

# SCC<sub>Mec</sub> TYPING AND DETECTION OF VISA-RELATED GENES IN METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* CLINICAL STRAINS FROM KOBE UNIVERSITY HOSPITAL, JAPAN

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**Abstract.** A total of 50 clinical strains of methicillin-resistant *Staphylococcus aureus* (MRSA) were collected from Kobe University Hospital in 2003. Molecular typing of SCC<sub>Mec</sub> was performed by multiplex polymerase chain reaction (PCR) and the presence of six genes (*vraR*, *vraG*, *vraA*, *vraF*, *fruA*, and *fruB*) associated with vancomycin (VCM) resistance was examined by simple PCR analysis. Out of 50 MRSA strains isolated 47 strains contained Type II SCC<sub>Mec</sub> and the remaining contained Type IV SCC<sub>Mec</sub>. Thirty seven strains contained pUB110 plasmid. *VraA* was present in 69% of the strains, *vraF* in 10%, *vraG* in 53%, and *vraR* in 16%. Noteworthy, strains without pUB110 contained *vraR* in relatively higher frequency (31%) compared with strains with pUB110 (11%).

## INTRODUCTION

*Staphylococcus aureus* causes a wide range of human infections and is a major cause of health care-associated infections. Furthermore, *S. aureus* strains can readily acquire tolerance to anti-bacterial agents (Atkinson and Lorian, 1984). After the initial success of penicillin in treating *S. aureus* infection, penicillin resistant *S. aureus* became a major threat in hospitals in the 1950s, requiring the use of methicillin and related drugs for treatment of *S. aureus* infections (Panillo *et al*, 1992). In the 1980s, methicillin-resistant *S. aureus* (MRSA) emerged, and became endemic in many hospitals, leading to the use of vancomycin (VCM).

Although VCM is frequently chosen for treatment of infections caused by MRSA, the incidence of resistance to VCM in *S. aureus* has been increasing for the last 8 years.

In 1987, *mecA* gene, a genetic factor of methicillin resistance, was cloned and sequenced from a Japanese MRSA strain (Matsuhashi *et al*, 1986; Song *et al*, 1987). *MecA* encodes the penicillin-binding protein 2 that has a low affinity for beta-lactam antibiotics and enables MRSA strains to continue cell wall synthesis despite the presence of beta-lactam antibiotics. *MecA* gene is carried by an exogenous DNA element inserted in the *S. aureus* chromosome, and is regulated by two genes, *mecR1* and *mecl*, which together with *mecA* have been referred to as the *mec* complex (Matsuhashi *et al*, 1986; Song *et al*, 1987). *Mec* complex is part of a larger region known as the *Staphylococcal* cassette chromosome *mec* (SCC<sub>Mec</sub>), which is a mobile genetic element inserted in a specific position (on *orfX*)

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near the initiation site of replication (Katayama *et al*, 2000).

SCC*mec* has been generally distinguished by four genetic types, type I-IV (Ito *et al*, 2001; Ma *et al*, 2002). Molecular structural typing of SCC*mec* has been used to characterize the MRSA lineages. Oliveira and Lencastre (2002) have developed a novel method to identify the structural types of SCC*mec* in MRSA strains based on a multiplex polymerase chain reaction (PCR) strategy.

Previous studies have described strains of coagulase-negative Staphylococci having intermediate resistance to VCM and teicoplanin (Bertin *et al*, 2004; Nakamura *et al*, 2004). A VCM-resistant *S. aureus* (VRSA) strain, Mu50, was first reported in 1997 (Hiramatsu *et al*, 1997). As Mu50 has clinically moderate resistance to VCM (MIC of 8 mg L<sup>-1</sup> or above), it is also called VCM-intermediate resistant *S. aureus* (VISA). Although Mu50 was reported to be absent of the VCM-resistance determinants (*vanA*, *vanB*, and *vanC1-3* genes), it was found to have both up- and down-regulated mutation genes (*vraR*, *vraS*, *vraA*, *vraB*, *vraC*, *vraD*, *vraE*, *vraF*, *vraG*, *fruR*, *fruA*, and *fruB*) (Kuroda *et al*, 2003).

In the present study, we have characterized the genetic features of 50 MRSA clinical strains in Kobe University Hospital, Japan, and also investigated the relationship between SCC*mec* molecular structural types and the presence of VISA-related genes.

## MATERIALS AND METHODS

### Bacterial strains

A total of 50 clinical strains of methicillin-resistant *S. aureus* were collected from Kobe University Hospital in 2003. Only one strain per patient was included. The *S. aureus* isolates were identified on the basis of colony pigment and clumping factor. Minimal inhibitory concentrations (MICs) were determined by broth micro-dilution procedure according to NCCLS guidelines, using cation-adjusted Mueller-

Hinton II broth of the same batch (Becton-Dickinson). Strains were incubated at 35°C for 18-24 hours and MICs were determined after 24 hours. Ampicillin, cefazolin, flomoxef, imipenem, gentamicin, erythromycin, clindamycin, minocyclin, levofloxacin, arbekacin, and vancomycin were tested. The breakpoint for the definition of antibiotic resistance in *S. aureus* was based on CDC guidelines.

### Genomic DNA isolation

Total genomic DNA was isolated using QIAamp Tissue Kit (QIAGEN GmbH, Germany).

### Multiplex PCR for SCC*mec* typing

Multiplex PCR included 8 loci (A through H) selected on the basis of previously described *mec* element sequence described in previous reports (Ito *et al*, 2001; Oliveira *et al*, 2001; Oliveira and Lencastre, 2002). Primers employed were based on those in previous reports (Oliveira *et al*, 2001) (see Table1). PCR was performed in 50 µl volume using Gene Amp PCR kit (Applied Biosystems, New Jersey, USA) and contained the following: 1x PCR buffer II; 200 µM (each) deoxynucleoside triphosphate; 400 nM primers CIF2 F2, CIF2 R2, *MEC1* P2, *MEC1* P3, RIF5 F10, RIF5 R13, pUB110 R1, and pT181 R1; 800 nM primers DCS F2, DCS R2, *MECA* P4, *MECA* P7, and IS431 P4; 200 nM primers KDP F1, KDP R1, RIF4 F3, and RIF4 R9; 1.25 U of Ampli Taq; and approximately 5 ng of template DNA. PCR amplifications were performed in ASTEC program temperature control system PC-701 DNA Thermocycler with the following parameters: 10 minutes at 95°C; 30 cycles of 30 seconds at 94°C, 30 seconds at 53°C, and 1 minute at 72°C; and 10 minutes at 72°C. Samples were kept at 4°C until the PCR products could be analyzed. Ten µl aliquots of the PCR products were electrophoresed in a 2.0% agarose gel for 30 minutes at 100 V. Gels were stained with ethidium bromide and photographed under ultraviolet light.

### PCR analysis of VISA-related genes

Six genes, *vraR*, *vraG*, *vraA*, *vraF*, *fruA*, and

Table 1  
Primers used in multiplex PCR.

Locus	Name	Oligonucleotide sequence (5'-3')	Amplicon size (bp)	Specificity (SCC <i>mec</i> type)
A	CIF2 F2	TTCGAGTTGCTGATGAAGAAGG	495	I
	CIF2 R2	ATTTACCACAAGGACTACCAGC		
B	KDP F1	AATCATCTGCCATTGGTGATGC	284	II
	KDP R1	CGAATGAAGTGAAAGAAAGTGG		
C	MECI P2	ATCAAGACTTGCATTACAGGC	209	II, III
	MECI P3	GCGGTTTCAATTCACTTGTC		
D	DCS F2	CATCCTATGATAGCTTGGTC	342	I, II, IV
	DCS R1	CTAAATCATAGCCATGACCG		
E	RIF4 F3	GTGATTGTTTCGAGATATGTGG	243	III
	RIF4 R9	CGCTTTATCTGTATCTATCGC		
F	RIF5 F10	TTCTTAAGTACACGCTGAATCG	414	III
	RIF5 R13	GTCACAGTAATCCATCAATGC		
G	IS431 P4	CAGGTCTCTTCAGATCTACG	381	pUB110
	pUB110 R1	GAGCCATAAACACCAATAGCC		
H	IS431 P4	CAGGTCTCTTCAGATCTACG	303	III
	pT181 R1	GAAGAATGGGGAAAGCTTCAC		
<i>mecA</i>	MECA P4	TCCAGATTACAACCTCACCAGG	162	positive control
	MECA P7	CCACTTCATATCTTGTAACG		

*fruB*, reported to be up-regulated in VISA, were selected for PCR analysis (Kuroda *et al*, 2003). PCR amplification of these six genes was performed using primers designed from published NCBI sequences (Table 2). PCR reactions were carried out in 13 ml reaction mixture containing 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM each deoxynucleotide triphosphates, 0.5 mM of each primer, and 2.5 U of Taq DNA polymerase (Applied Biosystems, New Jersey, USA). ASTEC program temperature control system PC-701 DNA Thermocycler was programmed as follows: 10 minutes at 95°C; 30 cycles of 1 minute at 94°C, 1 minute at 58°C, and 1 minute at 72°C; and 10 minutes at 72°C. Samples were kept at 4°C until the products could be analyzed. Ten µl aliquots of the PCR products were electrophoresed in 1.5% agarose gel for 30 minutes at 100 V. Gels were stained with ethidium bromide and photographed under ultraviolet light. Mu50 (ATCC; 700699) was used as positive control.

## RESULTS

### MRSA strains

We obtained 50 MRSA strains isolates that had a MIC of 2 mg L<sup>-1</sup> to VCM at Kobe University Hospital in 2003. Thirty percent of the MRSA were isolated from sputum (Table 1). Resistance to 11 antibiotics was tested, namely ampicillin, cefazolin, flomoxef, imipenem, gentamicin, erythromycin, clindamycin, minocyclin, levofloxacin, arbekacin, and vancomycin. All samples were sensitive to VCM.

### Structural typing of SCC*mec*

We detected that 47 out of 50 MRSA strains isolated contained the Type II SCC*mec*, and the remaining 3 strains contained the Type IV SCC*mec*. Thirty-seven strains contained pUB110 plasmid. Fig 1 shows the representative band patterns of the multiplex PCR products obtained for the SCC*mec* typing. All strains displayed the 162-bp internal fragment of the *mecA* gene. Type II strains displayed

Table 2  
Primers used in PCR analysis of VISA-related genes.

gene	Acc. No.	Forward	Reverse
<i>vraR</i>	AB035448	5'-CGTCATTCAAACGGTACAAAAG-3'	5'-CTTAAAAAAGACTAAACACCCAAC-3'
<i>vraG</i>	AB035453	5'-TATTAAGGAAGGCTCACAAGTC-3'	5'-ATGTTTCAAATACCGCCCT-3'
<i>vraA</i>	AB035450	5'-ATGAAAATGCAATAGCAGCC-3'	5'-AACATATCCTGTTGACGTCCC-3'
<i>vraF</i>	AB035453	5'-CCTCTGGATCTGGGAAAAC-3'	5'-CGTCAGCAAATATAATAGAAGGTAA-3'
<i>fruA</i>	AB035449	5'-CTTAATGAACGGTGTCTTAACAT-3'	5'-TACCACCAATAAATCCTGAACC-3'
<i>fruB</i>	AB035449	5'-AGATGTTGAGTCAACTGCCTT-3'	5'-CTTCCAGCAACAATAACTATATCTTC-3'

the 284-bp fragment (locus B), the 209-bp fragment (locus C), and the 342-bp fragment (locus D) with or without the 381 fragment (locus G) of pUB110 plasmid; and type IV strains displayed the 342-bp fragment (locus D) with or without the 381 fragment (locus G).

#### Presence of VISA-related genes

Of the six genes studied (*vraR*, *vraG*, *vraA*, *vraF*, *fruA*, and *fruB*), 69% of samples contained *vraA*, 10% contained *vraF*, 53% contained *vraG*, and 16% contained *vraR*. None of them contained *fruA* or *fruB*. Mu50 used as positive control contained all six genes. MRSA strains without pUB110 contained *vraR* and *vraF* genes at a higher frequency compared with the strains with pUB110 (Fig 2).

#### DISCUSSION

In the present study, we detected that 94% (47/50) of MRSA strains isolated from Kobe University Hospital in 2003 contained Type II *SCCmec*, and 6% contained Type IV *SCCmec*. When Imai and his colleagues (2003) examined the *SCCmec* type of 138 MRSA strains isolated in Japan in 1999 they found that 126 (91.3%) of these contained type II *SCCmec*, 6 strains (4.3%) contained type I *SCCmec*, and 5 strains (3.6%) contained type IV *SCCmec*. These results combined with the present findings indicate that type II *SCCmec* is the most common type of *SCCmec* in Japan, and that type III *SCCmec* has yet to be detected. However, type III *SCCmec* has been reported in European countries, Australia, New Zealand,

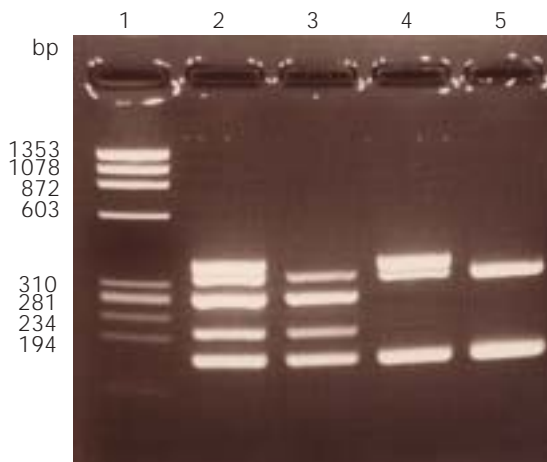


Fig 1—Representative band patterns of the multiplex PCR products for the *SCCmec* typing. Lane 1 is DNA M.W. standard marker (FX174-Hae III digest); lane 2, ribotype II with pUB110; lane 3, ribotype II without pUB110; lane 4, ribotype IV with pUB110; lane 5, ribotype IV without pUB110.

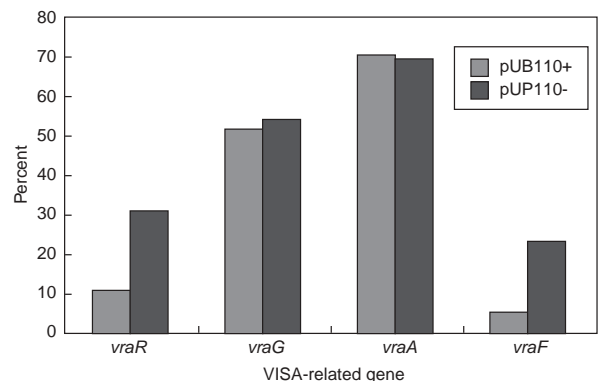


Fig 2—Comparison of the frequency of pUB110 among MRSA strains possessing VISA-related genes.

GENETIC ANALYSIS OF MRSA

Table 3  
MRSA strains isolated from Kobe University Hospital in 2003.

Sample No.	Origin	Rib Typing	pUB 110	Antibiotic drugs											VISA related gene
				ABPC	CEZ	FMOX	IPM	GM	EM	CLDM	MINO	LVFX	ABK	VCM	vraR
1	sputum	II	+	R	R	R	R	R	R	R	S	I	S	S	
2	sputum	II	-	R	R	R	R	R	R	R	I	R	S	S	+
3	drain tube	II	+	R	R	R	R	S	R	R	I	R	S	S	
4	drain tube	II	+	R	R	R	R	R	R	R	I	R	S	S	
5	blood	II	+	R	R	R	R	S	R	R	S	I	S	S	+
6	sputum	II	-	R	R	R	R	R	R	R	I	R	S	S	
7	pus	II	-	R	R	R	R	R	R	R	I	R	S	S	+
8	pus	II	+	R	R	R	R	R	R	S	S	S	S	S	
9	wound	IV	+	R	R	R	R	S	R	R	I	R	S	S	
10	nasal cavity	II	-	R	R	R	R	R	R	R	I	R	S	S	
11	sputum	II	-	R	R	R	R	R	R	R	I	R	S	S	
12	pus	II	+	R	R	R	R	S	R	R	S	R	S	S	
13	nasal cavity	II	-	R	R	R	R	R	R	R	S	R	S	S	+
14	pus	II	+	R	R	R	R	S	R	R	I	R	S	S	
15	urine	II	+	R	R	R	R	S	R	R	I	R	S	S	
16	sputum	II	+	R	R	R	R	S	R	R	I	R	S	S	
17	IPN tube	II	+	R	R	R	R	R	R	R	I	R	R	S	
18	wound	II	+	R	R	R	R	S	R	R	I	I	S	S	
19	other	II	+	S	R	R	R	R	R	R	I	R	S	S	
20	sputum	II	+	R	R	R	R	S	R	R	S	R	S	S	
21	broncho	II	+	R	R	R	R	S	R	S	S	R	S	S	
22	conjunctiva	II	-	R	R	R	S	S	S	S	S	S	S	S	
23	wound	II	-	R	R	R	R	R	R	R	I	R	S	S	
24	spinal	II	+	R	R	R	R	S	R	R	I	R	S	S	
25	broncho	II	+	R	R	R	R	R	R	R	I	R	S	S	
26	nasal cavity	II	+	R	R	R	R	S	R	R	I	R	S	S	
27	TPN tube	II	+	R	R	R	R	S	R	R	S	R	S	S	
28	urine	II	-	R	R	R	R	R	R	R	I	R	S	S	
29	sputum	II	+	R	R	R	R	R	R	R	I	R	S	S	
30	sputum	II	+	R	R	R	R	R	R	R	I	R	S	S	+
31	blood	II	+	R	R	R	R	R	R	R	I	R	S	S	
32	blood	II	+	R	R	R	R	R	I	I	S	S	S	S	
33	urine	II	+	R	R	R	R	S	R	R	I	I	S	S	
34	drain tube	II	+	R	R	R	R	S	R	R	I	R	S	S	+
35	sputum	II	+	R	R	R	R	S	R	R	S	I	S	S	
36	other	II	+	R	R	R	R	S	R	R	S	R	S	S	
37	nasal cavity	II	+	R	R	R	R	R	R	R	I	R	S	S	
38	nasal cavity	II	+	R	R	R	R	S	R	R	S	R	S	S	
39	wound	II	+	R	R	R	R	S	R	R	I	R	S	S	
40	blood	IV	-	R	R	R	R	R	R	R	S	I	S	S	
41	sputum	II	+	R	R	R	R	R	R	S	S	S	S	S	
42	sputum	II	+	R	R	R	R	R	R	R	I	S	S	S	
43	pus	IV	-	R	R	R	R	R	R	R	I	R	S	S	
44	anal fistula	II	+	R	S	R	R	S	R	R	I	R	S	S	
45	sputum	II	+	R	R	R	R	S	R	R	S	R	S	S	
46	stool	II	+	R	R	R	R	R	R	R	I	R	S	S	
47	sputum	II	+	R	R	R	R	S	R	R	I	R	S	S	
48	sputum	II	+	R	R	R	R	S	R	R	I	I	S	S	+
49	wound	II	-	R	R	R	R	S	R	R	S	R	S	S	
50	sputum	II	-	R	R	R	R	R	R	R	I	R	S	S	+

ABPC (ampicillin), CEZ (cefazolin), FMOX (flomoxef), IMP (imipenem), GM (gentamicin), EM (erythromycin), CLDM (clindamycin), MINO (minocyclin), ABK (arbakacin), VCM (vancomycin).  
R: Resistant, I: intermediated-resistant, S: sensitive. These breakpoints were based on CDC guidelines.

Thailand, Vietnam, Singapore, the Philippines, and elsewhere (Yamaguchi, 1997; Katayama *et al*, 2001).

In addition to classification of four structural types of *SCCmec*, we also examined the existence of pUB110 plasmid in *SCCmec* and found that 13 strains (26%) did not contain the pUB110 plasmid. While MRSA is known to cause nosocomial infectious diseases, MRSA infections have recently and increasingly been reported among groups of patients with no apparent connection to hospitals (Matsuhashi *et al*, 1986). Those strains, designated community-acquired MRSA strains, have been reported in various countries such as Australia (Maguire *et al*, 1998; Nimmo *et al*, 2000), New Zealand (Rings and Lang, 1998), the United Kingdom (Sacey *et al*, 1998), Canada (Embil *et al*, 1994), and the United States (Yamaguchi, 1997). Some investigators also reported that *SCCmec* of those community-acquired MRSA strains did not contain the pUB110 plasmid (Imai *et al*, 2003).

Of the six VISA-related genes (*vraR*, *vraG*, *vraA*, *vraF*, *fruA*, and *fruB*), none of the strains contained *fruA* or *fruB*. *VraA* gene is presumed to be a long chain fatty acid CoA ligase, and *vraF* and *vraG* are ABC transporter genes. These genes are up-regulated in VISA (Mu50) and may contribute to VCM resistance (Hanaki *et al*, 1998). Furthermore, VCM resistance is considered to be caused by enhanced cell-wall synthesis, and a role of its regulatory system has been postulated (Kuroda *et al*, 2003). The novel response regulatory system designated *vraSR* has been reported, and *vraR* of the two-component system seems to play a prior role in VCM-resistance, as introduction of *vraR* gene into a VCM-susceptible cell raised the level of resistance to VCM (Kuroda *et al*, 2003). Notably, we found that 16% of the clinical isolates of VCM-sensitive MRSA also contained *vraR* gene. Findings that VISA-related genes were found in VCM-sensitive MRSA clinical strains at a marked rate indicate the possible risk of a transition from VCM-

sensitive MRSA to VISA.

Noteworthy, we found that the 13 strains not containing the pUB110 plasmid, which has been reported to be a prospective community-acquired MRSA strain, possessed the *vraR* and *vraF* genes at a relatively higher frequency compared with the strains with pUB110. Further investigations of the relationship between the *SCCmec* typing, as an identification of the bacterial genetic background, and the presence of VISA-related genes are warranted to alert to the possibility of a transition from the VCM-sensitive MRSA to VISA in the MRSA clinical strains. In the present investigation, we applied the multiplex PCR assay to determine the *SCCmec* type, and the simple PCR assay to detect the presence of VISA-related genes. Both assays can be easily and rapidly performed in many hospitals and laboratories, and thus these assays should be considered as a useful tool for the investigations of MRSA clinical strains.

In summary, we have investigated the *SCCmec* types and the presence of VISA-related genes in clinical strains of MRSA isolated from Kobe University Hospital, Japan in 2003. Most of the strains contained Type II *SCCmec*, and a high frequency of the strains possessed VISA-related genes. In particular, strains considered as a community-acquired MRSA strain, namely those containing, *vraR*, were present at a relatively higher frequency compared with the other strains.

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