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Multiplex PCR for Identification of Methicillin-Resistant Staphylococci in the Clinical Laboratory

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A multiplex PCR assay for detection of the staphylococcal *mecA* gene (the structural gene for penicillin-binding protein 2a) was compared with agar dilution and disk diffusion susceptibility test methods for identifying methicillin resistance. The multiplex PCR assay combined two primer sets (*mecA* and 16S rRNA) in a single reaction. A total of 500 staphylococcal isolates (228 isolates of *Staphylococcus aureus* and 272 isolates of coagulase-negative staphylococci) from clinical specimens were studied. For *S. aureus*, 40 of 40 *mecA*-positive isolates and 4 of 188 *mecA*-negative isolates were oxacillin resistant (positive and negative predictive values of 100 and 98%, respectively). In 3 of 4 discordant isolates, resistance was due to hyperproduction of β-lactamase. For coagulase-negative staphylococci, 148 of 159 *mecA*-positive isolates and 0 of 113 *mecA*-negative isolates were oxacillin resistant (positive and negative predictive values of 93 and 100%, respectively). Twenty-six isolates were categorized as indeterminate because of the absence of a detectable 16S rRNA product. Four of these 26 isolates contained *mecA* when retested. The assay is designed to be incorporated into the work flow of the clinical microbiology laboratory and allows for the identification of intrinsic resistance in a timely and reliable manner.

Methicillin-resistant staphylococci are significant pathogens that have emerged over the past 30 years to now cause both nosocomial and community acquired infections (8, 11, 34, 44). Resistance is primarily mediated by the production of an altered penicillin-binding protein (PBP 2a) (7, 43). This cell wall enzyme, unlike the other constitutive PBPs, has a low affinity for β-lactam antibiotics and allows cell wall formation in concentrations of drug that render the other PBPs inactive. PBP 2a and the gene encoding it (*mecA*) have been found in all highly resistant staphylococci.

To date, the only standardized means of identifying methicillin resistance in the clinical microbiology laboratory are susceptibility tests, such as disk diffusion, agar or broth dilution, and agar screen methods (28, 29). The performance of these tests has been erratic because many factors such as inoculum size, incubation time and temperature, pH of the medium, salt concentration of the medium, and exposure to β-lactam antibiotics influence the phenotypic expression of resistance (13, 19, 35, 38, 46). To complicate matters further, methicillin resistance is often expressed heterogeneously, in that only 10^4 to 10^7 cells are phenotypically resistant (12). Taking into account these factors, the National Committee for Clinical Laboratory Standards (NCCLS) has set guidelines to maximize the phenotypic expression of resistance (28, 29). However, occasionally, organisms that are difficult to classify by these methods have been isolated. There is often discordance between results by disk diffusion and agar dilution. Although the NCCLS describes only one breakpoint for resistance (oxacillin MIC of >2 μg/ml), many investigators define a category of borderline or low-level resistance (1 to 8 μg/ml) that implies a mechanism other than that of PBP 2a (10, 23, 24, 35, 41).

The empirical approach of most clinicians has been to view all levels of methicillin resistance as being equivalent to intrinsic, high-level resistance (23). Intrinsic methicillin-resistant staphylococcal infections require vancomycin therapy (17, 27, 45). In addition, intrinsic methicillin-resistant *Staphylococcus aureus* infections in many hospitals require strict patient isolation procedures. Staphylococcal strains with borderline resistance, lacking PBP 2a or the *mecA* gene, have been effectively treated with β-lactam antibiotics (5). Furthermore, non-PBP 2a-bearing strains of *S. aureus* may not require expensive and inconvenient patient isolation procedures. Because intrinsic resistance of both *S. aureus* and coagulase-negative staphylococci (CNS) appears almost exclusively to be due to PBP 2a production, techniques have been developed to identify the *mecA* genetic determinant that codes for this protein. These assays utilize probe (2, 18) and PCR techniques (25, 31, 40). These techniques show a high degree of correlation among susceptibility tests and allow accurate classification of not only highly resistant but also borderline-resistant strains (10).

In this study, the largest of its kind to date, a multiplex PCR assay that uses primer sets specific for the *mecA* gene and a 16S rRNA universal target sequence was developed. The 16S rRNA gene, common to all bacteria (32), was used as an internal control to identify potential false-negative results. Isopsoralen compound 10 (HRI Associates, Concord, Calif.) was incorporated into the protocol to prevent false-positive PCR results. The test was designed to fit into the flow of daily susceptibility testing performed in the clinical microbiology laboratory. PCR results were compared with those of standard susceptibility methods (disk diffusion and agar dilution) (28, 29).

MATERIALS AND METHODS

Bacterial strains. The staphylococcal strains were clinical isolates from Mayo Clinic patients or were obtained through Mayo medical laboratories. The Clinical Microbiology Labo-

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ratory identified the organisms as *S. aureus* or CNS by colony morphology, Gram stain characteristics, and catalase and coagulase tests. Strains referred for quantitative susceptibility testing were collected for this study. Requirements for referral included recovery of organisms from normally sterile body sites or a specific request by the physician in attendance. From March through November 1992, 500 isolates were selected for genetic analysis. All methicillin-resistant *S. aureus* isolates were preferentially selected, with the remainder of the isolates being randomly assigned. *S. aureus* (ATCC 25923) was used as the *mecA*-negative control, and *Staphylococcus epidermidis* (ATCC 27626) was used as the *mecA*-positive control organism.

Agar dilution and disk susceptibility tests. The procedures routinely used in the Clinical Microbiology Susceptibility Laboratory were employed for this study. By agar dilution, MICs of oxacillin were determined in Mueller-Hinton agar without added NaCl inoculated with 10^4 CFU via a Cathra replicator system (Auto Med, Shoreview, Minn.). Plates were read at 24 and 48 h of incubation at 30°C. The MIC was defined as the lowest concentration of drug which inhibited visible growth of bacteria. The concentrations of oxacillin tested were 1 and 2 μg/ml. Disk diffusion tests were performed with 1 μg of oxacillin per disk placed on 25 ml of Mueller-Hinton agar without NaCl supplementation (28). The zone of inhibition was determined after 24 h of incubation at 35°C.

Strains were initially tested by the agar dilution method. For any staphylococci with an oxacillin MIC of 2 μg/ml or recovered from a normally sterile body site, the disk diffusion test was performed. Strains with an oxacillin MIC of >2 μg/ml or oxacillin disk zone diameter of ≤10 mm were classified as methicillin resistant (MIC of >2). Strains with an oxacillin MIC of <2 μg/ml and a disk test zone diameter of ≥14 mm were classified as susceptible (MIC = 1 μg/ml). The classification of strains with an oxacillin MIC of 2 μg/ml was based on the disk diffusion test result. If the zone diameter was >10 mm, the MIC was reported as 2 μg/ml. If the zone diameter was ≤10 mm, the MIC was reported as >2. For all *mecA*-negative Ox^r organisms, the actual MIC was determined up to 128 μg/ml.

β-Lactamase tests. β-Lactamase production was identified by using nitrocefin disks (BBL Microbiology Systems, Cockeysville, Md.). This test was performed on all *mecA*-negative Ox^r organisms.

Agar screen susceptibility tests. Agar screen tests for susceptibility to oxacillin were performed by a previously described method (29, 39). A total of 10^4 CFU was spot inoculated onto Mueller-Hinton agar with 4% NaCl supplementation containing 6 μg of oxacillin per ml. Plates were read at 24 h of incubation at 30°C. Agar screen tests were done on all discrepant isolates (*mecA* positive, Ox^s; *mecA* negative, Ox^r). If any colonies of growth were detected, the test was considered positive for resistance.

Amoxicillin-clavulanic acid disk tests. The amoxicillin-clavulanic acid (Augmentin) disk test was performed on the *mecA*-negative Ox^r isolates. A 6.35-mm-diameter disk containing 20 μg of amoxicillin and 10 μg of clavulanic acid was placed on Mueller-Hinton agar without NaCl supplementation (29). The breakpoint for susceptibility was a ≥20-mm-diameter zone of inhibition after 24 h of incubation at 30°C.

Preparation of bacterial lysates. A 0.25-ml aliquot of the organism suspended in indole broth was centrifuged at 12,000 × *g* for 5 min. The supernatant was discarded, and the pellet was resuspended in 100 μl of cell lysis solution {250 U of lysozyme (Sigma Chemical Co., St. Louis, Mo.) per ml, 25 U of lysostaphin (Sigma) per ml, 10 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid], 10 mM Tris (pH 8) in deionized H_2O}. This suspension was incubated for 30 min at 35°C, placed in a 95°C heat block for 10 min, cooled to room temperature, and then diluted in 900 μl of sterile water.

Oligonucleotides. All primers were synthesized on an Applied Biosystems (Foster City, Calif.) 320A DNA synthesizer. Two primer sets were used in a multiplex PCR procedure (3). The 16S rRNA primer sequences were synthesized according to the method of Relman et al. (32). Primer X (5'-GGA ATT CAA A[T/G, 1:1]G AAT TGA CGG GGG C) corresponded to nucleotides 911 to 930. Primer Y (5'-CGG GAT CCC AGG CCC GGG AAC GTA TTC AC) was complementary to nucleotides 1371 to 1390. These primers gave rise to a PCR product of 479 bp.

From a highly conserved region of the 2,456-bp *mecA* gene sequence (36, 37), two 25-bp primers were synthesized. Primer *mecA1* (5'-GTA GAA ATG ACT GAA CGT CCG ATA A) corresponded to nucleotides 318 to 342. Primer *mecA2* (5'-CCA ATT CCA CAT TGT TTC GGT CTA A) was complementary to nucleotides 603 to 627. Amplification with these primers gave rise to a 310-bp *mecA*-specific product. Following synthesis, the primers were purified by the Oligonucleotide Purification Cartridge procedure (Applied Biosystems).

DNA amplification procedure. The PCR reagent mixture consisted of 200 μM deoxynucleoside triphosphates, 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 μM $MgCl_2$, 0.5% Tween, 10% glycerol, 50 pmol of *mecA* primers, 5 pmol of 16S rRNA primers, 0.5% isopsoralen compound 10 (16), and water. Prior to adding 1.25 U of Ampli*Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) per 50 μl, the mixture was filtered through an ultra-free-DF filter unit (100,000 NMWL polysulfone membrane [Millipore Corp., Bedford, Mass.]) to eliminate possible exogenous bacterial DNA contamination. DNA extract solution (2 μl) was added to the PCR mixture for a 50-μl reaction mixture. DNA amplification was carried out in a Perkin-Elmer thermocycler, with the following thermal cycling profile: initial denaturation at 94°C for 4 min was followed by 30 cycles of amplification (denaturation at 94°C for 45 s, annealing at 50°C for 45 s, and extension at 72°C for 60 s) ending with a final extension at 72°C for 2 min.

Post-PCR isopsoralen inactivation was carried out by exposing the PCR amplified mixture to UV light at 365 nm at 4°C for 15 min (9, 16). The PCR products (10 μl) were separated by electrophoresis on a 3% agarose gel run at 100 V for 30 min followed by ethidium bromide staining for 30 min.

Statistical methods. Evaluation of the data comparing the *mecA* PCR assay results and the results from the standard susceptibility tests required a matched 2-by-2 table. Significance of the results was calculated by using the Sign and McNemars tests (15).

RESULTS

PCR detection of *mecA* gene. The PCR procedure took about 6 h to complete, requiring approximately 2 h of hands-on technologist time to evaluate up to 40 isolates. A total of 500 isolates were evaluated by this technique. For *mecA*-positive strains, two discrete DNA fragments, a 479-bp 16S rRNA-specific fragment and the *mecA*-specific product of 310 bp, were amplified. Because isopsoralen was used in a post-PCR inactivation procedure, the apparent molecular weights of these fragments were slightly above the expected values (30). This increase in molecular weight, however, was reproducible from run to run and did not interfere with the interpretation of the results (Fig. 1). For *mecA*-negative isolates, only the 16S rRNA-specific band of 479 bp was observed. In samples for

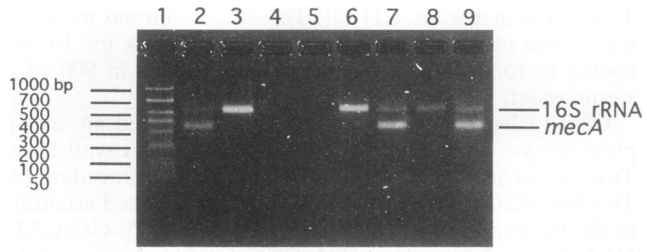

FIG. 1. Agarose gel electrophoresis for 16S rRNA and *mecA*. Lanes: 1, molecular weight standards; 2, positive control; 3, negative control; 4, water; 5, lysis solution, 6 to 9, clinical isolates.

which no product was observed, failure of either cell lysis or the PCR reaction itself (i.e., due to inhibition) was considered, and the test was repeated. From a total of 26 "no-product" reactions, 4 samples revealed a positive *mecA* result upon repeat testing.

No nonspecific background amplification products were encountered in this assay. Each reaction series included a positive control and negative control organism (as noted in Materials and Methods), a lysis solution blank, and a water blank (water used in diluting the specimens). Of 500 staphylococcal isolates evaluated, 199 (40%) were *mecA* positive (18% *S. aureus* and 58% CNS).

Correlation between genotypic and phenotypic susceptibility results. Of the 500 staphylococcal isolates tested, 228 were *S. aureus* and 272 were CNS. For *S. aureus*, 40 of 40 *mecA*-positive isolates were Ox^r and 184 of 188 *mecA*-negative isolates were Ox^s. This resulted in a positive predictive value (PPV) of 100% ($P = 0.25$) and negative predictive value (NPV) of 98% for the *mecA* assay. Further evaluation of the *mecA*-negative Ox^r organisms revealed actual oxacillin MICs of 2 to 4 μg/ml, lack of resistance on agar screen testing, and susceptibility to amoxicillin-clavulanic acid (Table 1). Thus, hyperproduction of β-lactamase was considered the most likely cause of borderline resistance in these cases. These isolates were also tested with an alternative set of *mecA* primers developed by Murakami et al. (25). The latter reagents, by using previously published PCR conditions (see Materials and Methods), also failed to detect the *mecA* gene in these organisms. For CNS, 134 of 159 *mecA*-positive isolates were initially Ox^r and 113 of 113 *mecA*-negative isolates were Ox^s. Fourteen of 25 (56%) isolates which were classified initially as Ox^s *mecA* positive expressed phenotypic resistance (MIC of >2 μg/ml) upon repeat susceptibility testing, leaving 148 of 159 Ox^r *mecA*-positive isolates. Thus, for CNS, the PPV and NPV were 93 ($P = 0.0001$) and 100%, respectively. The PPV of genotypic detection of Ox^r was relatively low because the discordant isolates could not be induced by agar screen testing to express phenotypic resistance. The Biolog (Hayward, Calif.) bacterial identification system was used to identify the discordant CNS isolates (*mecA* positive, Ox^s) to the species level: seven *S. epidermidis*, three *S. hominis*, and one *S. caprae* isolates (Table 2). None were inducible by agar screen testing, although several had intermediate (11- to 13-mm-diameter) zones by disk diffusion testing.

TABLE 1. Characteristics of *mecA*-negative Ox^r *S. aureus*[a]

Isolate no.	*S. aureus* source	Oxicillin MIC[b]	Agar screen[c]	Amoxicillin-clavulanic acid disk[d]	Interpretation of results
1	Urine	4	S	S	Hyperproduction of β-lactamase
2	Tracheal secretion	2[e]	S	S	Hyperproduction of β-lactamase
3	Hip tissue[f]	4	NG	NG	Indeterminate
4	Knee tissue[g]	2[e]	S	S	Hyperproduction of β-lactamase

[a] All isolates were β-lactamase positive.
[b] For *mecA*-negative Ox^r organisms, the MIC upper limit was determined.
[c] Mueller-Hinton agar was incubated 48 h at 30°C. S, oxacillin susceptible; NG, no growth.
[d] Amoxicillin-clavulanic acid disk interpretations: susceptibility (S), >19-mm diameter; resistance ≤19-mm diameter.
[e] Classified resistant on the basis of the disk diffusion result of ≥10-mm diameter.
[f] Tissue from total hip arthroplasty.
[g] Tissue from total knee arthroplasty.

TABLE 2. Characteristics of *mecA*-positive Ox^s CNS[a]

Organism	Source	Disk diffusion zone diameter (mm)	
		Test 1	Test 2
S. epidermidis	Elbow	22	27
S. hominis	Femur tissue	14	11
S. epidermidis	Urine	16	16
S. epidermidis	Knee tissue	12	14
S. epidermidis	Knee tissue	12	12
S. caprae	Urine	15	30
S. epidermidis	Urine	15	15
S. hominis	Femoral canal	17	18
S. epidermidis	Urine	16	18
S. hominis	Blood	15	20
S. epidermidis	Urine	13	25

[a] For all organisms tested, the oxacillin MIC was 1. Agar dilution and disk diffusion tests were performed twice (test 1 and test 2). By the agar screen test, which utilized 6 μg of oxacillin per ml in 4% NaCl-supplemented Mueller-Hinton agar, all organisms tested as susceptible.

DISCUSSION

The identification of methicillin-resistant staphylococci in the laboratory is sometimes complicated by the heterogenous expression of resistance and the variables that influence this expression (i.e., pH, temperature, and salt concentrations). The NCCLS provides a breakpoint MIC for resistance, but many investigators advocate recognition of degrees of resistance that are clinically important. Borderline methicillin resistance varies from study to study to include MICs of oxacillin from 1 to 16 μg/ml with disk diffusion zone diameters of 6 to 13 mm (10, 23, 24, 35, 39, 41). This category can involve non-PBP 2a-dependent mechanisms such as hyperproduction of β-lactamase (23, 41), the presence of other low-affinity PBPs (5, 41), or production of a newly described methicillinase (22). Chambers et al. (5) and Massanari et al. (21) have shown that differentiating high-level and borderline resistance is important in determining appropriate therapy. To date, the test sanctioned by the NCCLS is the salt-supplemented agar screen method to identify intrinsic resistance (29). Although this method may lack sensitivity (10), the agar screen method was used in this study to evaluate discrepant organisms.

The rate of methicillin resistance in staphylococci (oxacillin MIC of >2) varies widely among institutions. *S. aureus* resistance rates range from 5% to over 50%, and CNS resistance ranges from 50 to 80% (1). Because of this high rate and concern about unrecognized resistance, vancomycin is frequently used to treat CNS infections despite reported oxacillin susceptibility. For *S. aureus* infections, the management deci-

sions involve not only the choice of a treatment regimen but also the requirement for patient isolation. To better identify intrinsic resistance, genetic techniques have been used with satisfactory results. However, clinical laboratories have been slow to utilize these techniques for various technical reasons. The multiplex PCR assay described here was developed to fit into the daily work pattern of the laboratory. PCR was chosen because of its high sensitivity for identification of small numbers of resistant organisms.

For *S. aureus*, the PCR assay was comparable to the standard susceptibility methods in identifying intrinsic methicillin resistance. Its utility with this group of organisms was to characterize the borderline-resistant strains, which by the current NCCLS MIC breakpoints would have been considered intrinsically resistant for organism no. 1 and 3 (Table 1). Although the number of isolates for which this occurred was low (4 of 44), the implications that this may have for treatment options and the need for expensive patient isolation procedures may be significant. For CNS, the prevalence of *mecA* and expression of methicillin resistance was high (>50%). There were no *mecA*-negative Ox^r isolates. A significant finding was the number of *mecA*-positive Ox^s organisms; 56% of these 25 organisms with initial oxacillin MICs of ≤2 had MICs of >2 when retested. This finding suggests that the presence of the *mecA* gene is important in interpreting susceptibility data. The *mecA* gene is not consistently expressed, and there is evidence suggesting that certain auxiliary genes such as *femA*, *mecR*, and the gene encoding the β-lactamase plasmid may participate in control of its expression (4, 14, 20, 26, 33).

The so-called broad-range 16S rRNA gene detection method was originally developed to identify nonculturable bacteria in clinical specimens (32). The technique was applied in this multiplex PCR system as an internal control mechanism. Amplification of this portion of the 16S gene, which is common to all eubacteria, controls for DNA extraction as well as for failure of the PCR to amplify the target sequences. A no-product result at the position expected for the 16S rRNA product was interpreted as a sample extraction or PCR failure, and the test was repeated rather than being considered negative. A total of 26 isolates (10 *S. aureus* and 16 CNS) of 500 demonstrated poor extraction or amplification as determined by the absence of a 16S rRNA product; these specimens might have been judged *mecA* negative in the absence of such a control. Indeed, four false-negative reactions for the *mecA* locus would have arisen had this control not been included.

Isopsoralen was used in a post-PCR inactivation procedure (16, 30). Psoralens intercalate into double-stranded nucleic acids, forming covalent interstrand cross-links after photoactivation with UV light. Depending on the length and G-C content of the amplification products, >99% of the amplicons are inactivated, thus decreasing the problem of amplicon carryover and the resulting false-positive reactions (16). Incorporation of this step is important for an assay that is to be used daily in the clinical laboratory, especially when the rate of expected positive results is high.

Genotypic detection of drug resistance will undoubtedly become an important component of the diagnostic armamentarium of the clinical laboratory. Guidelines for interpretation of the *mecA* gene result will need to be formally addressed as more laboratories begin to use this method. We propose that *mecA*-positive organisms (both CNS and *S. aureus*) be regarded as intrinsically resistant and that *mecA*-positive results, which can be available well before the phenotypic results (6 versus 24 h, respectively), be reported immediately. Although some of these isolates may be phenotypically borderline resistant or even susceptible when tested in vitro, all have the potential to become highly resistant, and infections with these organisms may well become refractory to β-lactam antibiotic therapy (6, 26, 42). Most *mecA*-negative organisms will remain phenotypically susceptible to oxacillin. Those organisms with borderline resistance (*mecA* negative, Ox^r) may well respond to β-lactam antibiotics or β-lactam–β-lactamase inhibitor combinations similar to Ox^s organisms (5, 21). PCR assays for determination of the *mecA* gene in staphylococci appear to be a beneficial adjunct to standard susceptibility testing and allow for the identification of intrinsic resistance in a timely and reliable manner. The cost to the laboratory depends on the volume of specimens tested. For this assay, the technologist time required to run an assay of up to 40 specimens was approximately 2 h. Since vancomycin continues to be the only drug that reliably eradicates intrinsically methicillin-resistant staphylococci, preservation of this drug for only intrinsic resistance may help to control high costs associated with its overuse and slow the potential development of vancomycin resistance.

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