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ORIGINAL ARTICLE

COMPARATIVE PREVALENCE OF ANTIBIOTIC RESISTANCE, STAPHYLOCOCCAL CASSETTE CHROMOSOME *MEC* AND SUPERANTIGENIC TOXIN GENES IN COAGULASE-NEGATIVE STAPHYLOCOCCI

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Abstract Coagulase-negative staphylococci (CNS) are increasingly recognized to cause clinically significant infections. To investigate relationship of antibiotic resistance profiles, staphylococcal cassette chromosome *mec* (SCC*mec*) and superantigenic toxin genes in CNS, a total of 79 clinical CNS isolates from patients with surgical operations were comprehensively determined for minimum inhibitory concentration (MIC) of antibiotics, and searched for SCC*mec* and staphylococcal superantigenic toxin genes by novel multiplex PCR. Among 79 CNS isolates, at least one SCC*mec* gene was detected in 55 isolates (69.6%). The most of SCC*mec* type was type III (36.7%), and other detected types were type IVa (20.3%), type II (19.0%), type V (16.5%), type I (15.2%), and type IVb (3.8%). The isolates with type I or IVa gene showed highly resistance to clindamycin and gentamicin, the isolates with type II showed more resistance to teicoplanin, the isolates with type III or type V showed more resistance to clindamycin. *se* and *tst-1* genes were detected in none of the CNS isolates tested. These results suggested that there is high prevalence of SCC*mec* in clinical CNS isolates, and the SCC*mec* types are related to the resistances against specific antibiotics, but SCC*mec* do not related to superantigenic toxin genes in the CNS.

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Key words: staphylococci; antibiotic resistance; staphylococcal cassette chromosome *mec*; multiplex PCR; superantigen.

原　著

コアグラーゼ陰性ブドウ球菌の薬剤耐性，SCC*mec* およびスーパー抗原毒素遺伝子の検出と比較

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抄録　コアグラーゼ陰性ブドウ球菌(CNS)は，臨床上重要な感染症の原因となりうる．本研究では，SCC*mec* およびスーパー抗原毒素遺伝子を検出するマルチプレックス PCR 法を確立し，79株の臨床分離 CNS について SCC*mec* およびスーパー抗原毒素遺伝子の型別と薬剤耐性プロフィールを検討した．そのうち，69.6%(55株)から1種類以上の SCC*mec* が検出され，型別では SCC*mec*Ⅲが最も多く36.7%であり，次に多いⅣaが20.3%，Ⅱが19.0%，Ⅴが16.5%であった．SCC*mec* 型別と薬剤耐性プロフィールとの相関性を調べた結果，SCC*mec*ⅠまたはⅣaの保有株はクリンダマイシンとゲンタマイシンに対し強い抵抗性を示し，SCC*mec*Ⅱはテイコプラニン，ⅢまたはⅤはクリンダマイシンに対し強い抵抗性を示した．しかし，これらの菌株からスーパー抗原毒素遺伝子は検出されなかった．以上の結果から CNS 臨床分離株は SCC*mec* 保有率が高く，SCC*mec* 型と特定の薬剤耐性との関連が示唆された．

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キーワード：ブドウ球菌；薬剤耐性；SCC*mec*；マルチプレックスPCR；スーパー抗原．

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Introduction

Coagulase-negative staphylococci (CNS) are one of members of the normal microbial flora of the skin and mucous membranes of humans, and an important cause of nosocomial infections, particularly foreign device-related infections and infections among immunocompromised patients. CNS represent a serious concern in hospital-acquired infections including endocarditis and urinary tract diseases[1]. Methicillin resistance in staphylococci is caused by the expression of penicillin-binding protein PBP2a (PBP2′), which is encoded by the *mecA* gene located on a mobile genetic element called the staphylococcal cassette chromosome *mec* (SCC*mec*)[2]. Several different SCC*mec* types which are different in size (21 to 67 kbp) have been recognized, and characterized by a different set of *ccr* recombinase genes and *mec* gene complex[3-5]. Some of the types were further classified into subtypes according to differences in their J region DNA[4, 6].

SCC*mec* elements have been detected in CNS, which are isolated from patients, healthy humans, animals such as cattle and sheep, environment such as marine water and beach sand[7-10]. In addition to harboring SCC*mec* which is involved in methicillin-resistance, a considerable number of CNS isolates were resistant to non-β-lactam antibiotics, such as kanamycin, tobramycin, gentamicin and erythromycin[11]. The SCC*mec* has been identified exclusively among staphylococci, but its origin remains unknown. It has been suggested that *mecA* of methicillin-resistant *Staphylococcus aureus* (MRSA) originate from *mecA* homologue in *S. sciuri*[12]. The transfer of *mecA* from CNS to *S. aureus* has been suspected to occur in vivo[13].

Staphylococci, especially MRSA, produce one or more specific staphylococcal superantigenic toxins including staphylococcal enterotoxins (SEs), enterotoxin-like superantigens and toxic shock syndrome toxin-1 (TSST-1)[14]. To date, 21 SEs have been identified based on sequence homologies[15, 16]. Superantigenic toxins bypass normal antigen presentation and have strong T cell mitogenic activity by direct binding to the Vβ region of specific T cells and major histocompatibility complex class II molecules of antigen presenting cells. This leads to a massive release of proinflammatory cytokines such as tumor necrosis factor (TNF-α), interleukin (IL)-2, IL-6 and interferon-γ (IFN-γ), which are responsible for physiopathology of toxic shock syndrome and contributes to the severity of *S. aureus* sepsis[17]. Little information is available about the superantigen genes among populations of CNS isolates. It is still unclear whether CNS carry superantigenic toxin genes and how is prevalence of the superantigenic toxin genes in CNS isolates from clinical patients.

In the present study, in order to reveal prevalence and relationships among antibiotic resistance, SCC*mec* and superantigenic toxin genes of CNS, we investigated minimum inhibitory concentrations (MIC) of antibiotics, SCC*mec* types and superantigen genes, and correlation of them in clinical CNS isolates collected from different patients.

Materials and Methods

Bacteria isolates and culture conditions

A total of 79 CNS clinical isolates tested in this study were collected from patients of the Department of Cardiovascular Surgery, Hirosaki University Hospital, Hirosaki, Aomori, Japan. The isolates were stocked in trypticase soy broth with 15% glycerol at −80° C until use. The SCC*mec* typing standard MRSA control strains, including SCC*mec* type I (NCTC10442), type II (N315), type III (85/2082), type IVa (CA05), type IVb (8/6-3P), type IVc (MR108), type IVd (JCSC4469), and type V (WIS [WBG8318]-JCSC3624) (Table 1), were kindly provided by

Table 1 Primers used in this study

Primer	Oligonucleotide sequence	Product size (bp)	Specificity	Reaction mixture
Type I-F	GCTTTAAAGAGTGTCGTTACAGG	613	SCC*mec* I	A
Type I-R	GTTCTCTCATAGTATGACGTCC			
Type II-F	CGTTGAAGATGATGAAGCG	398	SCC*mec* II	A
Type II-R	CGAAATCAATGGTTAATGGACC			
Type III-F	CCATATTGTGTACGATGCG	280	SCC*mec* III	A
Type III-R	CCTTAGTTGTCGTAACAGATCG			
Type IVa-F	GCCTTATTCGAAGAAACCG	776	SCC*mec* IVa	B
Type IVa-R	CTACTCTTCTGAAAAGCGTCG			
Type IVb-F	TCTGGAATTACTTCAGCTGC	493	SCC*mec* IVb	B
Type IVb-R	AAACAATATTGCTCTCCCTC			
Type IVc-F	ACAATATTTGTATTATCGGAGAGC	200	SCC*mec* IVc	B
Type IVc-R	TTGGTATGAGGTATTGCTGG			
Type IVd-F	CTCAAAATACGGACCCCAATACA	881	SCC*mec* IVd	C
Type IVd-R	TGCTCCAGTAATTGCTAAAG			
Type V-F	GAACATTGTTACTTAAATGAGCG	325	SCC*mec* V	C
Type V-R	TGAAAGTTGTACCCTTGACACC			
MecA147-F	GTGAAGATATACCAAGTGATT	147	*mecA*	A, B, C
MecA147-R	ATGCGCTATAGATTGAAAGGAT			

K. Hiramatsu and T. Ito, Center of Excellent for Infection Control Science, Juntendo University Graduate School of Medicine, Tokyo, Japan[3-5, 18]. Eleven reference strains for *se* and *tst* gene typing (N315; DDB/GenBand/EMBL BA000018, Mu50; BA000017, MW2; BA000033) were used in this study[19-21].

Antibiotic susceptibility testing

The minimum amount of antibiotic that inhibited the visible growth of an isolate or MIC was determined. A serial two-fold dilution of the antibiotics was prepared in Mueller-Hinton agar. A volume of 2 μl of bacterial isolate was inoculated on the surface of agar containing various dilutions of antibiotics and incubated at 37℃ overnight. The lowest concentration of antibiotic that inhibited visible growth on surface of agar was taken as MIC. Ten antibiotic agents, that is, arbekacin sulfate (ABK), cefazolin sodium (CEZ), ciprofloxacin (CPFX), clindamycin phosphate (CLDM), gentamicin sulfate (GM), imipenem hydrate sodium (IPM), linezolid (LZD), teicoplanin (TEIC), sultamicillin tosilate (SBTPC), and vancomycin hydrochloride (VCM) were used for analyzing the susceptibility of CNS isolates.

DNA preparation

Template DNA for multiplex PCR was prepared as previously described[22]. Each CNS isolate was cultured on tryptic soy agar for 24 h and then one or two bacterial colonies were suspended in 50 μl of sterile distilled water. The suspension was heated at 100℃ for 10 min, and centrifuged at 20,000 g for 1 min. The supernatant was used as template DNA in multiplex PCR for detection of SCC*mec* and superantigenic toxin genes, or stored at −80℃ until use.

Multiplex PCR for typing of SCC*mec* genes

SCC*mec* type- and subtype-unique and specific primers as well as the specific primers for *mecA* genes (Table 1) were designed based on the comprehensive analyses and alignments

of *S. aureus* and MRSA genomes and SCC*mec* sequences currently available in the GenBank database. The oligonucleotide primers used in this study were synthesized and purchased from Greiner Japan (Greiner bio-one, Tokyo, Japan). SCC*mec* multiplex PCR typing assay contained 9 pairs of primers including the unique and specific primers for SCC*mec* types and subtypes I, II, III, IVa, IVb, IVc, IVd, and V, and the primers for *mecA* gene. Multiplex PCR assays were performed directly from bacterial suspensions obtained after the rapid DNA extraction method. An aliquot of 2 μl of the suspension was added to 23 μl of PCR mixture containing 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate (GeneAmp dNTP, Applied Biosystems, Tokyo, Japan), various concentrations of the respective primers, and 1.0 unit of Ampli *Taq* Gold polymerase (Applied Biosystems). The amplification was performed in iCycler (Bio-Rad, Tokyo, Japan) beginning with an initial denaturation step at 94℃ for 5 min followed by 32 cycles of 94℃ for 1 min, 50℃ for 1 min, and 72℃ for 2 min, ending with a final extension step at 72℃ for 10 min and followed by a hold at 4℃. For the single target amplification, PCR was run in 23 μl of PCR mixture but containing 0.2 μM of each primer, with cycling parameters beginning with an initial denaturation step at 94℃ for 5 min followed by 31 cycles of 94℃ for 1 min, 50℃ for 1 min, and 72℃ for 2 min, ending with a final extension step at 72℃ for 10 min. The PCR products were visualized using a transilluminator after electrophoresis in a 2% agarose gel and stained by 0.5 μg/ml ethidium bromide.

Multiplex PCR for detection of superantigenic toxin genes

For detection and typing of *se* and *tst* genes in CNS strains, multiplex PCR was performed as previously described[14, 20]. The primer sets were designed to anneal to unique regions and generate amplicons that would allow discrimination between each *se* or *tst* gene based on the molecular weight of its PCR product. Details of primer sets were as follows; Set1: *sea*, *seb*, *sec*, *sed*, *see*, *fem*A; Set2: *seg*, *seh*, *sei*, *selj*, *selp*, *fem*B; Set3: *selk*, *selm*, *selo*, *tst-1*, *fem*A; Set4: *sell*, *seln*, *selr*, *fem*B. Each of the primer sets was prepared as 10× primer master mixes containing 2 mM of each primer. Multiplex PCR was performed with QIAGEN multiplex PCR kit (QIAGEN, Tokyo, Japan) according to manufacturer's instructions. The amplification was performed using iCycler (Bio-Rad, Tokyo, Japan) beginning with an initial denaturation step at 95℃ for 15 min, followed by 35 cycles of 94℃ for 30 sec, 57℃ for 90 sec, and 72℃ for 90 sec, ending with a final extension step at 72℃ for 10 min. The PCR products were visualized using a transilluminator after electrophoresis in a 2.5% agarose gel and stained by 0.5 μg/ml ethidium bromide.

Results

Antibiotic resistance of CNS isolates from clinical specimen

Seventy-nine CNS isolates obtained from clinical specimens were studied for the resistance to ten antibiotics (Table 2). Of them, all of the CNS isolates were susceptible to arbekacin (MIC ≤0.25 μg/ml), 75.9% of the isolates were susceptible to imipenem, 67.1% of them were susceptible to ciprofloxacin, 62.0% were susceptible to sultamicillin tosilate and 57.0% were susceptible to gentamicin (MIC ≤ 0.25 μg/ml). In contrast to these antibiotics, the CNS isolates showed high resistance (MIC was ≥4 μg/ml) to clindamicin (68.3%) and teicoplanin (50.7%).

Table 2 Antibiotic resistance of CNS isolates (%)

Antibiotics	MIC (μg/mL)							
	≤0.25	0.5	1	2	4	8	16	≥32
Arbekacin	100.0	0	0	0	0	0	0	0
Cefazolin	43.0	17.7	17.7	8.9	0	3.8	0	8.9
Ciprofloxacin	67.1	2.5	2.5	1.3	13.9	7.6	2.5	2.5
Clindamycin	0	1.3	8.9	21.5	39.2	11.4	0	17.7
Gentamicin	57.0	3.8	1.3	2.5	3.8	11.4	11.4	8.9
Imipenem/Cilastatin	75.9	5.1	1.3	5.1	1.3	0	2.5	8.9
Linezolid	0	0	25.3	54.4	11.4	6.3	2.5	0
Teicoplanin	19.0	7.6	12.7	10.1	3.8	17.7	16.5	12.7
Sultamicillin tosilate	62.0	8.9	8.9	3.8	0	1.3	3.8	11.4
Vancomycin	12.7	32.9	24.1	10.1	15.2	5.1	0	0

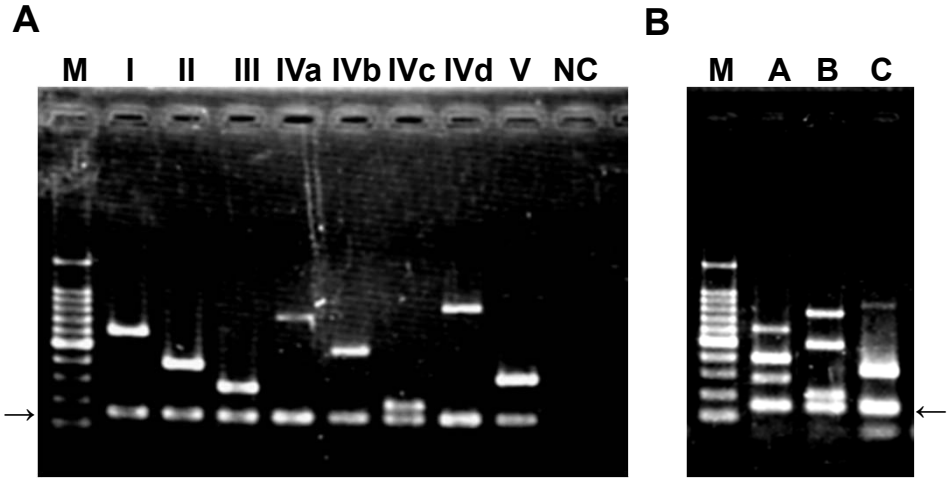

Figure 1 Multiplex PCR assay to optimize SCC*mec* types and subtypes I, II, III, IVa, IVb, IVc, IVd, and V, and simultaneously detect methicillin resistance (*mecA* gene). (A) Single PCR. I, Type I, strain NCTC10442; II, Type II, strain N315; III, Type III, strain85/2082; IVa, Type IVa, strain CA05; IVb, Type IVb, strain8/6-3P; IVc, Type IVc, strain MR108; IVd, Type IVd, strain JCSC4469; V, Type V, strain WIS-JCSC3624; M, molecular size markers; NC, negative control. The arrow shows *mecA*. (B) Multiplex PCR. A, primers specific for type I, II, III, and *mecA*; B, primers specific for type IVa, IVb, IVc, and *mecA*; C, primers specific for type IVd, V, and *mecA*; M, molecular size marker. The arrow shows *mecA*.

Prevalence of SCC*mec* genes in clinical CNS isolates

To construct the multiplex PCR for detecting the SCC*mec* genes in the CNS isolates, uniplex PCR using each primer set with total DNA of reference MRSA strains was firstly performed. The sizes of PCR products obtained by the uniplex PCR were identical to the predicted SCC*mec* and *mecA* genes from the design of the primers (Figure 1A). The combination of primer sets and reaction conditions for the multiplex PCR was optimized to ensure that all PCR products of target genes were satisfactorily amplified (Figure 1B). We ultimately constructed three optimized multiple primer sets. Reliable amplification of PCR products was observed in all multiplex PCR reactions using the three primer sets. The sizes of PCR products obtained from the positive control corresponded to their predicted sizes. A total

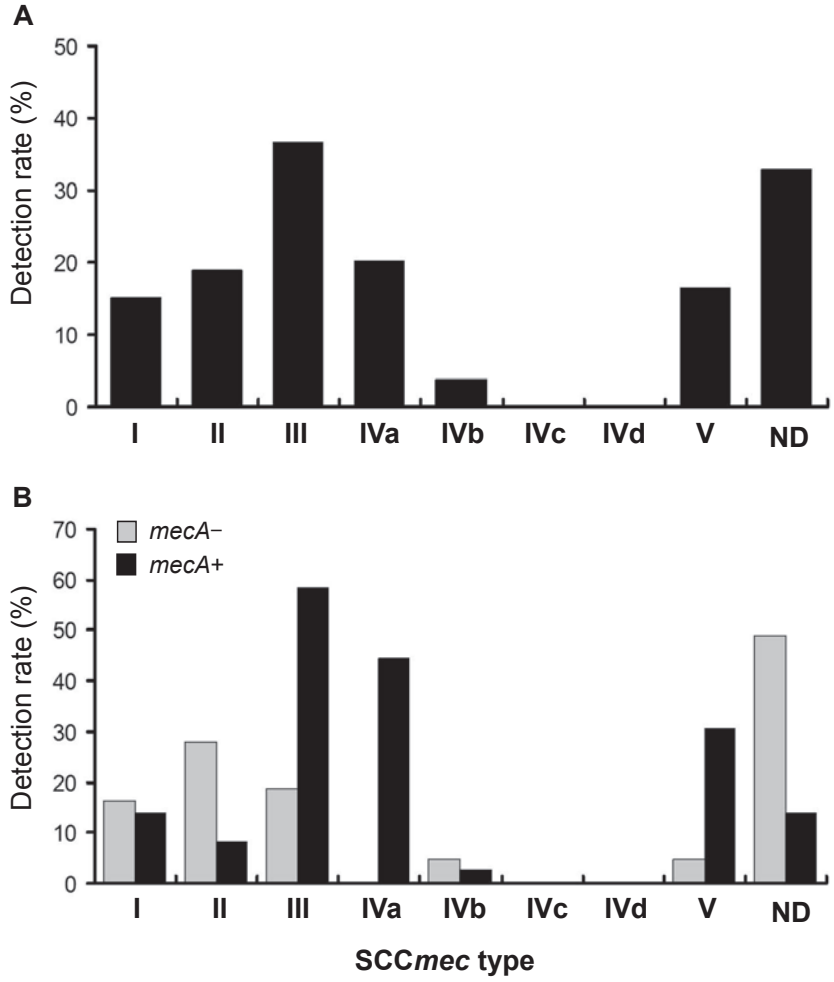

Figure 2 Detection of SCC*mec* genes of CNS isolates collected from clinical specimens. Total DNA of a CNS isolate was purified and 3 primer sets of SCC*mec* genes based on the molecular weight of its PCR product were used to anneal to unique regions and generate amplicons. The number of positive genes in each isolate was recorded and the percentage was calculated with the total isolates. (A) Percentage of SCC*mec* genetic elements in a total of 79 CNS isolates. (B) Percentage of SCC*mec* genetic elements in *mecA*− and *mecA*+ CNS isolates.

of 79 CNS isolates from clinical specimens were subjected to *mecA* and SCC*mec* typing analysis. Among these CNS isolates, at least one SCC*mec* gene was detected in 55 isolates (69.6%), and more than one SCC*mec* gene were detected in 24 of the 55 isolates. SCC*mec* type of the isolates were classified into type III (36.7%), type IVa (20.3%), type II (19.0%), type V (16.5%), type I (15.2%) and IVb (3.8%), and the highest proportion of SCC*mec* type (29/79 strains) was type III (Figure 2A). SCC*mec* IVc and IVd were not detected in any of the isolates. Thirty-six (45.6%) of the CNS isolates harbored the *mecA* genes (*mecA*+). We further compared SCC*mec* types between *mecA*+ and *mecA*− isolates (Fig. 2B). For the *mecA*+ isolates, 58.3% of the isolates carried SCC*mec* type III, 44.4% carried SCC*mec* type IVa and 30.6% carried SCC*mec* type V genetic element, and showed considerably higher than those of the *mecA*− isolates (Figure 2B). In contrast, for the *mecA*− isolates, 48.8% of them were not detected any SCC*mec* element tested in this study. SCC*mec* type patterns were considerably different between *mecA*+ isolates and *mecA*− isolates.

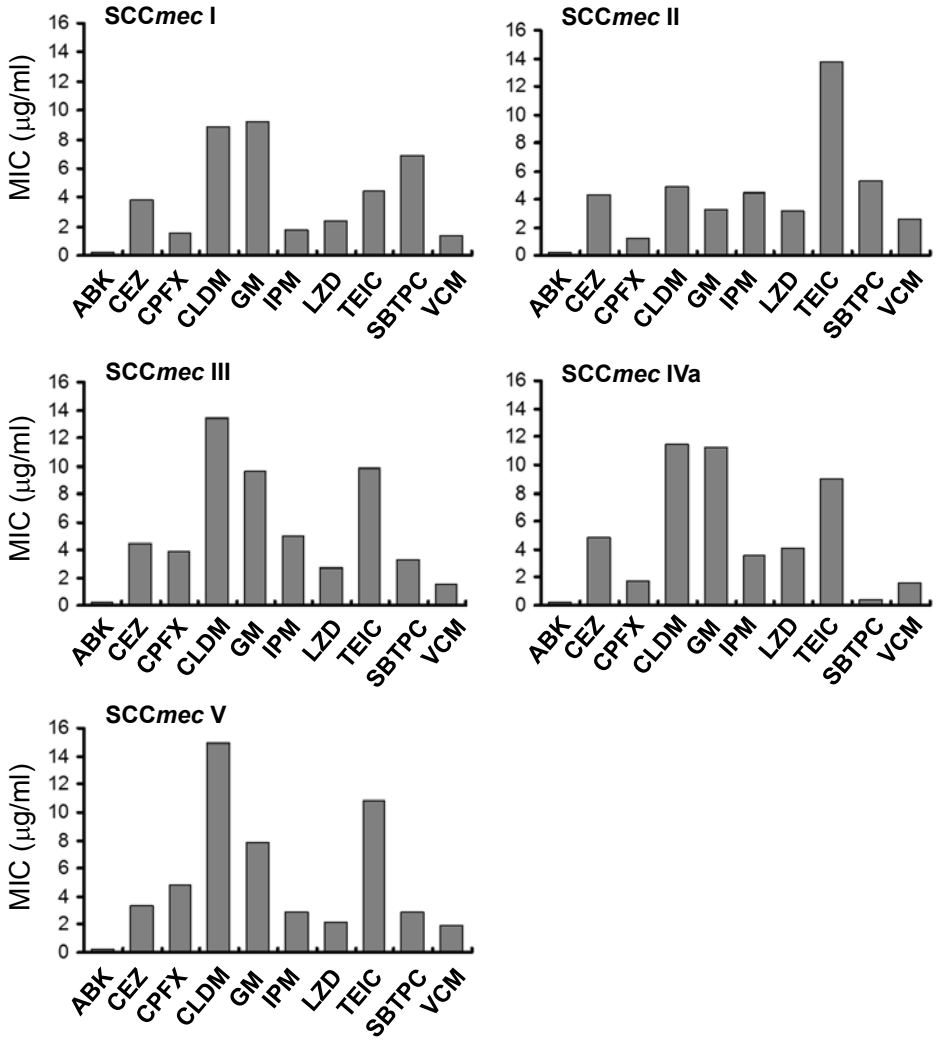

Figure 3 Relationship of SCC*mec* types and antibiotic resistance patterns. ABK, arbekacin; CEZ, cefazolin; CPFX, ciprofloxacin; CLDM, clindamycin; GM, gentamicin; IPM, imipenem; LZD, linezolid; TEIC, teicoplanin; SBTPC, sultamicillin; VCM, vancomycin.

Association between *mecA* gene and antimicrobial resistance of CNS isolates

We further compared antibiotic resistance patterns between *mecA*− and *mecA*+ CNS isolates. Although both of *mecA*− and *mecA*+ CNS isolates were susceptible (MIC ≤0.25 μg/ml) to arbekacin, the *mecA*− CNS isolates were more susceptible to cefazolin, ciprofloxacin, gentamicin and imipenem than *mecA*+ isolates. Specifically, rates of *mecA*− isolates for which MIC of cefazolin, ciprofloxacin, gentamicin and imipenem showed ≤0.25 μg/ml were 53.5%, 81.4%, 72.1% and 83.7%, whereas those of *mecA*+ isolates were 30.6%, 50.0%, 38.9% and 66.7%, respectively. Furthermore, *mecA*+ CNS isolates showed considerably resistant to clindamycin and gentamicin, specifically the isolates of MIC ≥4 μg/ml were 80.6% and 52.9%, respectively.

Relationship of SCC*mec* types and antibiotic resistance patterns of CNS isolates

To investigate the relationship between SCC*mec* type and antibiotic resistance pattern, SCC*mec* types and MIC of antibiotics of CNS isolates were compared and analyzed (Figure 3). The isolates harboring SCC*mec* type I

gene showed the high level resistance (the average of MICs >8 μg/ml) to clindamycin and gentamicin. The isolates harboring SCC*mec* type II were extremely resistant to teicoplanin (the average of MIC >12 μg/ml) compared with those of other antibiotics. For the isolates harboring SCC*mec* type III, MICs of clindamycin, gentamicin and teicoplanin showed the high level resistance (the average of MIC >8 μg/ml) than those of other seven antibiotics. The isolates harboring SCC*mec* type IVa gene showed high resistance (the average of MICs >8 μg/ml) to clindamycin, gentamicin and teicoplanin. MICs of clindamycin and teicoplanin for the isolates harboring SCC*mec* type V were considerably higher compared with those of other eight antibiotics (Figure 3).

Detection of superantigenic toxin genes in CNS isolates

To investigate the prevalence of superantigenic toxin genes, *se* and *tst* genes in a total of 79 clinical CNS isolates and 8 MRSA isolates (as positive control strains) were analyzed by multiplex PCR. The MRSA isolates were constantly detected *se* and/or *tst* genes. In contrast, any *se* or *tst* gene was not detected in all of the CNS isolates tested in this study.

Discussion

CNS are the main clinical isolates of blood-stream and central venous catheter related bloodstream infections among patients with hematological disorders. In this study, a total of 79 CNS isolates from patients with cardiovascular surgery were comprehensively determined for antibiotic resistance, SCC*mec* types and superantigenic toxin genes. Of them, 45.6% were *mecA*+ isolates, and SCC*mec* type III, IVa and V were detected in 21 (58.3%), 16 (44.4%) and 11 (30.6%) isolates, respectively. SCC*mec* genes were also detected in *mecA*− isolates at a high ratio of 51.2% (22/43 isolates) and showed SCC*mec* type II was 27.9%, type III was 18.6% and Type I was 16.3%. These results indicated that there are considerable differences of SCC*mec* type patterns between the *mecA*+ and *mecA*− CNS clinical isolates. Previous studies reported that 61.7% of MRCNS isolates from healthy Japanese children harbored SCC*mec* IVa[9], 52% of *mecA*+ CNS isolates from hospitalized patients harbored SCC*mec* type III, and 27.9% of the isolates harbored SCC*mec* type I[10]. Among the isolates from elderly patients in a long-term-care facility, SCC*mec* type IV was found in 33% and type V was found in 18% of the isolates[23]. For MRCNS isolates detected from outpatients, 29% was type IVa, 26% was type V[11]. Previous studies indicated that the majority of MRCNS isolates from humans other than hospitalized patients harbored SCC*mec* type IV or type V, but type IVa was also detected in comparatively many *mecA*+ isolates in this study. On the other hand, in the present study, SCC*mec* elements were also detected from *mecA*− CNS and rate of the isolates harboring SCC*mec* elements was 51.2%. The result suggested that SCC*mec* elements defective in *mecA* may exist.

The relationships between SCC*mec* types and antimicrobial resistance have been studied on MRSA, but little on CNS isolates. Kadlec et al. reported that MRSA isolates harboring SCC*mec* type V (43.4%) were resistant to macrolide and lincosamide including clindamycin[24]. MRSA isolates harboring SCC*mec* type V from bovine mastitis[25] and swine[24] were also significantly resistant to clindamycin. Nakaminami et al. reported that 90.8 % of the MRSA isolates from patients with impetigo, which are mainly composed of SCC*mec* type IV gene, were resistant to gentamicin[26]. Our study analyzed the relation of SCC*mec* genes and the antibiotic resistance of CNS isolates and showed that CNS harboring SCC*mec* genes were more resistant

to clindamycin, gentamicin and teicoplanin than other antibiotics tested in this study (Fig. 3). Mombach Pinheiro Machado et al. reported that 91.7% of CNS isolates harboring SCC*mec* type I and 89.2% of SCC*mec* type III were resistant to gentamicin, and 73.8% of the isolates with SCC*mec* type III were resistant to clindamycin[10]. Interestingly, our results showed that the isolates harboring SCC*mec* type II gene were considerably resistant to teicoplanin than other 9 antibiotics, and the CNS harboring type V was considerably resistant to clindamycin, and type IVa was more resistant to gentamicin. The mechanism between SCC*mec* types and antibiotic resistance profiles needs to be further investigated.

Staphylococcal superantigenic toxin genes in CNS have reported by some studies, but those results did not correspond. Previous studies reported that some CNS isolates from cheese had *se* genes[27], while CNS isolates from foodstuffs and patients did not carry *se* genes[28]. Seventeen (26.2%) of CNS isolates from cheese had any of *sea*, *seb*, and *sec*[27]. In 102 bovine CNS isolates from milk, none of the genes, or *sea* to *see*, *seg* to *seq*, *seu* and *tst* were detected[29]. Our results showed that *se* and *tst-1* genes were detected in none of the 79 CNS isolates from clinical specimen, suggesting that CNS does not carry superantigenic toxin genes. These results indicate that SCC*mec* types of CNS are strongly associated to their antimicrobial resistance profiles, but SCC*mec* do not directly related to superantigenic toxin genes.

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