Molecular Epidemiology of *Staphylococcus* spp. Contamination in the Ward Environment: Study on *mecA* and *femA* Genes in Methicillin-Resistant Strains

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Abstract

Staphylococcus spp. were isolated from the ward environment and antibiotic susceptibility tests were performed. Twenty-nine strains out of 274 isolates were *S. aureus*, and 41.4% of the *S. aureus* strains were methicillin resistant (MRSA). All 12 strains of MRSA were also resistant to oxacillin, ceftizoxime, ampicillin and clindamycin. Among the coagulase-negative staphylococci (CNS), methicillin-resistant (MR) strains of *S. epidermidis*, *S. capitis*, *S. warneri*, *S. haemolyticus*, *S. hominis*, *S. auricularis*, *S. saprophyticus* and *S. cohnii* were isolated. Eight of the 10 *S. haemolyticus* strains were methicillin resistant. The *femA* gene was detected in *S. aureus* (MSSA and MRSA), but not in CNS by polymerase chain reaction (PCR) analysis and Southern blot analysis. The *mecA* gene was found in all the MRSA and MR-*S. epidermidis* strains tested, and one of the two MR-*S. hominis* strains, but not in MSSA, MS-*S. epidermidis*, MS-*S. hominis*, or MS-*S. haemolyticus*. DNA from one strain of MR-*S. hominis* and 2 strains of MR-*S. haemolyticus* was not amplified by PCR using the *mecA* gene primer, or hybridized by Southern blotting. The ambiguity that *mecA* was detected in some MR-CNS strains, but not in others is discussed.

Introduction

Infection with *Staphylococcus aureus* is one of the most important nosocomial infections in the hospital, because a high frequency of methicillin resistance is found in *S. aureus*. Recently, genetic studies on the methicillin resistance have been encouraged^{1,2,3,4}. The resistance is evoked by the penicillin-binding protein-2' (PBP-2') which is encoded in the chromosomal *mecA* gene. It was also reported that the *mecA* gene present in coagulase-negative staphylococci (CNS) is horizontally transferred to *S. aureus*⁵. In this experiment, we isolated *Staphylococcus* strains from the general ward of Tottori University Hospital and tested their drug susceptibility. Then, the *mecA* gene and *femA* gene were investigated in *Staphylococcus* by the polymerase chain reaction (PCR) and Southern blot analysis.

Materials and Methods

1. Bacteria

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The *Staphylococcus* strains were collected from the University Hospital ward in December 1992 and April 1993. Sterile swabs were rubbed on 5 separate sites of the floor of the ward and on the surface of fixtures, and each swab was streaked directly on a nutrient agar plate. The plates were incubated for 24 to 48 hr at 37°C. Bacteria from each colony were transferred to a glass slide for gram staining, and gram-positive cocci were further selected by oxidation-fermentation and catalase tests. Bacteria from 4 to 5 colonies were separately picked from each plate. Species of *Staphylococcus* were identified with the N-ID test • SP-18 (Nissui Seiyaku Co. Ltd., Tokyo).

2. Antibiotic susceptibility

Antibiotic susceptibility testing was performed by the disk method described elsewhere (NCCLS)⁸⁾, using methicillin (DMPPC), oxacillin (MIPIC), ceftizoxime (CZX), ampicillin (ABPC), cefazolin (CEZ), cefmetazole (CMZ), imipenem (IPM), gentamicin (GM), clindamycin (CLDM), lomefloxacin (LFLX), minocycline (MINO), sulfamethoxazole-trimethoprim (ST) and vancomycin (VCM) (all from Showa Yakuhin Kako Co. Ltd., Tokyo). According to the manufacturer's instructions, methicillin-resistant *S. aureus* (MRSA) was identified by zone diameters of ≤ 20 mm for 30 μ g of DMPPC, ≤ 20 mm for 30 μ g of MIPIC, and ≤ 16 mm for 30 μ g of CZX, after incubation on Mueller-Hinton medium for 16–18 hr at 35°C. The strains categorized as giving an equivocal test result were further investigated by incubation at 32°C. Then, resistance was considered positive, when the inhibitory zone diameter decreased more than 4 mm at 32°C as compared with that at 35°C.

3. DNA preparation

DNA preparation was based on Marmur's method⁶⁾ with some modification. To a microtube was added 1.5 ml of an overnight culture of *S. aureus* in brain heart infusion broth (Difco Laboratories, Detroit, MI) and the tube was centrifuged at 12,000 rpm for 5 min. The precipitated cells were suspended in 400 μ l of saline-EDTA (0.1 M EDTA in 0.15 M NaCl, pH 8.0), and 6 μ l of 1% (w/v) of sodium dodecyl sulfate and 4 μ l of 2% (w/v) proteinase K (Wako Pure Chemical Industries Ltd., Osaka) in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) were added. Then the suspension was incubated at 37°C for 30 min, and treated with 5 μ l of 1% (w/v) RNase (Nippon Gene, Co. Ltd., Tokyo) at 37°C for 30 min. DNA was extracted with buffered phenol and then with chloroform-isoamylalcohol (24:1), then precipitated with ethanol containing one-ninth volume of 3 M sodium acetate. After centrifugation at 12,000 rpm, the precipitated DNA was washed with cold ethanol and dried *in vacuo*. DNA was resuspended in TE buffer for further experiments, and stored at 4°C until use.

4. Polymerase chain reaction analysis

Two oligonucleotide primers (sense, nucleotides 1282 to 1303 of *mecA* with the sequence 5'-AAAATCGATGGTAAAGGTTGGC-3', and antisense, nucleotides 1793 to 1814 of *mecA* with the sequence 5'-AGTTCTGCAGTACCGGATTTGC-3')¹⁾, and other nucleotide primers (sense, nucleotides 766 to 789 of *femA* with the sequence 5'-CGAGGTCATTGCAGCTTGCTTAC-3', and antisense, nucleotides 1736 to 1755 of *femA* with the sequence 5'-CTAGACCAGCATCTTCAGC-3')²⁾ were synthesized and supplied by Nippon Gene.

PCRs were performed in a DNA thermal cycler by using a Gene Amp Kit according to the manufacturer's recommendations. The PCR conditions were 94°C, 30 sec for denaturation; 60°C, 30 sec for annealing; 72°C, 2 min for primer extension; for 30 cycles in an MJ Research DNA thermal cycler. The PCR products were fractionated on 1% agarose gels.

5. Southern blot analysis

Extraction of the high molecular weight DNA and Southern blot analysis were performed according to the method described by Engler-Blum et al.⁷⁾ Filters prehybridized for 1 hr at 68°C were hybridized with probes at 68°C. The *mecA* and *femA* probes were labelled by a DIG DNA Labeling

Kit (Boehringer, Tokyo) and added to hybridization buffer. The *femA* probe was a 990-bp fragment¹⁾ from the PCR product of MRSA. The *mecA* probe was a 533-bp fragment²⁾ from the PCR product of MRSA. Filters were washed at 65°C in 0.1 M maleic acid, 3 M NaCl, 0.3% Tween 20 and exposed to film with a DIG Luminescent Detection Kit (Boehringer, Tokyo).

Results

1. Isolation of *Staphylococcus* spp.

Fifteen species of the genus *Staphylococcus* were isolated from the floor of the hospital ward in December 1992 and in April 1993, as shown in Table 1. In December 1992, 149 strains of *Staphylococcus* were isolated, of which only 2 strains were *S. aureus*. The isolation rate for *S. epidermidis* was the highest, and *S. hominis*, *S. haemolyticus*, *S. warneri* and *S. capitis* were main isolates. In contrast, there was a marked increase in the isolation rates for *S. aureus* and *S. caprae* in April 1993, and *S. epidermidis*, *S. haemolyticus*, *S. hominis* and *S. warneri* were also found (Table 1).

2. Antibigrams of the staphylococci

A total of 115 strains of the genus *Staphylococcus* were tested for susceptibility to 13 antibiotics (Table 2). MRSA was found to be resistant to many drugs. All 12 strains of MRSA were also resistant to ABPC and CLDM. The average number of resistance factors per strain was the highest in MRSA (7.9) and the next highest in *S. haemolyticus* (6.7). Eight of 10 strains of *S. haemolyticus* were resistant to methicillin. Only one strain of *S. simulans* was isolated throughout the experiment and it was resistant to 6 drugs.

Among the 13 drugs, ABPC resistance was observed in 47 of the 115 strains (40.9%). Thirty-nine, 36, and 43 of the 115 strains were resistant to DMPPC, MIPPC, and CZX, respectively. No strain resistant to MINO or VCM was isolated.

3. Detection of *femA* and *mecA* genes

PCR amplification of a segment of the *mecA* gene or *femA* gene from the staphylococci was performed (Fig. 1). The *femA* gene was observed in all the strains of *S. aureus* (MRSA and MSSA), but not in *S. epidermidis*, *S. hominis* or *S. haemolyticus*. The *mecA* gene was observed in all the 7

Table 1 Isolation of *Staphylococcus* in December 1992 and April 1993

Species	Stains isolated in :		Total (%)
	December	April	
<i>S. aureus</i>	2	27	29(10.6)
<i>S. epidermidis</i>	73	25	98(35.8)
<i>S. capitis</i>	10	5	15 (5.5)
<i>S. warneri</i>	12	12	24 (8.8)
<i>S. haemolyticus</i>	17	17	34(12.4)
<i>S. hominis</i>	18	13	31(11.3)
<i>S. auricularis</i>	4	4	8 (2.9)
<i>S. saprophyticus</i>	4	4	8 (2.9)
<i>S. cohnii</i>	4	3	7 (2.6)
<i>S. simulans</i>	1	0	1 (0.4)
<i>S. carnosus</i>	1	1	2 (0.7)
<i>S. sciuri</i>	1	0	1 (0.4)
<i>S. caprae</i>	2	12	14 (5.1)
<i>S. equorum</i>	0	1	1 (0.4)
<i>S. kloosi</i>	0	1	1 (0.4)
Total	149	125	274(100)

Table 2 Antibiotic susceptibility of the isolates to 13 antibiotics^{a)}

Species	(No.)	Number of strains resistant to :													TRF	(ratio) ^{b)}
		DMPPC	MPIPC	CZX	ABPC	CEZ	CMZ	IPM	GM	MINO	CLDM	LFLX	VCM	ST		
<i>S. aureus</i> (MSSA)	(17)				13										13	(0.8)
<i>S. aureus</i> (MRSA)	(12)	12	12	12	12	11	9	5	8		12	2			95	(7.9)
<i>S. epidermidis</i>	(10) ^{c)}	2	1	3	4	1	1				2				14	(1.4)
<i>S. capitis</i>	(10) ^{c)}	1		1	2						1				5	(0.5)
<i>S. warneri</i>	(10) ^{c)}	2	2	2	2										8	(0.8)
<i>S. haemolyticus</i>	(10) ^{c)}	8	8	8	9	8	8	4	9			5			67	(6.7)
<i>S. hominis</i>	(10) ^{c)}	3	3	4		2	3	1			1	1			18	(1.8)
<i>S. auricularis</i>	(8)	4	4	4		1	1					1			15	(1.9)
<i>S. saprophyticus</i>	(8)	4	4	4	4	4					2				22	(2.8)
<i>S. cohnii</i>	(7)	2	2	4		2					2				12	(1.7)
<i>S. simulans</i>	(1)			1	1				1		1	1		1	6	(6.0)
<i>S. carnosus</i>	(2)														0	(0)
<i>S. sciuri</i>	(1)														0	(0)
<i>S. caprae</i>	(7)	1													1	(0.3)
<i>S. equorum</i>	(1)														0	(0)
<i>S. kloosi</i>	(1)														0	(0)
Total	(115)	39	36	43	47	29	22	10	18	0	21	10	0	1	276	(2.4)

^{a)}DMPPC, methicillin; MPIPC, oxacillin; CZX, ceftizoxime; ABPC, ampicillin; CEZ, cefazolin; CMZ, cefmetazole; IPM, imipenem; GM, gentamicin; MINO, minocycline; CLDM, clindamycin; LFLX, lomefloxacin; VCM, vancomycin; ST, sulfamethoxazole-trimetoprim

^{b)}Total resistance factors (average number of resistance factors/strain)

^{c)}Ten strains each of these species were picked at random.

Fig. 1 PCR analysis of the *mecA* gene and the *femA* gene from staphylococci

A 533-bp segment of the *mecA* gene (upper) and a 990-bp segment of the *femA* gene (lower) were amplified from the lysate of *S. aureus* (A), and CNS (B). (A) M, size markers (in base pairs); lanes 1-7, MRSA; lanes 8-12, MSSA, (B) lanes 1-2, methicillin-resistant (MR) *S. epidermidis*; lanes 3-4, methicillin-sensitive (MS) *S. epidermidis*; lanes 5-6, MR-*S. hominis*; lanes 7-8, MS-*S. hominis*; lanes 9-10, MR-*S. haemolyticus*; lanes 11-12, MS-*S. haemolyticus*.

strains of MRSA tested, but not in 5 strains of MSSA. Two strains of methicillin-resistant (MR) *S. epidermidis* and one of 2 MR-*S. hominis* strains contained the *mecA* gene. No *mecA* gene was detected in the methicillin-sensitive strains of *S. epidermidis*, *S. hominis* or *S. haemolyticus*. Southern blot analysis using *mecA* and *femA* probes showed that *femA* and *mecA* genes were present in all the DNAs from the corresponding *femA*- or *mecA*-positive strains (Fig. 2).

Discussion

We collected *Staphylococcus* from the new ward environment of Tottori University Hospital over

Fig. 2 Southern blot analysis of *Staphylococcus* spp.

The DNAs from *S. aureus* (A) and from CNS (B) were hybridized with the *mecA* gene probe (upper) and the *femA* gene probe (lower). The strain and the lane number are the same as shown in Fig. 1.

one year from 1991 to 1992, and tested their drug susceptibility⁸⁾. The rates of isolation of *S. aureus* and of MRSA had gradually increased. The rates of isolation of *S. aureus* among *Staphylococcus* spp. and of MRSA among *S. aureus* strains in January 1992 were 22 and 43%, respectively. One year later, in this experiment, isolation rates for *S. aureus* and for MRSA among *S. aureus* were 10.6 and 41.4%, respectively (Table 1). As it was widely recognized in Japan that 50 to 60% of *S. aureus* strains isolated from inpatients was MRSA, it may have been reasonable to find that 40 to 45% of *S. aureus* strains in the environment were methicillin resistant.

A PCR-based test was developed for the detection of the *mecA* gene, which encodes methicillin resistance in *S. aureus*^{1,3)}. This technique was extensively used for the rapid identification of MRSA. However, it was found that the DNA from staphylococci other than *S. aureus* also encodes *mecA*. Other DNA regions of the *mecA* genes were also selected as a primer by some investigators^{4,9,10)}. Another gene, *femA*, has been characterized as essential for the expression of *S. aureus*¹¹⁾.

Yokoyama¹²⁾ used the DNA sequence encoding *mecA* and *femA* genes as a double primer. We had used a double primer encoding *mecA* and *femA* genes for identifying MRSA by a single PCR test. Unexpectedly, the strain which was positive in the PCR test with the primer for the *mecA* gene or *femA* gene alone was negative in the PCR with the double primer. A strategy for selection of the double primer may be needed.

Throughout these experiments, some strains of methicillin-resistant *Staphylococcus* other than *S. aureus* (MR-CNS) were found not to contain *mecA*. This might be due to the diversity of DNA sequences of the gene which encodes the *mecA* product, or to an other cellular DNA which encodes methicillin resistance. Alternatively, two regulatory *mecA* genes were found¹³⁾; one is the *mecI* gene which suppresses the expression of the *mecA* gene, and the other is the *mecR1* gene which acts as an inducer of the *mecA* gene. Unusual expression of these regulatory genes for *mecA* might be responsible for methicillin resistance. Resolution of these discrepancies will require further studies.

At any rate, staphylococci isolated from a hospital ward were revealed to possess methicillin resistance at relatively high frequency, and a similar *mecA* gene was detected in *S. aureus* and also in CNS. If a compromised patient is contaminated with MR-staphylococci directly from the ward environment or from the medical staff, methicillin resistance is transferred to the patient's resident *S. aureus*, and an opportunistic and devilish infection might occur. Therefore, extreme care against MRSA and MR-CNS is necessary for clinical treatment of the compromised inpatients.

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病棟における *Staphylococcus* 感染の分子疫学メチシリン耐性 *Staphylococcus* の *mecA* および *femA* の研究

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要 旨

鳥取大学医学部附属病院病棟の床、器具より *Staphylococcus* spp. の分離を試み、合計274株の分離株のうち29株が *S. aureus* で、*S. aureus* の41.4%がメチシリン耐性 (MRSA) であった。12株のMRSAはまたMIPIC, CZX, ABPC, CLMにも耐性であった。CNSの中にもメチシリン耐性 (MR) の *S. epidermidis*, *S. capitis*, *S. warneri*, *S. haemolyticus*, *S. hominis*, *S. auricularis*, *S. saprophyticus*, *S. cohnii* などが分離された。

PCR および Southern blot 分析で *femA* は

MRSA および MSSA にみられたが、CNS には認められなかった。*mecA* はMRSAとMR-*S. epidermidis*にみられ、MR-*S. hominis*の2株のうち1株に見られた。MSSA, MS-CNSには認められなかった。MR-*S. hominis*の1株と*S. haemolyticus*の2株のDNAには*mecA*が検出できなかった。原因について考察を試みたが、いずれにしても病棟環境中にMR-*Staphylococcus*が存在することを考えると、院内感染に十分な注意が必要である。