Evaluation of different methods for detecting methicillin (oxacillin) resistance in *Staphylococcus aureus*

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**Objectives**: To evaluate the performance of oxacillin, cefazolin, cefoxitin, cefotaxime and imipenem discs; Etest for oxacillin; microdilution; agar screening plates with 2 and 6 mg/L of oxacillin; and PBP2 agglutination for detection of methicillin-resistant *Staphylococcus aureus* (MRSA).

**Methods**: A total of 102 clinical *S. aureus* isolates, including 51 MRSA isolates, tested by PCR for the presence or absence of the mecA gene (gold standard method), isolated from different patients and at different times, were tested with: oxacillin (1 µg), cefazolin, cefoxitin, cefotaxime and imipenem (all 30 µg) discs; Etest for oxacillin; microdilution with oxacillin; agar screening tests (ORSAB medium) with 2 mg/L or 6 mg/L of oxacillin; and PBP2 agglutination with two different kits for detection of MRSA strains.

**Results**: The cefoxitin disc, ORSAB medium and PBP2 detection all showed 100% sensitivity. The cefoxitin, cefazolin and imipenem discs, Etest for oxacillin, microdilution and agar screening method with 6 mg/L at 24 h showed the highest specificity (100%), although variable degrees of sensitivity. The cefoxitin disc, which showed negative and positive predictive values of 100% and 98%, respectively was the best method for detecting MRSA isolates.

**Conclusions**: In the absence of availability of molecular biology techniques, the cefoxitin disc was the best predictor of methicillin resistance in *S. aureus* from among the techniques tested.

**Keywords**: MRSA, *S. aureus*, detection

**Introduction**

*Staphylococcus aureus* is one of the most common causes of nosocomial or community-based infections, leading to serious illnesses with high rates of morbidity and mortality. In recent years, the increase in the number of bacterial strains that show resistance to methicillin (MRSA) has become a serious clinical and epidemiological problem because this antibiotic is considered as the first option in the treatment of staphylococci infections, and because resistance to this antibiotic implies resistance to all β-lactam antibiotics. For these reasons, accuracy and promptness in the detection of methicillin resistance is of key importance to ensure correct antibiotic treatment in infected patients as well as control of MRSA isolates in hospital environments, to avoid them spreading.

MRSA strains harbour the mecA gene, which encodes a modified PBP2 protein (PBP2’ or PBP2a) with low affinity for methicillin and all β-lactam antibiotics. Phenotypic expression of methicillin resistance may alter depending on the growth conditions for *S. aureus*, such as temperature or osmolarity of the medium, and this may affect the accuracy of the methods used to detect methicillin resistance. Heteroresistant bacterial strains may evolve into fully resistant strains and therefore be selected in those patients receiving β-lactam antibiotics, thus causing therapeutic failure. From a clinical point of view, they should, therefore, be considered fully resistant.

There are several methods for detecting methicillin resistance⁷ including classical methods for determining MICs (disc diffusion, Etest, or broth dilution), screening techniques with solid culture medium containing oxacillin, and methods that detect the mecA gene or its protein product (PBP2’ protein). Detection of the mecA gene is considered as the reference method for determining resistance to methicillin. However, many laboratories throughout the world do not have the capacity...
Materials and methods

Strains

Between July and December 2003, 102 clinical isolates of *S. aureus* were collected (51 consecutive strains harbouring the *mecA* gene and 51 lacking this gene). All strains were identified by biochemical procedures. They belonged to different patients and were isolated from different anatomical locations. The methicillin-susceptible *S. aureus* strain ATCC 29213 was used as a control for the diagnostic procedures. They belonged to different patients and were isolated from different anatomical locations.

Detection of the *mecA* gene by PCR

We considered the presence of the *mecA* gene as the reference or ‘gold standard’ method for establishing the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of each of the techniques studied. In accordance with the published nucleotide sequence of the *mecA* gene, we designed the following oligonucleotides: mecA1, 5′-ATGAAAAAGATATTTAGTTC-3′; and mecA2, 5′-CTCATATGTTCCTGTATT-3′, which amplify an internal region of 447 bp of this gene. In order to avoid false negative results in those isolates that harbour the *mecA* gene, we used two specific primers, mecA1 and mecA2, to amplify the *mecA* gene. The chromosomal DNA of all *S. aureus* strains was used as internal control in all PCR assays with the following oligonucleotides used to amplify the *mecA* gene. A PCR analysis was carried out by using the chromosome of *E. faecalis* ATCC 407 as a template to obtain an amplicon of 941 bp, which was cloned into the Topo TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). This plasmid was used as internal control in all PCR assays with the aim of detecting the *mecA* gene. The chromosomal DNA of all clinical strains of *S. aureus* obtained following standard protocols was used as a template in the PCR. Only those reactions that yielded negative *mecA* gene amplification and positive amplification of the internal control were considered as significant. A total of 51 *mecA*-positive *S. aureus* and 51 lacking the *mecA* gene (MSSA) were selected for further studies as previously described in Materials and methods.

Antibiotic susceptibility tests

We evaluated the validity of the disc diffusion test by using oxacillin (1 μg), cefazolin (30 μg), cefoxitin (30 μg), cefotaxime (30 μg), and imipenem (30 μg) discs (Becton Dickinson and Company, Sparks, MD, USA). For this, Mueller–Hinton agar plates were overlaid with an inoculum (turbidity equivalent to that of a 0.5 McFarland Standard) of the *S. aureus* clinical strains. Zone diameters were measured at 24 and 48 h following NCCLS criteria. The oxacillin MICs were determined by Etest in all *S. aureus* isolates (AB Biodisk, Solna, Sweden) and microdilution with Mueller–Hinton broth supplemented with 2% NaCl following the NCCLS criteria. With cefoxitin disc diffusion, the new interpretative ranges, per NCCLS M100-S14, were used to distinguish MRSA from MSSA. *S. aureus* with a zone diameter of ≤ 19 mm were scored as resistant and those with a zone diameter of ≥ 20 mm were reported as susceptible.

The MICs obtained by Etest were read at 24 h and 48 h. Oxacillin was purchased from Sigma–Aldrich, Germany.

Agar screening

Agar screening plates have been standardized for *S. aureus* (using 6 mg/L of oxacillin; BD Diagnostics, Heidelberg, Germany), but not coagulase-negative staphylococci. We followed the manufacturer’s instructions in carrying out the screening tests.

We also evaluated ORSAB medium (oxacillin resistance screening agar base; Oxoid Ltd, UK) supplemented with 2 mg/L of oxacillin. This medium incorporates Aniline Blue for detection of mannitol fermentation, resulting in the presence of intense blue colonies of *S. aureus*. Both media were inoculated with a drop of a bacterial suspension (turbidity equivalent to that of a 0.5 McFarland Standard). Any bacterial growth after 24 h or 48 h was indicative of resistance to methicillin.

PBP2′ detection

In this study, we evaluated two slide latex agglutination kits for the rapid detection of PBP2′: (i) MRSA-Screen (Denka Seiken Co., Ltd, Japan); and (ii) Slidex MRSA Detection (bioMérieux), following the manufacturer’s instructions in each case.

Results

A total of 102 clinical strains of *S. aureus* were evaluated with the above-mentioned methods.

The *mecA* PCR assay including the internal control allowed us to classify 51 of the isolates as *S. aureus mecA*-positive and 51 as *S. aureus mecA*-negative (data not shown).

The overall results obtained with the different techniques are shown in Table 1.

As regards the disc diffusion test, sensitivity was slightly better at 48 h. The cefoxitin disc yielded the best sensitivity values (100%), followed by the oxacillin disc. Discs of first- and third-generation cephalosporins as well as imipenem scored lower sensitivity values. Regarding cefoxitin, the ranges of the inhibition zones around the antibiotic disc for the isolates were as follows: among the *mecA*-negative isolates, 50 isolates yielded halos higher than 25 mm whereas one isolate yielded a halo of 17 mm; among the *mecA*-positive isolates, 51 isolates yielded halos between 0 and 14 mm.

The Etest did not yield the level of sensitivity obtained with the cefoxitin disc, and was no better than the oxacillin disc, even when the incubation time was extended to 48 h. However, the oxacillin disc read at 24 h and the Etest both yielded 100% specificity values.

As regards the microdilution method (read only at 24 h), the results were identical to those obtained with the oxacillin disc (read at 24 h). It is important to emphasize that the three false negative results obtained with the oxacillin disc diffusion, Etest and microdilution corresponded to the same isolates.

Agar screening plates with oxacillin are frequently used in the determination of methicillin resistance. The agar screening plates used in our work each yielded different results. Of the two media used with different concentrations of oxacillin, the one with the higher concentration of oxacillin (6 mg/L) showed...
higher specificity, and therefore selected the mecA-positive strains, although sensitivity was slightly less than optimal (only two isolates provided false negative results). However, the ORSAB medium showed high sensitivity, but low specificity. As with the antibiotic oxacillin disc, reading at 48h gave lower specificity.

PBP2 detection carried out with both kits showed optimal and identical sensitivity values with only two false positive results.

Discussion

The accurate and early determination of methicillin resistance is of key importance in the prognosis of infections caused by S. aureus. Although multiple methods of detection of this resistance have been developed, they are often too slow or not sufficiently sensitive or specific to ensure appropriate treatment of the MRSA-infected patients. The sensitivity and specificity values of the methods used also vary depending on the person carrying them out and the techniques used.

Identification of the mecA gene is the most reliable method of detecting MRSA isolates, however not all laboratories can include molecular biology techniques in their routine clinical practice. For this reason, it is essential that phenotypic techniques able to detect MRSA isolates in a rapid and accurate manner are made available, in order to ensure correct antibiotic treatment and to avoid the spread of MRSA isolates in the hospital environment.

Methods based on disc diffusion and Etest, as well as microdilution with oxacillin, are often not entirely reliable at detecting some strains that harbour the mecA gene. Three out of the 51 clinical strains that were positive for the mecA gene yielded false negative results with the oxacillin disc diffusion, Etest for oxacillin, and microdilution. The lower sensitivity may be explained by the absence of, or reduced expression of, the mecA-encoded protein, PBP2. The results of this study confirm that those antibiotics able to induce expression of methicillin resistance, e.g. cefoxitin, are the most appropriate for detecting MRSA isolates, as all mecA-positive isolates were detected with the cefoxitin disc. The usefulness of the cefoxitin disc (30 μg) in predicting oxacillin resistance has recently been reported. However, other antibiotics with lower inducing capacities, such as cefazolin, cefotaxime and imipenem were less able to detect the resistance. The sensitivities of the disc diffusion and microdilution methods reported here are not consistent with previous reports of up to 100% sensitivity, although both of these studies included a lower number of isolates, which were therefore less likely to include isolates showing heteroresistance to methicillin. The extension of the incubation time to 48h, increased the sensitivity without significantly affecting specificity. However, the delay in obtaining the information reduces the usefulness of these procedures. It is important to point out that the use of systems that accurately determine the MIC (Etest or microdilution) do not increase sensitivity in MRSA detection.

With reference to oxacillin agar screening, the agar plate with 6mg/L showed higher specificity than the plate with 2mg/L (ORSAB). Moreover, these methods were more sensitive than the disc diffusion and microdilution methods (with the exception of cefoxitin), as only two isolates yielded false negative results. Similar sensitivity has previously been described for the 6mg/L plate, thus this technique appears to be an easily carried out and reliable option. The low specificity of the ORSAB medium prevents its use, at least alone, in predicting methicillin resistance in S. aureus. This medium has previously been reported to show good sensitivity, although in a study in which the agar base was supplemented with antibiotics.

Detection of the protein product of the mecA gene (PBP2 protein) is a rapid and easy method of predicting resistance to methillin. Both kits tested in this study showed high sensitivity (100%) and specificity (96%). In previous studies, the reported sensitivity of the MRSA-screen latex agglutination test kit ranged between 97% and 100%, and the specificity was optimal (100%) with S. aureus and coagulase-negative staphylococci. Even in studies including BORSA isolates (borderline phenotypic

### Table 1. Parameters of the different methods for detecting methicillin resistance

<table>
<thead>
<tr>
<th>Method</th>
<th>False negatives</th>
<th>False positives</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>NPV</th>
<th>PPV</th>
</tr>
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<tr>
<td>Oxacillin disc 24h&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3</td>
<td>0</td>
<td>94.1</td>
<td>100</td>
<td>94.4</td>
<td>100</td>
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<tr>
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<td>98.0</td>
<td>98.0</td>
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<tr>
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<td>7</td>
<td>0</td>
<td>86.2</td>
<td>100</td>
<td>87.9</td>
<td>100</td>
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<tr>
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<td>88.2</td>
<td>100</td>
<td>89.4</td>
<td>100</td>
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<tr>
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<td>1</td>
<td>100</td>
<td>98.0</td>
<td>100</td>
<td>98.0</td>
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<tr>
<td>Cefoxitin disc 48h</td>
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<td>1</td>
<td>100</td>
<td>98.0</td>
<td>100</td>
<td>98.0</td>
</tr>
<tr>
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<td>3</td>
<td>1</td>
<td>94.1</td>
<td>98.0</td>
<td>94.3</td>
<td>97.9</td>
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<tr>
<td>Cefotaxime disc 48h</td>
<td>2</td>
<td>1</td>
<td>96.0</td>
<td>98.0</td>
<td>96.1</td>
<td>98.0</td>
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<tr>
<td>Imipenem disc 24h</td>
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<td>0</td>
<td>86.2</td>
<td>100</td>
<td>87.9</td>
<td>100</td>
</tr>
<tr>
<td>Imipenem disc 48h</td>
<td>7</td>
<td>0</td>
<td>86.2</td>
<td>100</td>
<td>87.9</td>
<td>100</td>
</tr>
<tr>
<td>Etest oxacillin 24h</td>
<td>3</td>
<td>0</td>
<td>94.1</td>
<td>100</td>
<td>94.4</td>
<td>100</td>
</tr>
<tr>
<td>Etest oxacillin 48h</td>
<td>1</td>
<td>0</td>
<td>98.0</td>
<td>100</td>
<td>98.0</td>
<td>100</td>
</tr>
<tr>
<td>Oxacillin microdilution</td>
<td>3</td>
<td>0</td>
<td>94.1</td>
<td>100</td>
<td>94.4</td>
<td>100</td>
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<tr>
<td>OXA 6 mg/L 24 h</td>
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<td>0</td>
<td>96.0</td>
<td>100</td>
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<td>100</td>
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<td>ORSAB 2 mg/L 24h</td>
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<td>4</td>
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<td>100</td>
<td>92.7</td>
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<td>ORSAB 2 mg/L 48h</td>
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<td>17</td>
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<td>PBP2 agglutination&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>2</td>
<td>100</td>
<td>96.0</td>
<td>100</td>
<td>96.2</td>
</tr>
</tbody>
</table>

NPV, negative predictive value; PPV, positive predictive value.
<sup>a</sup>Interpretation at 24h and 48h with the exception of the microdilution and OXA agar screening of 6mg/L, which were read only at 24h.
<sup>b</sup>Identical results were obtained with both kits.

### Methods for detecting MRSA

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resistance to methicillin), the sensitivity reached 98.5%. The results of this study confirm that the Slidex MRSA Detection kit is a reliable method of detecting methicillin resistance. The fact that no false negatives were obtained with this method indicates that all S. aureus mecA-positive isolates produced at least low levels of PBP2' protein and thus the reduced sensitivity in the other techniques may be at least partly due to the low expression of the mecA product. Other agglutination kits (MRSA-Screen) yielded similar levels of sensitivity and specificity, thus confirming the usefulness of the agglutination system in detecting methicillin resistance.

In summary, in this study, cefoxitin disc diffusion and PBP2' detection were the most sensitive methods for detecting MRSA isolates. In addition, the cefoxitin disc, which showed negative and positive predictive values of 100% and 98%, respectively, was the best method for detecting MRSA isolates. Agar screening with 6 mg/L of oxacillin is an easy and cheap method, although less sensitive. Nevertheless, none of the techniques compared showed 100% sensitivity and specificity, although if the mecA gene detection method (PCR) is not accessible or available, it is advisable to combine two methods, one with high sensitivity and the other with high specificity.

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