Branched-DNA Assay for Detection of the \textit{mecA} Gene in Oxacillin-Resistant and Oxacillin-Sensitive Staphylococci


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The identification of methicillin-resistant staphylococcus isolates in the clinical laboratory has typically been performed by using methods that detect phenotypic expression of resistance determinants. However, these methods may be difficult to interpret and some isolates do not express resistance until selective pressure is administered. Assays that detect genetic determinants are not subject to these limitations and have been effective in distinguishing isolates that are capable of expressing the resistance phenotype. In this study, a novel branched-DNA (bDNA) hybridization assay was used to test for the \textit{mecA} gene in 416 clinical staphylococcal isolates. The results were compared with those obtained by a PCR-based assay and oxacillin disk diffusion. For 155 \textit{Staphylococcus aureus} and 261 coagulase-negative \textit{Staphylococcus} isolates, the bDNA assay and PCR results were 100% concordant. Among the \textit{S. aureus} isolates, 20 were \textit{MecA} and 135 were \textit{MecA}. For the coagulase-negative staphylococci, 150 were \textit{MecA} and 111 were \textit{MecA}. The results from the genotypic detection methods were compared with those obtained by oxacillin disk diffusion. No discrepancies were detected among the \textit{S. aureus} isolates; however, 10 coagulase-negative isolates were \textit{MecA} but oxacillin sensitive and 1 isolate was \textit{MecA} but oxacillin resistant. Oxacillin resistance was induced in 6 of the 10 \textit{MecA} isolates previously classified as oxacillin sensitive. These results suggest that the bDNA method described here is a sensitive and efficient method for detection of methicillin resistance in staphylococci and that genetic detection methods may be useful for detection of potential methicillin resistance in the clinical laboratory.

Methicillin resistance in clinical isolates of \textit{Staphylococcus} is thought to occur as a combined result of the expression of the \textit{mecA} gene, which codes for the cell wall surrogate enzyme penicillin binding protein (PBP) 2a or 2′ and several factors such as the fem gene series or auxiliary \textit{(aux)} genes (reviewed in reference 5). In clinical laboratories, antibiotic resistance is usually detected by using methods that require a viable culture of the organism and phenotypic expression of resistance genes. However, studies indicate that there is heterogeneous expression of PBP 2a that is dependent on environmental conditions (1, 10, 16). In addition, some isolates have been shown to exhibit low- or moderate-level methicillin resistance due to overproduction of β-lactamase, modifications in the PBP binding affinities, or the presence of expression factors not related to the \textit{mecA} gene (2, 9, 12, 20). Variations in laboratory reporting of high-level methicillin resistance, which requires treatment with vancomycin, may be responsible for unnecessary vancomycin usage. The current guidelines from the Centers for Disease Control and Prevention suggest restriction of vancomycin use in order to slow the occurrence of vancomycin resistance in staphylococci (4).

Molecular diagnostic assays, which detect genetic targets irrespective of expression level, have proven useful for the identification of isolates containing \textit{mecA}. In recent years, several genotypic detection methods have been described (3, 7, 11, 14, 17). Most are PCR based, and some are multiplexed with broad-range 16S ribosomal DNA primers to assess the lysis efficiency of each assay. While these assays are highly sensitive and specific, we have found that they are time-consuming and PCR failures may occasionally occur due to lysis inefficiency or inhibitory substances.

In the past, assays utilizing branched-DNA (bDNA) technology have been developed to detect antibiotic resistance markers as well as pathogenic agents in clinical samples (6, 19). This assay uses multiple probes that cause an amplification of chemiluminescent signal rather than the amplification of a genetic target that is observed in PCR-based assays. To avoid the complications observed with other tests, we developed a \textit{mecA}-specific assay that uses bDNA technology to detect the gene in lysates derived from bacterial colonies isolated on solid media and directly from blood cultures. The assay is performed in a 96-well microtiter plate format and takes approximately 6 h to complete, thereby allowing same-day results for cultures containing staphylococci.

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MATERIALS AND METHODS

\textbf{Bacterial isolates.} Staphylococcal isolates \((n = 433)\) were recovered from clinical samples that were taken from normally sterile anatomic sites. Consecutive isolates were recovered by inoculation onto 5% sheep erythrocyte agar plates (Becton Dickinson, Cockeysville, Md.) and incubation for 24 to 48 h at 37°C. Isolates exhibiting characteristics of gram-positive cocci by Gram stain reaction and morphology were initially identified as either \textit{Staphylococcus aureus} or coagulase-negative \textit{Staphylococcus} (CNS) species by using standard methods, and then all were assayed for routine antibiotic susceptibilities. With the exception of the discrepant isolates, gram-positive cocci such as \textit{Micrococcus} sp. and \textit{Stomatococcus} sp. were not distinguished from true staphylococci. Three bacterial cultures chosen to be controls for this study were first characterized by pheno-
typic detection methods, PCR (7), and by a previously described DNA hybrid-
ization assay (11). A MecA+ S. aureus (ATCC 33591), a MecA+ S. aureus
(ATCC 12600), and a MecA+ CNS (MC1387) were included in each assay run.
Discrepant coagulase-negative isolates were further identified by morphologic
features, catalase test, and by use of the Biolog (Hayward, Calif.) Microstation
system.

Phenotypic assay methods. Isolates were emulsified in Trypticase peptone
broth (Becton Dickinson) to a McFarland turbidity standard of approximately
1.0. They were then assayed for oxacillin susceptibility by using a standardized
disk diffusion method as described previously (7) with the exception that Mucl-
er-Hinton agar (MHA; Becton Dickinson) contained 2% NaCl. Positive and
negative control experiments were performed for each assay. When discrepan-
cies between phenotypic and genotypic methods occurred, the phenotypic assay
was repeated in an attempt to resolve the discrepancy.

In addition, the discrepant isolates were tested for the presence of β-lactamase
by the nitrocefin disk method (Cefinase; Becton Dickinson), and β-lactamase
overproduction was assessed by amoxacillin-clavulanic acid (20 µg/10 µg) disk
diffusion (Becton Dickinson). Next, a bacterial suspension with turbidity approx-
imately equal to a 1.0 McFarland standard was inoculated by being swabbed onto
agar medium containing 4% NaCl-6 mM MgCl₂. Positive and negative control
experiments were performed for each assay. When discrepan-
cies among the discrepant isolates were also
inoculated onto an in-house-prepared MHA medium containing 4% salt without
oxacillin (Becton Dickinson).

Induction of oxacillin resistance. Isolates that were MecA+ but sensitive to
oxacillin were inoculated onto a series of MHA plates containing increasing
concentrations of oxacillin. Sets of the agar plates were made by twofold dilutions
of oxacillin starting at 2.0 to 0.0625 µg/ml. Two MecA+ controls (ATCC 12600
and S. aureus ATCC 25923) were included to assess the media by providing an
end point for MecA+ isolates. Organisms were streaked for isolation on MHA
containing the lowest oxacillin concentration and incubated at 37°C for 24 h.
Colonies growing on the medium were inoculated onto MHA-oxacillin medium
of the next highest concentration. This procedure was repeated until isolates
were growing on medium containing a 2.0-µg/ml concentration of oxacillin.
Subsequently, the disk diffusion assay was repeated for each isolate to assess any
change in inhibition zone size (diameter).

PCR assay. All isolates were lysed and amplified by using a multiplex PCR
assay in accordance with the protocol described by Geha et al. (7).

bDNA assay. A 617-bp amplification product for use as a positive control was
created by using primers mecC72 (5'-TAATAGTTGTAGTTGTCGGGTTTG-
3') and mec765 (5'-GTTTTTAAGTGGAAACGAAAGGTAT-3'), which were
designed from the published mecA gene sequence for S. aureus (GenBank Ac-
cession no. X52593). Each primer (0.5 µM) was included in a 50-µl PCR mixture
along with 2.0 µl of S. aureus lysate, 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5
mM MgCl₂, 1.5 µM each deoxynucleoside triphosphate and 1.25 U of AmpliTag
DNA polymerase (Perkin-Elmer, Norwalk, Conn.). Amplification was per-
fomed in accordance with the following profile: initial denaturation at 94°C for
4 min, followed by 30 cycles of denaturation at 94°C for 45 s, 50°C for 45 s,
and 72°C for 1 min, and then 72°C for 2 min. Subsequently, the amplification
product was inserted into a plasmid vector in accordance with the manufacturer’s instruc-
tions for the TA cloning kit (Invitrogen, San Diego, Calif.). Plasmid DNA for use
in the bDNA assay was isolated from one of the positive transformants and
linearized with RsrII as described previously (18).

The MecA bDNA assay was performed as described in instructions provided
by Chiron Diagnostics (East Walpole, Mass.) (Fig. 1). As in the phenotypic and
PCR assays, both S. aureus and CNS controls were included in each run. The
target-associated luminescence was then measured in a luminometer (Chiron
Corporation, Emeryville, Calif.). The average relative light unit (RLU) value
for each isolate was then divided by the average value obtained from the negative
culture control to give a signal-to-noise (S/N) ratio. A S/N ratio of ≥3.0 indicated
the presence of the mecA gene. Based on the RLU values, a coefficient of
variance was determined for each sample replicate to assess the reproducibility
of each assay. In the event of a positive and a negative RLU value from a set of
replicates, assay of the sample was repeated.

Analytical sensitivity and specificity of the MecA bDNA assay. Dilution studies
were performed with known concentrations of MecA+ S. aureus with the intent
of determining the minimum concentration of organism needed to detect the
mecA gene. Eleven nonstaphylococcal isolates were assayed by using the MecA
bDNA assay to detect cross-reactivity that might occur with other microbial
species (Table 1).

RESULTS

To assess the analytical sensitivity of the MecA bDNA assay, serial dilutions of S. aureus containing the gene were analyzed
by using the MecA bDNA assay. The minimum dilution from which the mecA gene was detected contained a concentration of
10⁴ CFU/ml. No cross-reactivity with the mecA probes was
detected among the 11 nonstaphylococcal isolates that were tested (Table 1). Next, the clinical sensitivity and specificity of the MecA bDNA assay were assessed in comparison to those of other methods for detection of methicillin resistance. Four hundred thirty-three clinical staphylococcal isolates were assessed by bDNA, and the results were compared with those obtained by mecA-specific PCR and oxacillin disk diffusion assays. Seventeen isolates classified as CNS were removed from the study because of PCR failure, presumably because they were not lysed by our methods. Before the results for the unknown isolates were assessed, all control results were examined to ensure the validity of each assay. Our comparison was performed with 416 isolates, including 155 S. aureus, 261 CNS isolates. The results for bDNA were all corroborated by PCR (positive and negative predictive values = 100% [each]; sensitivity and specificity = 100% [each]) (data not shown). For S. aureus, 20 isolates were MecA⁺ by both genetic methods; 135 isolates were MecA⁻. For CNS, 150 isolates were MecA⁺ by both genetic methods; 111 isolates were MecA⁻.

The bDNA and PCR results were then compared with results obtained by the oxacillin disk diffusion test. No discrepancies occurred among the S. aureus isolates. For S. aureus, all 20 MecA⁺ isolates were resistant by disk diffusion; 135 isolates were MecA⁻ and sensitive by disk diffusion. For CNS isolates, 140 were MecA⁺ and resistant by disk diffusion; 110 were MecA⁻ and sensitive by disk diffusion. However, 10 CNS isolates were MecA⁺ but were oxacillin sensitive by disk diffusion (mean zone size = 15.1 mm) and one CNS isolate was MecA⁻ and oxacillin resistant by disk diffusion (zone size = 9 mm).

Subsequent to the initial antibiotic sensitivity testing, the discrepant CNS isolates were further identified to the species level by using the Biolog Microstation system, and the antibiotic susceptibility results were confirmed by repeating the oxacillin disk diffusion test. Among the 10 MecA⁺, oxacillin-sensitive isolates, 8 were identified as Staphylococcus epidermidis and two were identified as Staphylococcus hominis (Table 2). The isolate that was MecA⁻ and oxacillin resistant was found to be Micrococcus luteus. The results of the repeated disk diffusion tests for the MecA⁺, oxacillin-sensitive isolates were similar to the initial test results (mean zone size = 15.6 mm). Four of the MecA⁺, oxacillin-sensitive staphylococcal isolates produced β-lactamase; all of these were sensitive to the amoxicillin-clavulanic acid combination (mean zone size = 27 mm). Repeat disk diffusion testing of the MecA⁺, oxacillin-resistant isolate also gave a similar disk zone size (9 mm) and was negative for β-lactamase production, suggesting that another mechanism was responsible for the resistant phenotype.

The discrepant isolates were then assayed by screening with the combination of 6 µg of oxacillin per ml and 4% NaCl. As a control for growth under high-salt conditions, isolates were also inoculated onto MHA medium with the addition of 4% salt but no antibiotic. For the MecA⁺, oxacillin-sensitive isolates, one was inhibited by the 4% salt medium at both 24 and 48 h of incubation and nine grew on the control plates. At 24 h of incubation, none of the discrepant isolates grew on the oxacillin screening medium. At 48 h of incubation, 6 of 10 MecA⁺, oxacillin-sensitive isolates grew, indicating that they were highly resistant to oxacillin. Interestingly, only four of the six were also positive for β-lactamase production. The isolate that was MecA⁺ but oxacillin resistant did not grow at 24 h, but at 48 h, several tiny colonies grew on the test medium.

The ability of the MecA⁺, oxacillin-sensitive isolates to become phenotypically resistant during selective pressure was tested by inoculating colonies onto MHA medium with increasing concentrations of oxacillin. Neither of the MecA⁻ control isolates grew on medium with the highest concentration (2.0 µg/ml) of oxacillin. Among the 10 MecA⁺, oxacillin-sensitive isolates, the 8 S. epidermidis isolates grew on medium with the final concentration of 2 µg of oxacillin per ml; however, only 6 of these 8 isolates were subsequently resistant to

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**Table 1. Cross-reactivity of mecA probes as assessed by testing nonstaphylococcal clinical isolates**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Mean RLU value</th>
<th>S/N ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli</td>
<td>1.32</td>
<td>1.54</td>
</tr>
<tr>
<td>Corynebacterium sp.</td>
<td>0.94</td>
<td>1.10</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>0.77</td>
<td>0.90</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>0.93</td>
<td>0.90</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>1.11</td>
<td>1.29</td>
</tr>
<tr>
<td>Citrobacter freundii</td>
<td>0.73</td>
<td>0.85</td>
</tr>
<tr>
<td>Streptococcus pneumoniae</td>
<td>1.12</td>
<td>1.30</td>
</tr>
<tr>
<td>Streptococcus faecalis</td>
<td>0.98</td>
<td>1.14</td>
</tr>
<tr>
<td>Streptococcus bovis</td>
<td>0.87</td>
<td>1.01</td>
</tr>
<tr>
<td>Streptococcus agalactiae</td>
<td>0.97</td>
<td>1.12</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>0.88</td>
<td>1.10</td>
</tr>
</tbody>
</table>

* The average (mean) RLU value of two replicates was divided by the average RLU value of the negative control isolate to yield the S/N ratio. A S/N value of ≥3.0 was considered positive.

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**Table 2. Results of various confirmatory tests on the discrepant CNS isolates**

<table>
<thead>
<tr>
<th>No. of isolates and species identified</th>
<th>MecA status</th>
<th>Repeat OX disk zone diam (mm)</th>
<th>β-Lactamase production</th>
<th>Growth on OX-salt agar</th>
<th>AMOX-CLAV disk zone diam (mm)</th>
<th>OX zone size after induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>57 S. hominis</td>
<td>+</td>
<td>15</td>
<td>-</td>
<td>NG</td>
<td>ND</td>
<td>24</td>
</tr>
<tr>
<td>105 S. hominis</td>
<td>+</td>
<td>11</td>
<td>-</td>
<td>G</td>
<td>25</td>
<td>13</td>
</tr>
<tr>
<td>120 S. epidermidis</td>
<td>+</td>
<td>12</td>
<td>-</td>
<td>NG</td>
<td>ND</td>
<td>No zone</td>
</tr>
<tr>
<td>121 S. epidermidis</td>
<td>+</td>
<td>19</td>
<td>-</td>
<td>G</td>
<td>26</td>
<td>No zone</td>
</tr>
<tr>
<td>133 S. epidermidis</td>
<td>+</td>
<td>28</td>
<td>-</td>
<td>NG</td>
<td>ND</td>
<td>NG</td>
</tr>
<tr>
<td>198 S. epidermidis</td>
<td>+</td>
<td>16</td>
<td>-</td>
<td>NG</td>
<td>ND</td>
<td>NG</td>
</tr>
<tr>
<td>265 S. epidermidis</td>
<td>+</td>
<td>11</td>
<td>-</td>
<td>G</td>
<td>27</td>
<td>PPTS</td>
</tr>
<tr>
<td>266 S. epidermidis</td>
<td>+</td>
<td>13</td>
<td>-</td>
<td>G</td>
<td>27</td>
<td>9</td>
</tr>
<tr>
<td>336 S. epidermidis</td>
<td>+</td>
<td>15</td>
<td>-</td>
<td>G</td>
<td>27</td>
<td>No zone</td>
</tr>
<tr>
<td>412 S. epidermidis</td>
<td>+</td>
<td>17</td>
<td>+</td>
<td>G</td>
<td>30</td>
<td>No zone</td>
</tr>
<tr>
<td>91 M. luteus</td>
<td>-</td>
<td>9</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* OX, oxacillin; NG, no growth; ND, not done; PPTS, pinpoint colonies growing up to the antibiotic disk.

* Repeat OX disk, repeated oxacillin disk diffusion test.

* AMOX-CLAV disk, amoxicillin-clavulanic acid disk diffusion test.
oxacillin by disk diffusion. Four of the six had no zone of bacterial inhibition on the media, one had a zone of 16 mm with pinpoint colonies up to the disk, and one had a zone size of 9 mm. Although the two *S. hominis* isolates grew on the final concentration of 2 μg/ml, they were both sensitive by repeat disk diffusion (mean zone size = 18.5 mm).

**DISCUSSION**

In a previous study, we evaluated a mecA-specific assay that used a paramagnetic particle-labeled probe to separate the target-probe duplexes from solution and an acridinium ester-labeled probe to detect the hybridized mecA gene (11). However, the assay was limited by low sensitivity and high concentrations of organisms were needed to obtain a valid result. The MecA bDNA assay affords a significant increase in sensitivity that is probably due to the inherent signal amplification properties of the assay. When the PCR was used as the gold standard, the bDNA assay was 100% sensitive and specific for both *S. aureus* and CNS.

Among 416 isolates, only 11 gave discrepant results between genotypic and phenotypic assays. Of these discrepant isolates, 10 contained the mecA gene yet were phenotypically sensitive to oxacillin and 1 did not contain the mecA gene but was oxacillin resistant. Eight of these MecA− isolates were later identified as *S. epidermidis*, and two were identified as *S. hominis*. The MecA− discrepant isolate was found to be *M. luteus*, and its phenotypic resistance can possibly be explained by other mechanisms not addressed in this study.

The ability of several oxacillin-sensitive isolates to become resistant after selective pressure underscores the importance of genotypic screening for antibiotic resistance markers. Presumably, such acquired resistance could occur in vivo during antibiotic therapy. Six of the 10 MecA− discrepant isolates grew on the commercially prepared oxacillin screening medium prior to the induction experiment, 1 seemed to be inhibited by the high salt concentration, and 3 did not grow in the presence of 6 μg of oxacillin per ml. Such results are not surprising, considering that all of these isolates were sensitive to oxacillin before the induction experiment was performed. The appearance of growth after 48 h but not after 24 h might represent an induction of mecA expression during the extended incubation period. Indeed, similar results were observed by Ramotar et al. (15), with the conclusion that many mecA-positive isolates for which MICs are below breakpoint would not be detected by screening with the oxacillin-salt agar medium.

The problems associated with using phenotypic methods for identification of methicillin resistance have been well defined, and many researchers use direct detection of the mecA gene or PBP 2a as the gold standard for comparison (7, 8, 11, 13, 21). On the other hand, most phenotypic detection methods take little hands-on time and are inexpensive, thus allowing them to easily fit into the daily work flow of the clinical laboratory. Most clinical laboratories do not routinely assess the genetic status of oxacillin-resistant isolates. Isolates that contain the mecA gene yet are sensitive to oxacillin may be induced to express resistance by exposure to weak concentrations of oxacillin. Therefore, genotypic methods would be helpful in accurately assessing the potential of an isolate to become resistant during therapy. Furthermore, in light of these data, it may be possible to adjust MIC breakpoints for the coagulase-negative staphylococci to improve the sensitivity of detection of mecA-containing isolates. Additional studies are needed to determine the clinical implications of unexpressed antibiotic resistance genes within pathogenic agents and the impact of this a priori knowledge on treatment regimens.

In our hands, the MecA bDNA assay was a highly accurate alternative to other genetic detection methods and gave reproducible results for all samples, including the isolates from which the PCR assay failed to amplify the internal control. The MecA bDNA assay has also been shown to be useful for direct detection of the mecA gene in broth taken from blood culture bottles without the need for an involved DNA extraction process (22). Currently, we are investigating cost-effective methods for the integration of bDNA technology in the clinical laboratory.

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**REFERENCES**

14. McDougal, L. K., and C. Thornsbury. 1986. The role of β-lactamase in staphylococcal resistance to penicillinase-resistant penicillins and cephalo-