin MICs were 4 mg/L for seven of the strains and 8 mg/L for another two strains. All were mecA-negative and were inhibited by cefoxitin at 4 mg/L.

Our observations with the use of cefoxitin discs were very encouraging. Using the NCCLS disc diffusion criteria\(^22\) to define resistance (cefoxitin zone diameters of ≤ 19 mm for resistance and ≥ 20 mm for susceptibility), the sensitivity and specificity were 100% in the 598 strains tested in the study. Skov et al.\(^22\) tested cefoxitin 30 mcg discs on Oxoid IsoSensitest agar and reported that a zone diameter of < 29 mm would be appropriate for methicillin-resistant strains. We have tested a few nmrRSA

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**Table 2.** Comparison of cefoxitin MICs with cefoxitin zone diameters for MSSA, nmrMRSA, MRSA and RSA

<table>
<thead>
<tr>
<th>Organism (no. tested)</th>
<th>Number of strains</th>
<th>Oxacillin MICs (mg/L)</th>
<th>Cefoxitin MICs (mg/L)</th>
<th>Cefoxitin zones (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSSA 394</td>
<td>NT</td>
<td>0.25–4</td>
<td>23–35</td>
<td></td>
</tr>
<tr>
<td>nmrMRSA 95</td>
<td></td>
<td>2–256</td>
<td>8–256</td>
<td>0–17</td>
</tr>
<tr>
<td>MRSA 85</td>
<td>NT</td>
<td>32–256</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>RSA 24</td>
<td>0.25–4</td>
<td>2–4</td>
<td>22–28</td>
<td></td>
</tr>
</tbody>
</table>

NT, not tested.

Total number of strains tested: 598.

Cefoxitin MIC results are presented in Table 1. All 575 strains of MSSA were inhibited by cefoxitin at concentrations of ≤ 4 mg/L; 520 of these strains (90%) had an MIC of 4 mg/L. Similarly, all 24 RSA strains were also inhibited by 2–4 mg/L of cefoxitin (and were mecA-negative). However, nine of these 24 strains were resistant to oxacillin and methicillin by the NCCLS breakpoints using agar dilution testing. All MRSA strains were highly resistant to cefoxitin; 95% of the strains had an MIC between 128 and 256 mg/L. Cefoxitin MICs for the nmrMRSA strains were generally lower, and ranged between 8 and 256 mg/L. Only one of these strains had an MIC of 8 mg/L; this strain was mecA-positive but appeared oxacillin-susceptible (MIC 2 mg/L) and methicillin-susceptible (8 mg/L) on agar dilution testing.

Disc susceptibility tests were performed on 598 strains (Table 2). Cefoxitin zone diameters for the 394 MSSA strains ranged from 23 to 35 mm. Using the NCCLS guidelines for susceptibility (zone diameter ≥ 20 mm), all of the MSSA strains and all of the 24 RSA strains were clearly susceptible. None of the 85 MRSA strains had zones around the cefoxitin disc. With the nmrMRSA strains, while the oxacillin MICs ranged between 2 and 256 mg/L, they all tested cefoxitin resistant (i.e. zone diameter ≤ 19 mm). Cefoxitin zone diameters for these strains ranged between 0 and 17 mm, and did not appear to correspond well to the cefoxitin MICs.

The influence of different media and some of the test conditions on cefoxitin MICs for nmrMRSA strains are shown in Table 3. The ratios of MICs obtained are compared with the MICs obtained on BBL Mueller–Hinton agar. Addition of 2% NaCl generally lowered the MICs of cefoxitin by two-fold.

**Table 3.** Effect of media on MICs of cefoxitin for nmrMRSA

<table>
<thead>
<tr>
<th>Media compared</th>
<th>Antibacterial</th>
<th>Number of strains with cefoxitin MIC ratios (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.125</td>
</tr>
<tr>
<td>Mueller–Hinton BBL + NaCl/Mueller–Hinton BBL</td>
<td>Cefoxitin</td>
<td>5</td>
</tr>
<tr>
<td>Mueller–Hinton Oxoid/Mueller–Hinton BBL</td>
<td>Cefoxitin</td>
<td>1</td>
</tr>
<tr>
<td>Columbia Oxoid/Mueller–Hinton BBL</td>
<td>Cefoxitin</td>
<td>8</td>
</tr>
<tr>
<td>Mueller–Hinton BBL + NaCl/Mueller–Hinton BBL</td>
<td>Oxacillin</td>
<td>12</td>
</tr>
</tbody>
</table>

Number of strains tested: 95.

\(^a\)One MIC divided by the corresponding MIC (e.g. a cefoxitin MIC of 8 mg/L with BBL + NaCl compared with a cefoxitin MIC of 16 mg/L with Mueller–Hinton BBL equals a cefoxitin MIC ratio of 0.5).
strains on BBL Mueller–Hinton agar and on Oxoid IsoSensitest agar in parallel, and have noted that the zone diameters are generally larger on Oxoid IsoSensitest agar. If NCCLS recommendations are to be used, it would be necessary to adhere to the 20 mm cut-off recommendation.

NCCLS has not made recommendations for using cefoxitin to define methicillin resistance using agar dilution tests. The data presented here suggest that, if a breakpoint of cefoxitin 4 mg/L is used, then the sensitivity and specificity for detecting methicillin resistance would be 100% on the diverse selection of clinical isolates included in this study. The addition of 2% NaCl to Mueller–Hinton agar is recommended when testing oxacillin and methicillin, and has been shown to improve sensitivity when testing community-acquired strains. However, this does not appear to be necessary when testing using cefoxitin, and testing can be performed at standard temperatures (i.e. 35–37°C). In addition, it may be possible to use such plates to identify the presence of methicillin-resistant S. aureus in specimens submitted for screening purposes as well.

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References

C. J. Fernandes et al.


