Evaluation of Different Phenotypic Methods for Detection of Methicillin (Oxacillin) Resistance in Coagulase-Negative Staphylococci

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Coagulase-negative staphylococci (CNS) are one of the major causes of nosocomial infections. Methicillin (oxacillin) resistant strains are particularly important because they narrow therapeutic options. Detecting methicillin resistance among CNS has been a challenge for years. The objective of this study was to evaluate the ability and accuracy of four phenotypic methods, disk diffusion (DD); agar screening plate with 6µg of oxacillin per ml (OXA); E-test and the MRSA-Screen latex agglutination test (Denka-Seiken, Tokyo, Japan), to determine the susceptibility of CNS to oxacillin. The methods were evaluated by using the presence of the \textit{mecA} gene, as detected by PCR, as the “gold standard”. One hundred and ninety seven strains of CNS of 7 species were analysed. 49.2\% were \textit{mecA} positive. For the different methods evaluated, the sensitivities and specificities were as follows: for disk diffusion, 93.8 and 93\%, respectively; for the agar screen test 95.9 and 98\%, respectively; for E-test, 100 and 95\%, respectively; and for the slide latex agglutination test, 96.9 and 100\%, respectively. The latex agglutination test sensitivity was increased to 100\% when retested after induction. In conclusion, all of the phenotypic methods evaluated in the present study appeared to perform very well for the detection of oxacillin resistance in CNS. The MRSA-Screen latex agglutination test was not only the most sensitive, specific and accurate method but also rapid and technically simple method to be applied in routine laboratories for the detection of oxacillin resistance which is mediated by the \textit{mecA} gene.

INTRODUCTION

Coagulase-negative staphylococci (CNS) have been the major cause of nosocomial infections (20,34). The rates of resistance to methicillin among CNS have increased in the last two decades, according to the National Nosocomial Infectious Surveillance System (NNIS) (27) and the empirical treatment of choice for infections caused by these organisms is often vancomycin. The most common mechanism of methicillin resistance is the production of a penicillin-binding protein 2a (PBP2a), with low affinity for \beta-lactams. PBP2a is coded by the \textit{mecA} gene and can be detected by PCR, which is considered the gold-standard test to identify methicillin resistance (3,30) but it is not feasible for the busy clinical microbiology laboratory.

The detection of methicillin resistance among CNS isolates has been a challenge for years (9) because these microorganisms present a hetero-resistant pattern (3). In 1999, the National Committee for Clinical Laboratory Standards (NCCLS) (23) lowered the oxacillin breakpoints for CNS strains (from $\geq 4$ to $\geq 0.5 \mu g/ml$). Although these recent breakpoints have shown an acceptable correlation with the presence or absence of the \textit{mecA} gene in \textit{Staphylococcus epidermidis}, \textit{Staphylococcus hominis} and \textit{Staphylococcus haemolyticus} isolates, species with borderline MICs and less common species impair test accuracy (5,9,15). In addition, the use of the agar screening method was no longer recommended for this group of organisms, although some investigators found the test to be a sensitive method for the detection of oxacillin resistance in CNS (2). Rapid and accurate identification of methicillin (oxacillin) resistance is essential in order to determine the most appropriate antimicrobial therapy. The NCCLS (23) has therefore recommended the detection of PBP2a for these pathogens. Moreover, a slide latex agglutination test (MRSA-Screen), (Denka-Seiken, Tokyo, Japan), for the detection of this protein has recently been developed. It is a simple 15 min test that uses latex particles sensitized with a monoclonal antibody against PBP 2a (2). This test was
evaluated for *S. aureus* with overall favourable results for sensitive and specific detection of methicillin-resistant *S. aureus* (MRSA) even in Egypt (21). However, its accuracy for the detection of oxacillin resistance in CNS strains has been assessed, but not extensively, in some studies (11,13,17,35).

In the study described here, we evaluated the latex agglutination test (MRSA-Screen) for its ability to detect PBP2a in clinical strains of CNS. In addition, an evaluation of other test methods, including the disk diffusion, oxacillin agar screen test and E-test (AB Biodisk, Solna, Sweden), was also performed; and the results obtained by those tests were compared to those obtained by PCR detection of the *mecA* gene.

**MATERIALS AND METHODS**

**Bacterial isolates.** 197 clinical isolates of CNS were selected for testing. The strains were isolated between January 2004 and December 2005 from patients attending the in-patients and out-patients clinics at Zagazig University Hospitals from the different clinical specimens: blood (18 samples), surgical wound (109 samples), burns wound (27 samples), catheter tips (31 samples), urine (8 samples) and body fluids (4 samples). Only one strain from each patient was analyzed. CNS strains were identified to the species level by their characteristic growth morphologies, Gram staining, reaction to catalase and coagulase non production as detected with Staphaurex Plus system (Murex Biotech Ltd, Dartford, United Kingdom) and API Staph (bioMerieux sa, France). Isolates were kept frozen at -20 °C in tryptic soy broth containing 20% glycerol (vol/vol) (11).

**PCR detection of *mecA* gene.** DNA of CNS isolates were extracted using the high pure PCR template preparation kit (Boehringer Mannheim, Germany) according to the manufacturer’s instructions. Then 5 µl of the extracted DNA was used for each 50-µl reaction mixture. PCR was performed by using the conditions described previously (2) with the following primers, *mecA* 1 (5'-GTA GAA ATG ACT GAA CGT CCG ATA A) and *mecA* 2 (5'-CCA ATT CCA CAT TGT TTC GGT CTA A) previously designed by Geha et al. (8). DNA amplification was carried out in a Perkin-Elmer thermal cycler, with the following thermal cycling profile: initial denaturation at 94 °C for 5 min, followed by 30 cycles of amplification (denaturation at 94 °C for 15 s, annealing at 55 °C for 15 s, extension at 72 °C for 30 s), followed by a final extension at 72 °C for 2 min. A positive result was indicated by the presence of the 310-bp amplified DNA fragment revealed by the electrophoresis on a 2% agarose gel stained with ethidium bromide at 100 V for 45 min (figure 1). *Staphylococcus aureus* ATCC 33591 (methicillin-resistant) and *S. aureus* ATCC 25923 (methicillin-susceptible) were included as control organisms.

**Disk diffusion test (DD).** The disk test was performed as described by NCCLS (24,26) with a 1-µg oxacillin disk and Muller-Hinton agar (Oxoid) without additional NaCl. The plates were incubated in ambient air at 35 °C, and zone diameters were read with transmitted light at 24 and 48 h. Any growth, including light growth, within the 17mm-diameter zone around the disk was considered indicative of resistance.

**Oxacillin salt agar screen method (OXA).** Muller-Hinton agar prepared in house supplemented with 4% NaCl and 6 µg of oxacillin per ml were inoculated as a streak in three directions by using a cotton swab dipped into a direct colony suspension equivalent to a 0.5 McFarland standard in tryptic soy broth (26). As a control, the same medium containing 4% NaCl without oxacillin was inoculated first. Plates were incubated in ambient air at 35 °C and were read at 24 and 48 h. Any growth was recorded as indicating oxacillin resistance.

**E-test.** The MICs of oxacillin were determined by the E-test (AB Biodisk, Solna, Sweden) according to the manufacturer’s recommendations (Figure 2). Muller-Hinton agar plates containing 2% NaCl, which enhance the growth of microcolonies and the expression of the resistance, were inoculated by swabbing the surfaces with a 0.5 McFarland suspension of the isolates. E-test strips were placed on the medium, and the plates were then incubated at 35 °C for 24 h. The MIC was read at the point of intersection between the zone edge and the E-test strip. Resistance to oxacillin was determined to be indicated by a MIC of ≥0.5 µg/ml, according to the guidelines of the NCCLS (23).

**MRSA-Screen latex agglutination test (LA).** The test was performed in accordance
with the manufacturer’s protocol (Denka Seiken Co., Niigata, Japan). But with one modification: a large, “heaping” inoculum of test organism (approximately 30 to 50 colonies, depending on the colony size) was used instead of the standard 1-µl loopful (11,17). Briefly, this large inoculum of bacterial growth was suspended in 200 µl of extraction reagent 1 (to be at least a McFarland no.6 standard) and subsequently lysed by boiling for 3 min. After cooling to room temperature, 50 µl of extraction reagent 2 was added to the lysate. The tubes were centrifuged at 1,500 X g for 5 min; 50 µl of the supernatant was used for testing agglutination with sensitized latex particles (1 drop) and another 50 µl of the supernatant was used for testing with the control latex particles (1 drop). The test slides were mixed by rotating them for 3 min, after which agglutination was assessed visually. *S. aureus* ATCC 33591 (methicillin-resistant) and *S. aureus* ATCC 25923 (methicillin-susceptible) were included as positive and negative controls of the test, respectively.

**RESULTS**

![Fig. (1): A 2% agarose gel containing the PCR products of mecA gene of CNS.](image1)

Lane 1 and 15 had the MW marker and revealed DNA bands of 50,150,300,500,750 and 1000 bp respectively. Lane 2 shows negative control, lane 3 shows mecA gene positive control, lanes 5, 7, 8, 11 and 14 show positive results (each has one DNA band of approximately 310 bp) and lanes 4, 6, 9, 10, 12 and 13 are negative.

![Fig. (2): E-test of oxacillin.](image2)
Table 1: Identification of CNS isolates and the oxacillin susceptibility profile based on the presence or absence of mecA gene.

<table>
<thead>
<tr>
<th>CNS species</th>
<th>No. (%)</th>
<th>mecA gene No. (%)</th>
<th>mecA positive</th>
<th>mecA negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.epidermidis</td>
<td>143 (72.6)</td>
<td>70 (35.5)</td>
<td>73 (37.1)</td>
<td></td>
</tr>
<tr>
<td>S.hominis subsp.hominis</td>
<td>30 (15.2)</td>
<td>13 (6.6)</td>
<td>17 (8.6)</td>
<td></td>
</tr>
<tr>
<td>S.haemolyticus</td>
<td>14 (7.2)</td>
<td>9 (4.6)</td>
<td>5 (2.5)</td>
<td></td>
</tr>
<tr>
<td>S.auricularis</td>
<td>3 (1.5)</td>
<td>1 (0.5)</td>
<td>2 (1)</td>
<td></td>
</tr>
<tr>
<td>S.lugdunensis</td>
<td>3 (1.5)</td>
<td>0 (0)</td>
<td>3 (1.5)</td>
<td></td>
</tr>
<tr>
<td>S.simulans</td>
<td>2 (1.0)</td>
<td>2 (1)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>S.capitis subsp.urealyticus</td>
<td>2 (1.0)</td>
<td>2 (1)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>197 (100)</td>
<td>97 (49.2)</td>
<td>100 (50.8)</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Sensitivities, specificities, positive and negative predictive values and accuracy for phenotypic methods in comparison with the results of PCR for detection of oxacillin susceptibility among 197 CNS isolates.

<table>
<thead>
<tr>
<th>Method</th>
<th>PCR for mecA gene</th>
<th>mecA positive (n=97)</th>
<th>mecA negative (n=100)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>True +ve False -ve</td>
<td>True -ve False +ve</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DD</td>
<td></td>
<td>91</td>
<td>6</td>
<td>93</td>
<td>7</td>
<td>93.8</td>
<td>93</td>
<td>92.9</td>
</tr>
<tr>
<td>OXA</td>
<td></td>
<td>93</td>
<td>4</td>
<td>98</td>
<td>2</td>
<td>95.9</td>
<td>98</td>
<td>97.9</td>
</tr>
<tr>
<td>E-test</td>
<td></td>
<td>97</td>
<td>0</td>
<td>95</td>
<td>5</td>
<td>100</td>
<td>95</td>
<td>95.1</td>
</tr>
<tr>
<td>LA B</td>
<td></td>
<td>94</td>
<td>3</td>
<td>100</td>
<td>0</td>
<td>96.9</td>
<td>100</td>
<td>97.1</td>
</tr>
<tr>
<td>LA A</td>
<td></td>
<td>97</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

DD = Disk diffusion test, OXA = Oxacillin salt agar screen method, LA B = MRSA-Screen latex agglutination test before induction and LA A = MRSA-Screen latex agglutination test after induction.

Table 3: Susceptibilities to oxacillin of the 19 CNS isolates that presented discrepancies by one or more phenotypic methods.

<table>
<thead>
<tr>
<th>CNS species(n = 19)</th>
<th>Presence of mecA gene</th>
<th>LA</th>
<th>DD a</th>
<th>OXA</th>
<th>E test MIC(µg/ml) b</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.epidermidis (2)</td>
<td>+</td>
<td>+</td>
<td>R</td>
<td>-</td>
<td>3 (R)</td>
</tr>
<tr>
<td>S.epidermidis (2)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>3 (R)</td>
</tr>
<tr>
<td>S.epidermidis (2)</td>
<td>+</td>
<td>-/+</td>
<td>S</td>
<td>+</td>
<td>2 (R)</td>
</tr>
<tr>
<td>S.hominis subsp.hominis (1)</td>
<td>+</td>
<td>+</td>
<td>R</td>
<td>-</td>
<td>1 (R)</td>
</tr>
<tr>
<td>S.hominis subsp.hominis (1)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>S</td>
<td>1 (R)</td>
</tr>
<tr>
<td>S.capitis subsp.urealyticus (1)</td>
<td>+</td>
<td>-/+</td>
<td>S</td>
<td>+</td>
<td>1 (R)</td>
</tr>
<tr>
<td>S.epidermidis (3)</td>
<td>-</td>
<td>-</td>
<td>R</td>
<td>-</td>
<td>0.25 (S)</td>
</tr>
<tr>
<td>S.epidermidis (1)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>R</td>
<td>4 (R)</td>
</tr>
<tr>
<td>S.hominis subsp.hominis (1)</td>
<td>-</td>
<td>-</td>
<td>R</td>
<td>-</td>
<td>0.19 (S)</td>
</tr>
<tr>
<td>S.haemolyticus (1)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>R</td>
<td>0.25 (S)</td>
</tr>
<tr>
<td>S.lugdunensis (3)</td>
<td>-</td>
<td>-</td>
<td>S</td>
<td>-</td>
<td>0.5-1 (R)</td>
</tr>
<tr>
<td>S.auricularis (1)</td>
<td>-</td>
<td>-</td>
<td>R</td>
<td>+</td>
<td>4 (R)</td>
</tr>
</tbody>
</table>

LA = MRSA-Screen latex agglutination test, DD = Disk diffusion testing and OXA = Oxacillin salt agar screen method.

a) S=susceptible and R= resistant.
b) Items in parentheses are NCCLS interpretation categories (S= susceptible and R= resistant).
-/+ Negative before induction and positive after induction.
A total of 197 CNS strains belonging to 7 species were identified, including *S. epidermidis* (143 strains), *S. hominis* subsp. *hominis* (30 strains), *S. haemolyticus* (14 strains), *S. auricularis* (3 strains), *S. lugdunensis* (3 strains), *S. simulans* (2 strains) and *S. capitis* subsp. *urealyticus* (2 strains). Ninety seven (49.2%) strains were mecA positive and one hundred (50.8%) were mecA negative by PCR (Tables 1).

The sensitivities, specificities, positive and negative predictive values and accuracy of the assays evaluated were presented in Table (2). The disk diffusion test failed to detect six mecA-positive strains and seven mecA-negative strains, showing 93.8% sensitivity and 93% specificity. Also agar screening plate test failed to detect four mecA-positive strains and only two mecA-negative strains, showing 95.9% sensitivity and 98% specificity. On the other hand, the E-test was reliable in the detection of oxacillin resistance in mecA-positive strains (100% sensitivity) where the oxacillin MICs for all of these strains were >0.5 μg/ml. However, it failed to detect five strains lacking the mecA gene (95% specificities) (Table 2). These five isolates, including three *S. lugdunensis* strains, one *S. auricularis* strain and one *S. epidermidis* strain, were classified as oxacillin resistant according to NCCLS guidelines (25), with MICs ranging from 0.5 to 4 μg/ml.

The slide latex agglutination method showed sensitivity, specificity, PPV, NPV and accuracy of (96.9, 100, 100, 97.1 and 98.5%) respectively, for the detection of oxacillin resistance in the CNS isolates. None of the mecA-negative strains showed oxacillin resistance, while oxacillin resistance was not detected in three of the mecA-positive isolates (false negative) in initial testing. After retesting of these 3 isolates after induction (stimulation of PBP2a expression), by inoculating the bacteria around the oxacillin disk, the sensitivity, NPV and accuracy of the slide latex test were 100,100 and 100%, respectively (Table 2).

The susceptibilities to oxacillin of all 19 isolates that showed discrepant results by one or more phenotypic tests are given in Table (3). Six strains of *S. epidermidis*, two strains of *S. hominis* subsp. *hominis* and one *S. capitis* subsp. *urealyticus* strain that contained the mecA gene showed false-negative results by at least one of the oxacillin susceptibility tests evaluated. On the other hand, mecA-negative *S. epidermidis* (4 strains), *S. hominis* subsp. *hominis* (1 strain), *S. haemolyticus* (1 strain), *S. lugdunensis* (3 strains), and *S. auricularis* (1 strain) isolates presented false-positive results by at least one of the phenotypic methods analyzed.

**DISCUSSION**

In this study we compared five different phenotyping and genotyping methods to evaluate the oxacillin susceptibilities of CNS strains. A total of 197 CNS strains belonging to 7 species were identified. Ninety seven (49.2%) strains were mecA positive and one hundred (50.8%) were mecA negative by PCR (Table 1). These results were similar to that in other studies (10,28), on the other hand they were higher than that reported by El-Nawawi et al. (4) (28%) because their study was done on low number of strains in comparison to this study.

Detection of oxacillin resistance among CNS isolates is difficult, mainly because it is often heterogeneous (3). To overcome this problem, different methods have been used. In this study, the disc diffusion method showed the lowest sensitivity (93.8%) for the detection of oxacillin resistance in CNS strains (Table 2). This result was agreed with those obtained in other studies (1,4,5,6,17). Six CNS isolates (four *S. epidermidis*, one *S. homonis subsp.hominis* and one *S. capitis subsp.urealyticus* isolates) that contained the mecA gene were found to be susceptible to oxacillin by this method (Table 3). This finding has already been reported and can be associated with the heteroresistance of the strains to oxacillin, as well as the absence of mecA gene expression in these isolates (7). However, the sensitivity and specificity of the disc diffusion in this study (93.8 and 93%, respectively) were higher than that reported by El-Nawawi et al. (4) (88.8 and 69.2%, respectively) because their study was done on only 40 strains of CNS.

According to the present oxacillin breakpoints, our E-test MIC results correctly classified all CNS strains with the mecA gene as oxacillin resistant ,100% sensitivity (Table 2), but at the same time misclassified five mecA-negative CNS isolates (one *S. epidermidis*, one *S. auricularis* (with MIC 4
mC, and all the three S. lugdunensis strains (with MIC 0.5-1 μg/ml)\textsuperscript{1} (Table 3) as oxacillin resistant. These findings were similar to those from other studies (5,9,15,17). Such results might be explained by the presence of mechanisms of oxacillin resistance other than that mediated by the mecA gene, specially for S. epidermidis and S. auricularis strains, such as other altered penicillin-binding proteins, as described by Suzuki et al. (29), or the overproduction of β-lactamases (3) by these isolates but these were not investigated further. Alternatively, some of these strains may be susceptible to oxacillin and the present NCCLS breakpoint guidelines may need to be re-evaluated for certain staphylococcal species, such as S. lugdunensis (15,31). At present, the NCCLS guidelines (25) do not recommend the use of the E-test method with S. saprophyticus and S. lugdunensis isolates, although this test is still recommended for use with other more uncommon CNS species. For this reason other studies that analyze a higher number of CNS species are necessary to establish a more reliable breakpoint.

Since the breakpoints for oxacillin resistance among CNS strains were lowered (23), use of the agar screening test with 6 μg of oxacillin per ml and 48 h of incubation is no longer recommended by the NCCLS. However, several studies observed that this technique is sensitive and can be used as an additional test to confirm the results obtained by the disk diffusion test (2,4,7,14,17). In this study, the results obtained by this method showed a good correlation with those obtained by PCR (95.9% sensitivity and 98% specificity) (Table 2). However, in order to raise the sensitivity of this method, Ferreira et al. (5), in their recent study, reported that the agar screening test with 4 μg of oxacillin per ml (instead of 6 μg) and 48 h of incubation presented 100% sensitivity and 100% specificity. Besides that, this test was easy to perform and cheap, allowing it to be used as a good alternative to the PCR technique. However, we did not use this concentration in this study so, we recommend making a comparison between the two concentrations of oxacillin in the agar screening test in a next study to confirm this issue.

The slide latex agglutination (MRSA-Screen latex agglutination test) was initially developed for the rapid detection of PBP 2a in oxacillin-resistant S. aureus isolates (2). However, several studies have also observed that this method has good accuracy for the detection of PBP 2a in CNS isolates (11,12,13,17). In the present study, the latex agglutination test showed a high sensitivity (96.9%) and a very high specificity (100%) (Table 2).

Induction of oxacillin resistance (stimulation of PBP2a expression) was not required in most of the isolates in this study, as was previously reported by Hussain et al. (12) due to the use of large inoculum of the organism (a heaping inoculum) in initial testing which also did not result in any loss of specificity. This modification was made on the basis of previous experience with the MRSA-Screen test and testing of S. aureus isolates (21) and also according to the recommendation of other investigators (17,18).

However, the three mecA-positive CNS isolates, two S. epidermidis strains and one S. capitis subsp. urealyticus strain (Table 3) which did not show visible agglutination in the primary testing were retested after the induction and all of these CNS strains yielded positive results, raising the sensitivity to 100% (Table 2). This result was in agreement with that reported by other investigator who tested the influence of induction of resistance in the slide latex agglutination test. Hussain and colleagues (13) observed an increase in sensitivity from 50 to 100% (their low initial sensitivity, 50%, was because they used small number of colonies, 3-5 colonies, in their testing). While Ferreira et al. (5) reported an increase in sensitivity from 96.1 to 99% after induction. However, another advantage from the use of oxacillin disc in the induction of resistance is the stronger agglutination reaction observed after induction, which makes the interpretation of the test results easier (13) but we prefer using a rather heavy inoculum, as oxacillin induction of PBP2a requires sub-cultivation and could delay the results by 24 h unless the oxacillin disk is generally performed in primary inoculum in the routine laboratory.

In summary, compared to PCR as the "gold standard," the MRSA-Screen latex agglutination test was able to rapidly and accurately determine the presence of oxacillin resistance mediated by the mecA gene in S. epidermidis and most other species of CNS. Our findings suggest that induction of
oxacillin resistance may be unnecessary if a large initial inoculum is used. All of the phenotypic methods evaluated in the present study appeared to perform very well for the detection of oxacillin resistance.

Future studies of oxacillin susceptibility testing for certain species of CNS (such as *S. lugdunensis*) may indicate the need for re-evaluation of the NCCLS breakpoints. Until such breakpoints are reassessed, the MRSA-Screen latex agglutination test may be considered an accurate, rapid and technically simple method for confirmation of an oxacillin MIC of $\geq 0.5$ µg/ml in routine laboratories, regardless of the phenotypic method used for susceptibility testing, for the detection of resistance which is mediated by the *mecA* gene.

**REFERENCE**


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لا يوجد نص يمكن قراءته بشكل طبيعي من الصورة المقدمة.