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Identification of Methicillin-Resistant Strains of Staphylococci by Polymerase Chain Reaction

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A simple and reliable method using a polymerase chain reaction (PCR) was devised to identify methicillin-resistant staphylococci. By using lysates of the strain to be tested as templates and 22-mer oligonucleotides as primers, a 533-bp region of *mecA*, the structural gene of a low-affinity penicillin-binding protein (PBP 2'), was amplified by PCR and detected by agarose gel electrophoresis. Results obtained by this method were compared with those obtained by broth microdilution MIC determination for 210 and 100 clinical isolates of *Staphylococcus aureus* and coagulase-negative staphylococci, respectively. Of 99 *mecA*-negative *S. aureus* isolates, 100% of the strains were methicillin susceptible and 98% of the strains were oxacillin susceptible. Three strains (3%) of 111 *mecA*-positive *S. aureus* isolates exhibited almost the same susceptibility to β-lactams as the *mecA*-negative ones and did not produce detectable amounts of PBP 2' despite the presence of the *mecA* gene. One of them yielded typically methicillin-resistant variants at a low frequency with concomitant recovery of PBP 2' production. The *mecA* gene was also found in coagulase-negative *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus sciuri*, *Staphylococcus saprophyticus*, and *Staphylococcus caprae* and conferred resistance on most of the bacteria.

Nosocomial infections caused by methicillin-resistant *Staphylococcus aureus* pose a serious problem in many countries. Appropriate therapy of these infections requires rapid and reliable identification of methicillin-resistant strains. Standardized methods of susceptibility tests have been developed for this purpose (3, 23). However, phenotypic expression of methicillin resistance is usually heterogeneous (8, 10). In addition, methicillin resistance is influenced by culture conditions such as temperature, medium pH, and NaCl content in the medium (17). These factors complicate the detection of methicillin resistance, especially for strains with low-level resistance.

Methicillin-resistant *S. aureus* produce a low-affinity penicillin-binding protein (PBP 2' or PBP 2a) in addition to the usual PBPs (7, 26). Available data show that the structural gene of this PBP (*mecA*) is present in the resistant strains but not in the susceptible ones (19). Also, the nucleotide sequence of this gene has been reported (20). These achievements have enabled the development of an alternative method for identifying methicillin-resistant *S. aureus* by detecting the *mecA* gene. Recently, a method based on this principle, in which a DNA probe derived from the *mecA* gene was hybridized with bacterial chromosomal DNA, was reported (2). In the present study, the polymerase chain reaction (PCR) (18) was used to detect the methicillin resistance determinant by amplifying a 533-bp region of the *mecA* gene. This method revealed the presence of *mecA* sequences in *S. aureus* strains that exhibited β-lactam susceptibility similar to that of *mecA*-negative strains.

MATERIALS AND METHODS

Organisms. Most of the staphylococcal strains used in this study were clinical isolates from Japan. Staphylococcal species were identified by Staphyogram (Terumo, Tokyo, Japan), an identification kit, and the coagulase test. *S. aureus* SR3626, SR3633, SR3636, SR3639, SR3665, SR3681, and SR3716 were used as *mecA*-positive control organisms (12), and *S. aureus* ATCC 25923, 209P JC-1, and Smith were used as *mecA*-negative control organisms.

β-Lactam antibiotics and reagents. Methicillin and oxacillin were purchased from Sigma Chemical Co. (St. Louis, Mo.), and cefazolin was from Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan). The chemicals used were reagent grade and were obtained from commercial sources.

Oligonucleotides. Primers for PCRs and a probe for Southern hybridization were synthesized with a GENET A-II DNA synthesizer (Nippon Zeon, Tokyo, Japan). One of the primers (5' AAAATCGATGGTAAAGGTTGGC) corresponded to nucleotides 1282 to 1303, and the other (5' AG TTCTGCAGTACCGGATTTGC) was complementary to nucleotides 1793 to 1814. The probe for Southern hybridization (5' ATCTGTACTGGGTTAATC) was complementary to nucleotides 1581 to 1598 of the PBP 2' coding frame (20).

Preparation of bacterial lysates. A bacterial colony was suspended in 10 mM Tris-HCl–1 mM EDTA (pH 8.0) at a density of about 3×10^8 CFU/ml. Ten microliters of achromopeptidase (10,000 U/ml; Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added to 240 μl of the bacterial suspension and incubated at 55°C for 30 min. Next, 250 μl of the buffer described above and 2.5 μl of 20% sodium dodecyl sulfate (SDS) were added to lyse the bacterial cells. After 10 min of incubation at 100°C, the lysate was centrifuged at $9,500 \times g$ for 5 min, and 5 μl of supernatant containing bacterial DNA was used as the template in the PCR.

PCR. By using 5 μl of template DNA prepared as described above, 0.25 μM (each) primer, and the Gene Amp DNA amplification kit for PCR (Takara Shuzo, Kyoto, Japan), DNA amplification was carried out for 40 cycles in 100 μl of reaction mixture as follows: denaturation of 94°C

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for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min with a final extension at 72°C for 5 min. Ten microliters of PCR products was analyzed by 2% agarose gel electrophoresis.

Southern blot analysis of PCR products. PCR products separated on a 2% agarose gel were transferred to a Hybond-N membrane (Amersham International plc., Aylesbury, Buckinghamshire, England) under alkaline conditions (16) as recommended by the manufacturer. After being baked at 80°C for 15 min, the membrane was prehybridized at 42°C for 4 h in 10 ml of hybridization solution consisting of 3× SSPE (0.54 M NaCl, 0.03 M sodium phosphate, and 0.003 M EDTA, pH 7.7), 5× Denhardt's solution (0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone), 0.2 mM Tris-HCl (pH 8.0), 0.5% SDS, and 30% formamide and hybridized at 42°C for 18 h in 10 ml of hybridization solution with 10 pmol of the probe, which was labeled by using T4 polynucleotide kinase (Takara Shuzo) with [γ-^{32}P]ATP (DuPont, NEN Research Products, Boston, Mass.). The membrane was washed three times in 200 ml of 2× SSPE and 0.1% SDS at 42°C for 20 min and exposed to New RX film (Fuji Photo Film Co., Ltd., Tokyo, Japan) to detect hybridization.

Susceptibility test. MICs were determined by broth microdilution with cation-supplemented Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) containing 2% NaCl as recommended by the National Commitee for Clinical Laboratory Standards (13). Bacteria were inoculated at a final bacterial density of about 5×10^5 CFU/ml and incubated at 35°C for 24 h before MICs were determined.

Induction and analysis of PBP 2'. PBP 2' was induced and detected as described previously (12). Briefly, bacteria were grown with shaking in L broth (10 g of tryptone [Difco] per liter, 5 g of yeast extract [Difco] per liter, and 5 g of NaCl per liter [pH 7.2]) at 32°C to the logarithmic phase, and cefazolin was added to give a final concentration of 1 μg/ml. Cultivation was continued for an additional 90 min at 32°C to induce PBP 2'. Bacterial cells were harvested and disrupted by repetition of the incubation with 50 μg of lysostaphin (Sigma) at 30°C and sonic disintegration. The membrane fractions collected by ultracentrifugation were suspended at a protein concentration of 4 mg/ml and solubilized with 1% Sarkosyl (Ciba-Geigy Corp., Summit, N.J.). Membrane proteins were then subjected to SDS-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue R-250. The membrane proteins of *S. aureus* SR3636, a well-defined PBP 2' producer, were run on the same gel as a reference.

RESULTS

Detection of *mecA* gene by PCR. The PCR technique was applied to seven strains of methicillin-resistant *S. aureus*, all of which were PBP 2' producers (12), and three strains of methicillin-susceptible *S. aureus*. The DNA fragment of 533 bp was amplified from DNA of all seven PBP 2' producers and was absent from the susceptible strains (Fig. 1). No background DNA bands were observed on the gel. Under the reaction conditions employed, bacterial suspensions with concentrations of as low as 4×10^5 CFU/ml, which was used for the preparation of bacterial lysate, gave a positive reaction for PBP 2' producers.

Correlation between presence of the *mecA* gene and resistance level in the clinical isolates of *S. aureus*. Testing for the *mecA* gene and determination of MICs of oxacillin and methicillin were performed for 210 clinical isolates of *S. aureus*. Table 1 summarizes the results. Two (2%) of 99 *mecA*-negative *S. aureus* isolates were classified as oxacillin resistant despite borderline MIC values (oxacillin MIC, 4 μg/ml). Methicillin susceptibilities were completely consistent with PCR results for these strains. On the other hand, 3 (3%) of 111 *mecA*-positive isolates yielded results in the oxacillin and methicillin susceptibility tests which were discrepant from those of PCR analysis. The susceptibilities of these three strains, SR3615, SR3648, and SR3679, to β-lactams were similar to those of *mecA*-negative strains (the MIC of oxacillin is shown in Table 2).

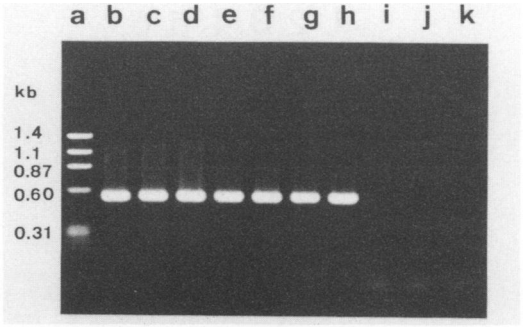

FIG. 1. Agarose gel electrophoresis of amplified 533-bp DNA fragment. PCR was performed with a lysate of methicillin-resistant (R) or -susceptible (S) *S. aureus* as a template. Production of PBP 2' in methicillin-resistant strains used in this figure has been described elsewhere (12). Lanes: a, size markers (in kilobases); b, strain SR3626 (R); c, SR3633 (R); d, SR3636 (R); e, SR3639 (R); f, SR3665 (R); g, SR3681 (R); h, SR3716 (R); i, ATCC 25923 (S); j, 209P JC-1 (S); k, Smith (S).

Three *mecA*-positive strains that were susceptible to β-lactams. To confirm the presence of the *mecA* gene in the three strains described above, we tested five colonies of each strain by PCR analysis; all colonies yielded positive results. Similar experiments using three *mecA*-negative strains were carried out at the same time as negative controls; all colonies yielded negative results. Furthermore, Southern hybridization of PCR products of strains SR3615, SR3648, and SR3679 with a probe complementary to nucleotides 1581 to 1598 of the *mecA* gene (20) yielded positive reactions (Fig. 2). These results excluded the possibility of contamination of resistant bacterial cells or nonspecificity of primers used in PCR analysis. In spite of their phenotypic similarities to susceptible strains, typical methicillin-resistant variants of one of these *mecA*-positive strains, SR3648, occurred at a low frequency when a bacterial suspension of high density was spread on an oxacillin plate (Table 2). The other two *mecA*-positive strains and two *mecA*-negative strains yielded no such resistant variants. PBP 2' analysis revealed that these three *mecA*-positive strains did not produce a

TABLE 1. Correlation of *mecA* gene PCR result and methicillin resistance of *S. aureus*

Characteristic	No. of strains[a]			
	Oxacillin		Methicillin	
	S	R	S	R
mecA negative	97	2	99	0
mecA positive	3	108	3	108

[a] S, susceptible; R, resistant. MICs were determined by broth microdilution assay. The breakpoints for susceptibility and resistance were ≤2 and ≥4 μg/ml for oxacillin and ≤8 and ≥16 μg/ml for methicillin, respectively.

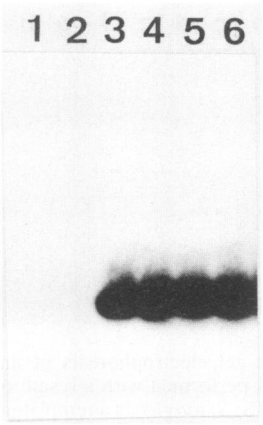

FIG. 2. Southern blot analysis of PCR products of *mecA*-positive strains of *S. aureus* that were susceptible to β-lactam antibiotics, using an 18-mer oligonucleotide as a probe. Lanes: 1, *Hae*III digest of φX174 DNA; 2, a *mecA*-negative strain; 3, strain SR3615; 4, strain SR3679; 5, strain SR3648; 6, a *mecA*-positive and methicillin-resistant strain.

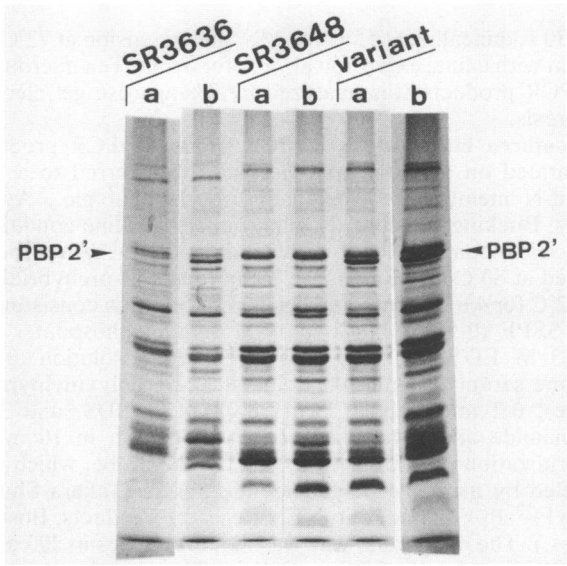

FIG. 3. Production of PBP 2′ in *S. aureus*. Membrane proteins were separated by SDS-polyacrylamide gel electrophoresis and stained with Coomassie blue. PBP 2′ was induced for 90 min at 32°C with no antibiotic (lanes a) or 1 μg of cefazolin per ml (lanes b). Strains SR3636 and SR3648 were methicillin resistant and cryptically resistant, respectively. The variant was derived from strain SR3648 and was highly methicillin resistant.

detectable amount of PBP 2′ constitutively or inducibly, while a resistant variant of SR3648 did (Fig. 3). Therefore, the high antibiotic susceptibility of these *mecA*-positive strains could be ascribed to their inability to produce enough PBP 2′.

Detection of the *mecA* gene in coagulase-negative staphylococci. The presence of the *mecA* gene in 100 strains of coagulase-negative staphylococci stocked in our laboratory, which included nine species, was tested. Among them, *mecA* could be detected in *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus sciuri*, *Staphylococcus saprophyticus*, and *Staphylococcus caprae* (Table 3). Of 36 *mecA*-positive *S. epidermidis* isolates, 4 (11%) were classified as susceptible (oxacillin MIC, 1 or 2 μg/ml). Two strains of *S. haemolyticus* that tested negative for the *mecA* gene demonstrated resistance to oxacillin at or near the breakpoint (MICs, 4 and 8 μg/ml).

TABLE 2. Properties of the *mecA*-positive strains that were susceptible to β-lactam antibiotics

S. aureus strain[a]	Presence of *mecA* gene	MIC of oxacillin[b] (μg/ml)	Frequency of occurrence of resistant cells[c] (10^{-9})
SR3680	−	1.0	0
SR3662	−	0.5	0
SR3615	+	2.0	0
SR3648	+	1.0	4[d]
SR3679	+	1.0	0
SR3636	+	64	ND[e]

[a] Two *mecA*-negative strains and a typical resistant strain, SR3636, were included in the experiments as controls.
[b] MICs were determined by broth microdilution assay.
[c] About 5×10^8 cells were spread on sensitivity test agar (Nissui, Tokyo, Japan) containing 10 μg of oxacillin per ml and incubated at 30°C for 2 days. Similar results were obtained with 6-μg/ml oxacillin plates containing 4% NaCl.
[d] Resistance of each colony was confirmed by streaking the colonies on the same plate or by MIC determination.
[e] ND, not determined.

DISCUSSION

The PCR technique described here required a bacterial suspension of more than 4×10^5 CFU/ml for the preparation of template DNA for PCR to detect the *mecA* gene, which was equivalent to 2×10^3 bacterial cells per PCR tube. Because of the relatively high limit for *mecA* detection, it was unlikely that contamination of a small number of the resistant bacterial cells led to the false-positive results. This detection limit seemed to be due to the low yield of template DNA prepared from methicillin-resistant *S. aureus*, since 10 PFU of λ phage per reaction tube yielded positive results for phage sequence detection by PCR performed under conditions similar to those for *mecA* detection. In testing for the *mecA* gene, we used a bacterial suspension of 3×10^8 CFU/ml to prepare bacterial lysate, which was sufficient to obtain very clear results, as shown in Fig. 1. Moreover, one

TABLE 3. Presence of *mecA* gene in coagulase-negative staphylococci and their resistance level

Species	Presence of *mecA* gene	No. of strains[a]	
		S	R
S. epidermidis	−	14	0
	+	4	32
S. haemolyticus	−	4	2
	+	0	9
S. sciuri	−	0	0
	+	0	1
S. saprophyticus	−	11	0
	+	0	4
S. caprae	−	6	0
	+	0	2

[a] S, susceptible; R, resistant. Oxacillin MICs were determined by broth microdilution assay. The breakpoints for susceptibility and resistance were ≤2 μg/ml and ≥4 μg/ml, respectively.

colony 1 mm in diameter was enough to prepare a bacterial suspension of this density, though there was a possibility that a single colony from a clinical specimen yielded false-negative results in clinical laboratories. Furthermore, definite results could be obtained within several hours after isolation of the colony to be tested. Another potential advantage of this method is that agarose gel electrophoresis for identification of amplified DNA fragments enables detection of several genetic markers in the same experiment by using appropriate primers which amplify different sizes of DNA regions.

Examination of *mecA* genes in 210 clinical isolates of *S. aureus* showed that, while there was a gross correlation between the presence of the gene and the level of bacterial resistance to oxacillin and methicillin, three *mecA*-positive strains could not be distinguished from the *mecA*-negative ones by the susceptibility test beeause of their inability to produce PBP 2′ (Table 2 and Fig. 3). Despite these phenotypic similarities to the *mecA*-negative strains, one of these *mecA*-positive strains yielded typically resistant variants at a low frequency, but the negative strains did not. A recent study found non-PBP 2′-producing strains to be resistant to cephamycin-type but not to cephem-type antibiotics and to yield typical methicillin-resistant variants with concomitant recovery of PBP 2′ produced after incubation with cephamycin-type antibiotics (14). We did not find such strains in the bacterial collection used in our study, and the *mecA*-positive strains listed in Table 2 were not cephamycin resistant. The occurrence of the methicillin-resistant variant described above implied the possibility that, during chemotherapy with β-lactam antibiotics, a typical resistant subpopulation occurred in a cryptically methicillin-resistant *S. aureus* strain (a *mecA*-positive but non-PBP 2′-producing strain), even when strains could not be identified as resistant by conventional susceptibility tests in clinics. For this reason, all *mecA*-positive strains, including cryptically methicillin-resistant strains, must be detected precisely, and this requirement was fulfilled by direct detection of the methicillin resistance determinant by methods such as those described previously (2) and in this study. Some of the cryptically methicillin-resistant strains could be detected by spreading a bacterial suspension of high density on a plate containing antibiotic to observe the appearance of the resistant colonies.

The cryptically methicillin-resistant strains were most probably derived from typically resistant strains; that is, they were first selected as methicillin-resistant strains by β-lactam antibiotics but later stopped the production of PBP 2′ with loss of their resistance. The unstable nature of methicillin resistance has been reported previously (1, 6), and a recent report described *mecA*-positive but phenotypically susceptible subclones, as well as *mecA*-negative ones, that arose from a methicillin-resistant strain after penicillinase plasmid elimination (9). We also isolated a similar strain upon elimination of the penicillinase plasmid. Furthermore, insertion of Tn*551* into a site other than the *mecA* gene was reported to make methicillin-resistant *S. aureus* susceptible to β-lactams (4, 11). These findings support the derivation of cryptically methicillin-resistant strains from typically resistant ones. Another possible explanation is that the cryptic strains continued to be methicillin susceptible even after acquisition of the *mecA* gene but were later selected by resistance to drugs other than β-lactams.

Methicillin-resistant coagulase-negative staphylococci strains have been reported to produce PBP 2′ (5, 15, 21, 22, 24, 25). We also detected the gene for this protein in the coagulase-negative staphylococci listed in Table 3, proving that our method was effective for these species as well. Two strains of *S. haemolyticus* which did not have the *mecA* gene were moderately resistant to oxacillin, suggesting a resistance mechanism other than the production of PBP2′.

In conclusion, methicillin-resistant staphylococci could be successfully detected by the PCR technique employed here. This method could also be used to detect cryptically methicillin-resistant strains which yielded a typically methicillin-resistant subpopulation. From the viewpoint of clinical practice, these cryptic strains should not be classified as methicillin susceptible in spite of their susceptibility to β-lactam antibiotics, because of the possibility that typically methicillin-resistant variants appeared during chemotherapy with β-lactam antibiotics.

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