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Comparison of a Polymerase Chain Reaction Assay and a Conventional Microbiologic Method for Detection of Methicillin-Resistant *Staphylococcus aureus*

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The presence or absence of a methicillin resistance gene in 58 clinical isolates of *Staphylococcus aureus* was examined by the polymerase chain reaction (PCR) and Southern blot analyses. The results were analyzed in relation to those of the MIC assay of methicillin and oxacillin. PCR assay results were identical to those of Southern blot analysis of genomic DNA digested with *Hin*dIII (positive, 28 strains; negative, 30 strains). Among the 28 PCR-positive strains, 6 strains showed methicillin susceptibility by the conventional susceptibility test (MICs, ≤8 μg/ml). Culturing of the six strains with ceftizoxime led to an increase in the phenotypic level of resistance to methicillin and oxacillin, indicating that these strains should be classified as methicillin-resistant *S. aureus* (MRSA). The PCR assay was found to be a sensitive and reliable procedure for the rapid diagnosis of MRSA infection, even in cases in which the conventional MIC assay failed to detect MRSA.

The β-lactam resistance of methicillin-resistant *Staphylococcus aureus* (MRSA), which is an intrinsic resistance of the cells to all β-lactam antibiotics, is mediated by the methicillin resistance determinant. An additional low-affinity penicillin-binding protein (PBP) designated PBP 2′ is encoded by the MRSA-PBP gene (10) and is the main factor responsible for the expression of methicillin resistance (20). PBP 2′ is the only PBP which functions during cell wall synthesis at high β-lactam concentrations that are sufficient to inhibit the growth of susceptible strains (13).

The MICs of β-lactam antibiotics for MRSA are influenced frequently by growth conditions such as temperature (3), pH (14), and salt concentration (4). Thus, routine susceptibility tests may fail to detect MRSA (2). Direct demonstration of the MRSA-PBP gene provides unequivocal evidence for the methicillin resistance of the bacteria. We have previously demonstrated that the polymerase chain reaction (PCR) is a sensitive and specific assay for the detection of MRSA (18). In this study, we screened the MRSA-PBP gene in clinical isolates of *S. aureus* by PCR and Southern blot hybridization, and the results thus obtained are discussed in relation to the MICs of methicillin and oxacillin.

MATERIALS AND METHODS

Bacterial strains. The *S. aureus* strains used in this study are listed in Table 1. Fifty-eight strains of *S. aureus* isolated in five hospitals in the Tohoku district of Japan and in the Laboratory of Drug Resistance in Bacteria, School of Medicine, Gunma University, Maebashi, Japan, were used in the present experiment.

Antibiotics and susceptibility testing. The following antibiotics were used in this experiment: methicillin and oxacillin, Banyu Pharmaceutical Co., Ltd., Tokyo, Japan, and ceftizoxime, Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan.

MICs were determined by the standard broth microdilution method (7) in Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) supplemented with 2% NaCl (17). The MIC of an antibiotic was defined as the lowest drug concentration which inhibited the visible growth of bacteria after 24 h of incubation at 37°C.

Probe and primers. A 30-mer oligonucleotide probe and two 20-mer PCR primers were synthesized with a Cyclone DNA synthesizer (Biosearch, San Rafael, Calif.). These oligonucleotides are complementary to the target segment of the MRSA-PBP gene sequence, as follows: probe, 5′ (1031)-ATGATTATGGCTCAGGTACTGCTATCCACC-(1060) 3′; primer MR1, 5′ (478)-GTGGAATTGGCCAATACAGG-(497) 3′; and primer MR2, 5′ (1816)-TGAGTTCTGCAGTACCG GAT-(1797) 3′. The sequence numbers indicate their positions as described by Song et al. (15).

Preparation of DNA. Cells from 5 ml of culture were washed with and suspended in 2 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 5 mM EDTA and 50 mM NaCl. Lysostaphin and RNase A (Sigma Chemical Co., St. Louis, Mo.) were added to make final concentrations of 20 and 200 μg/ml, respectively, and the suspension thus prepared was incubated with mild shaking at 37°C for 30 min. Then, 400 μl of 0.5% sodium dodecyl sulfate, 50 mM Tris, 0.4 M EDTA, and 1 mg of proteinase K (Sigma) per ml were mixed, and the pH was adjusted to 7.5. The resulting mixture was heated at 50°C for 60 min. The DNA extract was precipitated with ethanol and resuspended in 50 to 100 μl of 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA.

PCR. The DNA extract (1 μg) was amplified by PCR in 100 μl of a reaction mixture containing 200 μM (each) deoxynucleoside triphosphates, 1 μM (each) primers, 2.5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, and 0.01% gelatin. The procedural steps were as follows: denaturation, 30 s at 94°C; annealing, 30 s at 55°C; primer extension, 2 min at 72°C. Each step was repeated 25 times. Ten microliters of the reaction mixture was loaded onto a 1.0% agarose gel with ethidium bromide. The band of amplified DNA was visualized under UV light.

Southern blot hybridization of DNA. The bacterial genomic

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TABLE 1. Presence or absence of MRSA-PBP gene in relation to MICs against *S. aureus*

Strain no.	MIC (μg/ml)		MRSA-PBP gene	
	Methicillin	Oxacillin	PCR	Southern blot
1	1	0.125	−	−
2	1	0.125	−	−
3	1	0.125	−	−
4	1	0.125	−	−
5	1	0.125	−	−
6	1	0.125	−	−
7	1	0.125	−	−
8	1	0.125	−	−
9	1	0.125	−	−
10	1	0.125	−	−
11	1	0.25	−	−
12	1	0.25	−	−
13	1	0.25	−	−
14	2	0.125	−	−
15	2	0.125	−	−
16	2	0.125	−	−
17	2	0.125	−	−
18	2	0.25	−	−
19	2	0.25	−	−
20	2	0.25	−	−
21	2	0.25	−	−
22	2	0.5	−	−
23	2	0.5	−	−
24	2	0.5	−	−
25	2	0.5	+	+
26	2	1	−	−
27	2	1	+	+
28	2	2	+	+
29	2	1	+	+
30	2	2	+	+
31	2	32	+	+
32	4	0.25	−	−
33	4	0.25	−	−
34	4	0.25	−	−
35	4	2	−	−
36	8	1	−	−
37	16	16	+	+
38	32	32	+	+
39	128	64	+	+
40	>128	128	+	+
41	>128	128	+	+
42	>128	128	+	+
43	>128	128	+	+
44	>128	128	+	+
45	>128	128	+	+
46	>128	128	+	+
47	>128	>128	+	+
48	>128	>128	+	+
49	>128	>128	+	+
50	>128	>128	+	+
51	>128	>128	+	+
52	>128	>128	+	+
53	>128	>128	+	+
54	>128	>128	+	+
55	>128	>128	+	+
56	>128	>128	+	+
57	>128	>128	+	+
58	>128	>128	+	+

DNA was digested with *Hin*dIII, electrophoresed on a 1.0% agarose gel, and transferred to a nitrocellulose membrane by the method of Southern (16). The 30-mer oligonucleotide probe was labeled with [γ-^{32}P]ATP by the end-labeling method (9). Hybridization, washing, and autoradiography were carried out as described previously (18).

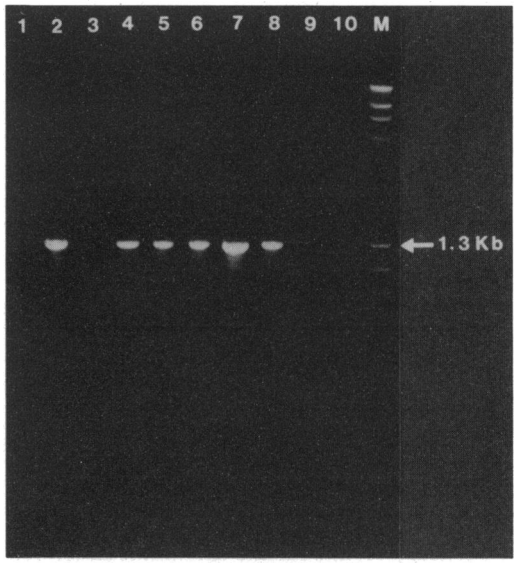

FIG. 1. Agarose gel analysis of PCR products after amplification. Lanes: 1, strain 24; 2, strain 25; 3, strain 26; 4, strain 27; 5, strain 28; 6, strain 29; 7, strain 30; 8, strain 31; 9, strain 32; 10, strain 33; M, DNA marker fragments. All strains were methicillin susceptible (MICs, ≤4 μg/ml) by susceptibility testing. In six strains (strains 25 and 27 through 31), however, amplified DNA fragments of the MRSA-PBP gene (1,339 bp) were detected.

Influence of β-lactam antibiotics on the level of resistance to methicillin. The synthesis of PBP 2′ has been shown to be inducible by β-lactam antibiotics (19). Therefore, methicillin-susceptible strains were cultured and subcultured overnight for 2 consecutive days each in Mueller-Hinton broth containing 5 μg of ceftizoxime per ml. The MICs were then determined as described above.

RESULTS

As shown in Table 1, the results of the PCR assay were the same as those of Southern blot analysis (positive, 28 strains; negative, 30 strains). Six of the PCR-positive strains (strains 25 and 27 through 31) were classified as methicillin susceptible (MICs, ≤8 μg/ml) by susceptibility tests. Five of these six PCR-positive strains (strains 25 and 27 through 30) were also susceptible to oxacillin (MICs, ≤2 μg/ml).

Figure 1 shows representative data of the PCR assay for 10 strains. The amplified DNA fragment of the MRSA-PBP gene, the predicted size of which was 1,339 bp, could be detected in strains 25 and 27 through 31 (lanes 2 and 4 through 8, respectively). No DNA amplification was seen in strains 24, 26, 32, or 33 (lanes 1, 3, 9, and 10, respectively).

The *Hin*dIII digest of genomic DNA was checked by Southern blot hybridization, and a single band of 4.3 kb was detected in DNA from strains 25 and 27 through 31 (lanes 2 and 4 through 8, respectively, in Fig. 2). This finding is compatible with the report that the MRSA-PBP gene is included in the 4.3-kb fragment of the *Hin*dIII digest (10).

The six strains described above were not classified as MRSA by susceptibility testing alone, because the MICs of β-lactam antibiotics for them were slightly higher than those for common methicillin-susceptible strains and it was somewhat more difficult to increase the level of methicillin resistance in those six strains after incubation at 30°C in the presence of 3% NaCl (data not shown).

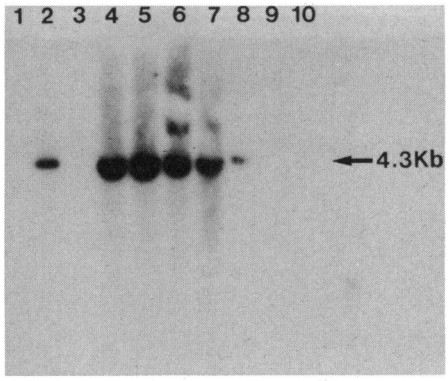

FIG. 2. Southern blot analysis of *Hin*dIII digest of the *S. aureus* genomic DNA. Lanes are the same as those described in the legend to Fig. 1. A single band of 4.3 kb was detected in DNA from six strains (strains 25 and 27 through 31).

The PCR assay results for the six strains described above were incompatible with the MIC assay findings. Therefore, the strains were cultured with β-lactam antibiotics in order to determine whether the level of methicillin resistance could be increased after incubation with the antibiotics (Table 2). One PCR-negative strain was also examined as a control. The level of resistance to methicillin and oxacillin rose in all six of the PCR-positive strains (strains 25 and 27 through 31) after incubation. The level of resistance to methicillin remained at the same level in the PCR-negative strain (strain 26) even after incubation with β-lactam antibiotics.

DISCUSSION

MRSA infections have recently become a serious problem in antibacterial chemotherapy. Early and specific diagnosis of MRSA infections is important in preventing their spread. A rapid and reliable test for the identification of MRSA would be desirable so that appropriate therapy could be initiated without delay.

Archer and Pennell (1) reported that a DNA probe for the gene encoding the determinant for methicillin resistance could hybridize to crude staphylococcal lysates of different species.

On the other hand, the PCR assay makes it possible to amplify a specific target segment of a single DNA molecule and has been used for the diagnosis of viral infections and genetic diseases. The PCR assay has recently been used to detect bacterial and viral pathogen-specific nucleic acid sequences such as those in toxigenic *Escherichia coli* (11), *Shigella* spp. (6), *Mycobacterium tuberculosis* (5), human immunodeficiency virus type 1 (8), and human cytomegalovirus (12).

In our previous study (18), a PCR-amplified DNA fragment was hybridized with a specific oligonucleotide probe, and the result of the PCR assay was the same as that of Southern blot analysis of genomic DNA digested with *Hin*dIII. In addition to its specificity, the PCR assay has advantages in that (i) it is simple and can be performed rapidly, (ii) it can be performed without radioisotopes, and (iii) a much smaller amount of DNA is sufficient for the detection of the MRSA-PBP gene compared with that needed for DNA probe analysis.

In our previous study, all of the methicillin-resistant strains (MICs, ≥16 μg/ml) were PCR positive, while none of the methicillin-susceptible strains (MICs, ≤8 μg/ml) were PCR positive (18). In the present study, however, six PCR-positive strains were classified as methicillin susceptible by the conventional susceptibility test. In interpreting the results of the PCR assay, the following two possibilities of obtaining a false-positive PCR result should be excluded. (i) The strains which harbored a nonfunctional, partial gene containing the region enclosed by the PCR primer used in the present study may be evaluated as PCR positive; and (ii) the presence of the MRSA-PBP gene alone was not responsible for the degree to which resistance was expressed, but some other factors also influenced the phenotypic level of resistance.

In the six strains described above, however, because the level of resistance to methicillin and oxacillin was elevated by ceftizoxime, they should be classified as MRSA. Therefore, demonstration of the MRSA-PBP gene by the PCR assay provides unequivocal evidence for methicillin resistance, even when the conventional MIC assay fails to detect MRSA.

The PCR assay appears to be more reliable than routine susceptibility testing and as accurate as DNA probe hybridization. As described above, the PCR assay is a simpler, more rapid test for the detection of the MRSA-PBP gene than Southern blot hybridization. It should be noted, however, that until more evidence indicating that the PCR assay is as reliable as Southern blotting is accumulated, the Southern blot method will remain the reference to which the results of other tests for the demonstration of methicillin resistance can be compared.

TABLE 2. Influence of ceftizoxime on the level of methicillin resistance in *S. aureus*

Strain no.[a]	MIC (μg/ml)			
	Methicillin		Oxacillin	
	Before[b]	After[c]	Before	After
25	2	32	0.5	32
26	2	2	1	1
27	2	16	1	16
28	2	64	2	64
29	2	32	1	32
30	2	32	2	32
31	2	>128	32	128

[a] Strains 25 and 27 through 31 were PCR positive; strain 26 was PCR negative.
[b] Before, before incubation with ceftizoxime.
[c] After, after incubation with ceftizoxime.

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