Identification of Methicillin-Resistant Staphylococci by Multiplex Polymerase Chain Reaction Assay

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A multiplex polymerase chain reaction (PCR) assay using oligonucleotide primers to detect *mecA* and 16S ribosomal RNA gene was developed to aid in identification of methicillin-resistant staphylococci. Validation included 99 isolates of staphylococcus grouped into one of five categories: methicillin-susceptible coagulase-negative staphylococcus (MSCNS), methicillin-resistant coagulase-negative staphylococcus (MRCNS), methicillin-susceptible *Staphylococcus aureus* (MSSA), high β-lactamase producing *S aureus* (HiBSA), and methicillin-resistant *S aureus* (MRSA). *mecA* was detected in MRSA (21/21), and in MRCNS (20/20), but not in MSSA (0/20). *mecA* was occasionally detected in HiBSA (1/19) and MSCNS (3/19). This multiplex PCR assay was also used to test 30 clinical isolates of coagulase-negative staphylococci with discrepancies between results of in vitro tests for susceptibility to oxacillin and was found to be valuable when a more definitive determination of intrinsic methicillin-resistance was desired. (Key words: Methicillin-resistant staphylococci; Multiplex polymerase chain reaction assay) Am J Clin Pathol 1997;107:368–373.

Staphylococci are ubiquitous gram-positive cocci normally found as colonizers of human skin and mucous membranes. At least 31 species of staphylococci exist, and of these, 15 have been recovered from humans. *Staphylococcus aureus* is an especially virulent species and is the only species commonly found in humans that produces coagulase, an enzyme that coagulates plasma. All other species of staphylococci not producing coagulase are collectively called the *coagulase-negative staphylococci* (CNS), and some of these are now recognized as opportunistic pathogens.

Infections with staphylococci are controlled with antibiotics, and this has selected for antibiotic resistance. More than 90% of staphylococci are resistant to penicillin, and resistance to penicillinase-resistant antibiotics such as nafcillin, oxacillin, and methicillin has also developed. Isolates resistant to any of these penicillinase-resistant antibiotics are classified as *methicillin resistant* and are considered cross-resistant to all β-lactams, including β-lactam/β-lactamase inhibitor combinations. To date, essentially all these isolates remain susceptible to vancomycin, the drug of choice for treatment of infections caused by methicillin-resistant staphylococci. Patients with methicillin-resistant *S aureus* (MRSA) infections are placed in isolation to prevent the spread of this organism.

Detection of methicillin resistance in the laboratory has been problematic.[1–9] Methicillin-resistant strains typically express their resistance heterogeneously with only a few cells, 1 in 10^4 or 10^6, expressing the phenotype. Other variables, including the pH, inoculum size, incubation time, temperature, and salt concentration can also influence the expression of resistance. Despite many publications on the subject, the definitive determination of intrinsic methicillin resistance remains elusive. The disk diffusion (Difco Laboratories, Detroit, Mich) and Vitek GPS-SB (bioMérieux Vitek Inc, Hazelwood, Md) automated methods currently used in our laboratory occasionally give discrepant results. During this study, 3.3% of the non–*Staphylococcus saprophyticus* CNS isolates gave discrepant results. Occasional MRSA isolates had atypical susceptibility patterns or low-level resistance with minimum inhibitory concentrations (MICs) of 4 to 8 μg/mL. Three mechanisms[10,11] have been proposed to account for this low-level resistance in *S aureus*: (1) heterogeneous expression of *mecA*, (2) β-lactamase-mediated resistance, and (3) resistance associated with modified penicillin-binding proteins 1, 2, or 4. When methicillin-resistant isolates are resistant through a chromosomal gene designated *mecA*, they are regarded as "intrinsically" methicillin resistant and may be predicted to fail β-lactam therapy.

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Molecular investigations[12] show *mecA* to be a 2456 base pair (bp) region that codes for a distinct 78 kd penicillin-binding protein, PBP 2a. Normal penicillin-binding proteins are enzymes responsible for the synthesis of the bacterial cell wall. When β-lactam antibiotics covalently bind to them, cell wall synthesis is interrupted with eventual cell death. PBP 2a has a very low affinity for β-lactam antibiotics, allowing for continued cell wall synthesis and cell survival even in the presence of a β-lactam antibiotic. We hypothesized that genotypic detection of *mecA* would provide a more definitive determination of intrinsic methicillin resistance by avoiding the phenotypic variables of the current in vitro test methods and the heterogeneous expression of *mecA*. The multiplex polymerase chain reaction (PCR) assay described in this study provides a genotypic approach for the detection of *mecA* that does not depend on the unpredictable phenotypic expression of *mecA*-mediated methicillin resistance, and provides an internal control for the presence of amplifiable bacterial DNA, thereby avoiding false-negative results. The assay can be accomplished in 4 hours, allowing for the timely and reliable identification of intrinsic methicillin resistance.

MATERIALS AND METHODS

Preparation of Purified Staphylococcus aureus *DNA*

Purified *S aureus* DNA was obtained from methicillin-susceptible *S aureus* (ATCC 25923) and methicillin-resistant *S aureus* (UWHC 4702) as described by Tokue et al[13] with the addition of a phenol-chloroform-isoamyl alcohol extraction before the ethanol precipitation. This purified control DNA was stored at -70°C at 30 μg/mL in 10 mmol/L Tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 7.5).

Identification of Staphylococcal Isolates and Susceptibility Testing

The staphylococcal isolates used in this study were identified as *S aureus* or coagulase-negative staphylococcus by colony morphology and catalase and coagulase reactions. Methicillin resistance was detected using Vitek GPS-SB cards (bioMérieux Vitek Inc, Hazelwood, Md) and disk diffusion, following published guidelines.[14] Isolates with an oxacillin MIC ≥4 μg/mL and oxacillin disk zone diameter ≤10 mm were classified as methicillin resistant. Isolates with penicillin MICs from 0.06 to 0.12 μg/mL were tested for β-lactamase production with BBL Cefinase nitrocefin disks (Becton Dickinson, Cockeysville, Md). *Staphylococcus aureus* isolates resistant to oxacillin by one or both methods but failing to show resistance to clindamycin, erythromycin, gentamicin, or imipenem/cilastatin were tested for susceptibility to ticarcillin/clavulanate potassium, ampicillin/sulbactam, amoxicillin/clavulanate potassium, ampicillin, and ticarcillin by disk diffusion. *Staphylococcus aureus* isolates that were susceptible to ticarcillin/clavulanate potassium (≥20 mm), ampicillin/sulbactam (≥15mm), and amoxicillin/clavulanate potassium (≥20 mm) were defined as high β-lactamase producing *S aureus* (HiBSA). The remaining staphylococcal isolates were categorized using the aforementioned criteria as methicillin-susceptible coagulase-negative staphylococcus (MSCNS), methicillin-resistant coagulase-negative staphylococcus (MRCNS), methicillin-susceptible *S aureus* (MSSA), MRSA, or as discrepant staphylococcus.

Preparation of Bacterial Lysates

Lysates of the 34 isolates shown in Figure 1 were prepared by sequential enzymatic digestion with lysostaphin and proteinase K. Parallel lysates were also prepared by heating with Chelex-100 (Bio-Rad, Anaheim, Calif). All remaining isolates were solely prepared by sequential enzymatic digestion with lysostaphin and proteinase K, and assays were performed in singleton. For the enzymatic digestion, bacteria were harvested from blood agar plates and suspended in saline to match a McFarland 3 standard. One hundred microliters of this suspension was centrifuged and the supernatant was removed and replaced with 50 μL of 100 μg/mL lysostaphin (Sigma, St Louis, Mo) in water. After mixing and incubating for 10 minutes at 37°C, 50 μL of proteinase K (100 μg/mL in water) and 150 μL of 0.1 mol/L Tris buffer (pH 7.5) were added, followed by another incubation for 10 minutes at 37°C. The enzymes were inactivated by incubation for 5 minutes in a boiling water bath. Lysates were centrifuged before use and were prepared on the day of assay. For the Chelex treatment, bacteria were harvested from blood agar plates as described. Two-hundred fifty microliters of 5% Chelex-100 resin in water was added to the cell pellet and the suspension was placed in a boiling water bath for 5 minutes. Lysates were centrifuged before use and were prepared on the day of assay.

PCR Amplification

Oligonucleotide primers were obtained from a commercial source (Research Genetics Inc, Huntsville, Ala). The primer pair to detect *mecA* were those as described by Predari et al,[15] and produce a 528 bp

product (primer mec1, nucleotides 516 to 536: 5′-GGG-ATC-ATA-GCG-TCA-TTA-TTC-3′; primer *mec*2, nucleotides 1044 to 1024: 5′-AAC-GAT-TGT-GAC-ACG-ATA-GCC-3′). The sequence numbers for the (*mecA*) primer pair are those reported by Song et al.[16] Universal primers were modified from Relman et al[17,18] to match *S aureus* 16S ribosomal RNA (rRNA) sequences (Genbank accession number X70648) and produce an 876 bp product (primer UP-1: 5′-GTG-CCA-GCA-GCC-GCG-GTA-A-3′; primer UP-2: 5′-AGA-CCC-GGG-AAC-GTA-TTC-AC-3′). Other reagents were obtained from various suppliers: *Taq*, $MgCl^2$, and PCR buffer (Perkin Elmer, Norwalk, Conn) and dNTPs (Promega, Madison, Wis). The final PCR conditions were 50 mmol/L KCl, 10 mmol/L Tris pH 8.3, 3.75 mmol/L $MgCl^2$, 50 pmol *mecA* primers, 5 pmol universal primers, 200 µmol/L dNTPs, and 1.25 U *Taq* polymerase in 50 µL-reaction volumes. This final volume included 15 µL of the bacterial lysate, purified control DNA, or water. The PCR amplifications were performed on a Perkin Elmer GeneAmp PCR System 9600 with the following cycling profile: (95°C, 5 minutes) 1 time; (94°C, 30 sec → 55°C, 30 sec → 72°C, 30 sec) 24 times; and (94°C, 30 sec → 55°C, 30 sec → 72°C, 2 min) 1 time. The amplified products were separated by 1% agarose gel electrophoresis, visualized with ethidium bromide staining, and photographed with UV illumination using Polaroid-type 667 film. Standard precautions,[19] were used to prevent contamination.

Isolate	Phenotype	16S rRNA	*mecA*	Agarose gel
	MM			
	water	–	–	
	cMRSA	+	+	
	cMSSA	+	–	
1	MRSA	+	+	
2	MSSA	+	–	
3	HiBSA	+	–	
4	MRSA	+	+	
5	MRCNS	+	+	
6	xCNS	+	+	
7	MSSA	+	–	
8	MRSA	+	+	
9	MSCNS	+	–	
10	MRCNS	+	+	
11	MRSA	+	+	
12	MRSA	+	+	
13	MSSA	+	–	
14	MSSA	+	–	
15	HiBSA	+	–	
16	MSCNS	+	–	
17	xCNS	+	+	
18	MSSA	+	+	
19	MRSA	+	–	
20	HiBSA	+	–	
21	MRSA	+	+	
22	MSCNS	+	–	
23	MRCNS	+	+	
24	MRSA	+	+	
25	MRSA	+	+	
26	HiBSA	+	–	
27	MRSA	+	+	
28	MSSA	+	–	
29	xCNS	+	+	
30	MRSA	+	+	
31	MSSA	+	–	
32	MRSA	+	+	
33	MSSA	+	–	
34	MRSA	+	+	

FIG 1. Validation study. MM = molecular weight markers; cMRSA = purified control DNA from methicillin-resistant *Staphylococcus aureus* (MRSA); cMSSA = purified control DNA from methicillin-susceptible *S aureus* (MSSA); MSCNS = methicillin-susceptible coagulase-negative staphylococcus; MRCNS = methicillin-resistant coagulase-negative staphylococcus; MRSA; methicillin-resistant *S aureus*, HiBSA = high β-lactamase producing *S aureus*; xCNS = discrepant CNS isolate; + = PCR product detected; – = PCR product absent.

RESULTS

Our initial PCR experiment is shown in Figure 2 and was performed using purified DNA from MRSA or MSSA using *mecA* and universal primers singly and in combination. Only the expected amplified products were produced from *mecA* (528 bp) and 16S rRNA gene (876 bp). Amplification of the 16S rRNA gene fragment was significantly more robust than the *mecA* fragment, and the ratio of *mecA* and universal primers was subsequently titrated. A ratio of 50 pmol of *mecA* primers (1.0 µmol/L) to 5 pmol of universal primers (0.1 µmol/L) produced bands of approximately equal intensities (data not shown). To further optimize the reaction conditions, $MgCl_2$ concentration was titrated from 1.00 to 4.00 mmol/L. $MgCl_2$ concentration below 1.25 mmol/L quenched the PCR reaction from both 16S rRNA gene and *mecA* sequences, while a concentration of 3.75 mmol/L gave sharply resolved bands without nonspecific products (data not shown).

Validation of this *mecA* multiplex PCR was initially performed and interpreted on 31 well-characterized (nondiscrepant) clinical isolates. Enzyme-prepared lysate results are shown in Figure 1. No failed reactions

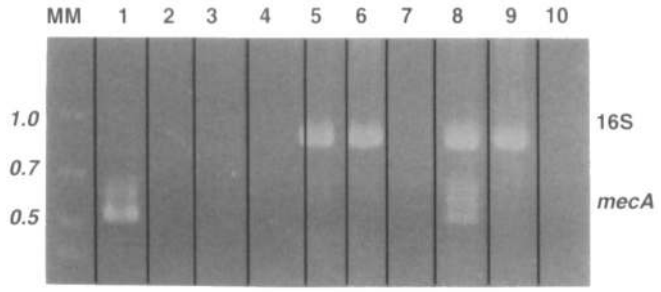

FIG 2. Development of a multiplex polymerase chain reaction (PCR) assay. MM = molecular weight markers in kb; 16S = amplified product from 16S ribosomal RNA (rRNA) gene; *mecA* = amplified product from *mecA*. Reactions were performed using *mecA* primers (lanes 1,2,3), universal primers (lanes 4,5,6,7), or with both *mecA* and universal primers (lanes 8,9,10). Samples were purified methicillin-resistant *Staphylococcus aureus* (MRSA) DNA (lanes 1,5,8), purified methicillin-susceptible *S aureus* (MSSA) DNA (lanes 2,6,9), or water (lanes 3,4,7,10).

were observed with the enzyme-prepared lysates as determined by the presence of 16S rRNA gene product. The results of three discrepant CNS isolates (lanes 6,17, and 29) were excluded from the validation study and were included in the discrepant CNS study described later in this section. *mecA* was detected in MRSA (13/13), and in MRCNS (3/3), but not in MSSA (0/8), HiBSA (0/4), or MSCNS (0/3). Chelex-prepared lysates gave identical results except for four isolates that failed to amplify (data not shown).

Additional well-characterized isolates were tested by PCR, bringing the total in each category of the validation study to 20. When the results of this testing were combined with those described in the preceding paragraph, *mecA* was detected in all MRSA (20/20) and MRCNS (20/20), but not in MSSA (0/20). *mecA* was unexpectedly detected in HiBSA (2/20) and MSCNS (4/20). These six strains were reisolated and retested by PCR and in vitro methods. *mecA* was confirmed in the two HiBSA isolates and three of four MSCNS isolates. The *mecA* PCR result on the final MSCNS was equivocal, ie, the band was very weak, and this strain was eliminated from further calculations. In vitro retesting revealed one strain of HiBSA that was phenotypically reclassifiable to MRSA based on resistance to amoxicillin/clavulanate potassium by disk diffusion at 30°C. The remaining strains yielded the same phenotypic pattern as demonstrated on initial testing. Using phenotypic testing as the accepted standard, *mecA* was detected by PCR in 21 of 21 MRSA, 20 of 20 MRCNS, 0 of 20 MSSA, 1 of 19 HiBSA, and 3 of 19 MSCNS, yielding 100% sensitivity, 93% specificity, 91% positive predictive value, and 100% negative predictive value.

The *mecA* multiplex PCR was used to test 30 discrepant clinical isolates of CNS collected from 29 patients. These isolates represented 3.3% of all non–*S saprophyticus* CNS isolated during that period. Of the 30 discrepant isolates, 26 were resistant and two had intermediate resistance to oxacillin by disk diffusion and were susceptible by Vitek. Two isolates were resistant by Vitek and susceptible by disk diffusion. Twenty-four of 28 isolates that were resistant or had intermediate resistance by disk diffusion and two of two isolates that were resistant by Vitek were confirmed to carry *mecA* by the PCR assay. Using the presence of *mecA* to define intrinsic methicillin resistance in these coagulase-negative staphylococci, two isolates were falsely susceptible and four isolates were falsely resistant to methicillin by disk diffusion. Conversely, 24 isolates were falsely susceptible and none were falsely resistant to methicillin by Vitek.

DISCUSSION

This multiplex PCR assay was developed to help our laboratory detect intrinsic (*mecA*-mediated) methicillin resistance in *S aureus* when the conventional methods had given equivocal results. The PCR method is useful because, while methicillin-resistant or questionably methicillin-resistant isolates are believed to arise by various mechanisms, only those due to *mecA* are believed to preclude use of nafcillin or a congener for therapy and to require isolation of the patient. Heterogeneous or inducible expression of methicillin resistance is strain specific and seems to be regulated by additional loci, such as the flanking mec genes *mecR* and *mecI*, as well as other chromosomally located factors termed fem (factors essential for the expression of methicillin resistance) or aux (auxiliary) factors.[12,20–24] As a consequence, detection of truly methicillin-resistant isolates and the discrimination from isolates that are "hyperproducers" of β-lactamase, for example, can be difficult. The correct diagnosis of the resistance mechanism is important, because it allows us to reserve the recommendation of vancomycin treatment for only those truly methicillin-resistant organisms.

In this study, one (2%) of 39 phenotypically MSSA and three (16%) of 19 methicillin susceptible coagulase-negative staphylococci had detectable *mecA*. The calculated positive and negative predictive values (91% and 100%) are similar to those reported elsewhere.[25] The disagreement between susceptibility testing and genotype

analysis for the four strains may be because of nonproduction of the PBP2a due to the multiple regulatory mechanisms affecting *mecA* expression. Alternatively, the expression of a resistance phenotype is highly dependent on growth conditions and cutoff values, and those used in this study may not provide optimal sensitivity, particularly for the coagulase-negative staphylococci.[8] We currently regard the presence of *mecA* as indicative of intrinsic methicillin resistance regardless of expression class.

Two routine methods used in our laboratory (Vitek and disk diffusion) have identified several discrepant results. These were noted especially with coagulase-negative staphylococci. The use of the *mecA* PCR for the definitive detection of intrinsic methicillin resistance has identified false susceptibility (Vitek, and to a much lesser extent disk diffusion) and false resistance (disk diffusion) in our laboratory, and further investigation of these problems is warranted.

The current study describes a multiplex PCR assay capable of detecting *mecA* in crude bacterial lysates. We have determined that the cost of performing the *mecA* PCR is about $70 to $75. This is cost-effective compared with the additional health care cost of maintaining isolation of a patient, along with the additional costs of using vancomycin (vancomycin, $25.24/day; nafcillin, $12.78/day), and the risks of additional adverse drug reactions and development of further antibiotic resistance. Previous overuse of vancomycin was a major contributing factor in the appearance of vancomycin-resistant enterococcus.

In our hands, the PCR assay has proved very robust with the control MRSA and MSSA purified DNA testing *mecA* positive and *mecA* negative, respectively, in 20 of 20 independent runs. We also use this assay to provide hands-on experience for students rotating through the molecular diagnostics laboratory and found very reproducible results even with inexperienced technologists. A number of recent studies have reported PCR-based detection of *mecA*, using a variety of amplification and detection formats.[8,13,15,25-33] While no standardized procedure has been proposed, molecular approaches to detection have demonstrated the importance of genotypic detection of intrinsic methicillin resistance. Our method is rapid, relatively inexpensive, and reliable, and we have found PCR testing to be most useful as an adjunct to routine susceptibility testing (disk diffusion, Vitek) when discrepant results cannot readily be resolved or during an MRSA outbreak, when rapid recognition of MRSA and isolation of the patient dictates that time is of the essence.

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