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Combination of Multiplex PCRs for Staphylococcal Cassette Chromosome *mec* Type Assignment: Rapid Identification System for *mec*, *ccr*, and Major Differences in Junkyard Regions[∇]

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Staphylococcal cassette chromosome *mec* (SCC*mec*) typing, in combination with genotyping of the *Staphylococcus aureus* chromosome, has become essential for defining methicillin-resistant *S. aureus* (MRSA) clones in epidemiological studies. We have developed a convenient system for SCC*mec* type assignment. The system consists of six multiplex PCRs (M-PCRs) for identifying the *ccr* gene complex (*ccr*), the *mec* gene complex (*mec*), and specific structures in the junkyard (J) regions: M-PCR with primer set 1 (M-PCR 1) identified five types of *ccr* genes; M-PCR 2 identified class A to class C *mec*; M-PCRs 3 and 4 identified specific open reading frames in the J1 regions of type I and IV and of type II, III, and V SCC*mec* elements, respectively; M-PCR 5 identified the transposons Tn*554* and ΨTn*554* integrated into the J2 regions of type II and III SCC*mec* elements; and M-PCR 6 identified plasmids pT181 and pUB110 integrated into J3 regions. The system was validated with 99 MRSA strains carrying SCC*mec* elements of different types. The SCC*mec* types of 93 out of the 99 MRSA strains could be assigned. The SCC*mec* type assignments were identical to those made with a PCR system that uses numerous primer pairs to identify genes or gene alleles. Our system of six M-PCRs is thus a convenient and reliable method for typing SCC*mec* elements.

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains have become prevalent in health care facilities and in the community worldwide (3, 4). MRSA strains produce penicillin binding protein 2′ or 2a, which is poorly acylated by β-lactam antibiotics (5, 22, 25). The *mecA* gene, encoding PBP2a, is carried on a peculiar type of mobile genetic element inserted into the staphylococcal chromosome, designated staphylococcal cassette chromosome *mec* (SCC*mec*) elements (12, 14, 24).

SCC*mec* elements typically share four characteristics: first, they carry the *mec* gene complex (*mec*) consisting of the methicillin resistance determinant *mecA* and its regulatory genes and insertion sequences; second, they carry the *ccr* gene complex (*ccr*) consisting of *ccr* genes that are responsible for the mobility of the element and its surrounding sequences; third, they have characteristic directly repeated nucleotide sequences and inverted complementary sequences at both ends; and last, they integrate into the 3′ end of an open reading frame (ORF), *orfX*.

Despite these similarities, the structures of SCC*mec* elements are rather divergent. Allotypic differences that are used for SCC*mec* type definitions have been identified in both *ccr* and *mec*. Five types of *ccr* and four classes of *mec* have been reported. *ccr* types 1 to 4 carry the *ccrA* and *ccrB* genes, which share approximately 80% identity with each other, and the type 5 *ccr* carries the *ccrC* gene (10, 11, 17, 19). Four classes of the *mec* gene complexes have been identified among methicillin-resistant staphylococcal strains of various species: class A *mec*, consisting of IS*431mec*-*mecA*-*mecR1*-*mecI*; class B *mec*, consisting of IS*431mec*-*mecA*-Δ*mecR1*-IS*1272*; class C *mec*, consisting of IS*431mec*-*mecA*-Δ*mecR1*-IS*431*; and class D *mec*, consisting of IS*431mec*-*mecA*-Δ*mecR1* with no insertion sequences downstream of Δ*mecR1* identified by PCR as of yet (13). In *S. aureus* strains, *mec* classes A, B, and C have been identified. Insertion sequences have sometimes been found to be integrated in or around the class A *mec*. A class A *mec* carrying IS*431* downstream of *mecI* was found in *Staphylococcus haemolyticus* (13). Recently, Shore et al. identified MRSA strains carrying class A *mec* with an insertion of IS*1182* in and around the *mecI* gene and designated them classes A3 and A4 (23).

The SCC*mec* element type has been defined by the combination of *ccr* type and *mec* class. In MRSA strains, six types of SCC*mec* elements, that is, six combinations of *ccr* and *mec*, have been reported (Table 1). These six SCC*mec* elements have been further classified by differences in regions other than *ccr* and *mec*, which are designated junkyard (J) regions. The J regions comprise three parts: J1 (the region between *ccr* and the right-flanking chromosomal region), J2 (the region between *mec* and *ccr*), and J3 (the region between *orfX* and *mec*). The J regions are not always specific to each SCC*mec* type, but certain J regions are commonly shared among certain types of SCC*mec* elements. Of the three regions, we regard J1 as being the most fundamental, because we presume that it reflects the original

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TABLE 1. List of SCC*mec* elements and strains used for standard strains

Reported SCC*mec* type	Name of SCC*mec* type used in this study[a]	Combination of *ccr* and *mec*	Specific features used for discrimination of J regions			GenBank accession no. or reference	Strain used as standard
			J1 region	J2 region	J3 region		
I	I.1.1.1	1A	E007 in J1 of type I SCC*mec*	NT[b]	pUB110 (−)	AB033763	NCTC10442
IA	(I.1.1.2)	1A	Locus A in J1 of type I SCC*mec*	NT	pUB110 (+)	19	
IIa	II.1.1.1	2A	*kdp* operon (*kdpB*) in J1 region of type II.1 SCC*mec*	Tn*554* and its flanking region	pUB110 (+)	D86934	N315
II variant	(II.1.n.2)	2A	Locus B in J1 region of type II.1 SCC*mec*	ND[c]	pUB110 (−)	1	
IIb	II.2.1.2	2A	S01 in J1 region of type II.2 SCC*mec*	Tn*554* and its flanking region	pUB110 (−)	AB127982	JCSC3063
IIA	II.3.1.1	2A	Same as that of type IV.2 SCC*mec* of 8/6-3P	Tn*554* and its flanking region	pUB110 (+)	23	BK351[d]
IIB	(II.3)	2A	Same as that of type IV.2 SCC*mec* of 8/6-3P	Tn*554* negative	pUB110 (+)	AJ810123	
IIC	(II.3)	2A	Same as that of type IV.2 SCC*mec* of 8/6-3P	Tn*554* positive, short J2 region	pUB110 (+)	23	
IID	(II.3)	2A	Same as that of type IV.2 SCC*mec* of 8/6-3P	Tn*554* and its flanking region	pUB110 (−)	23	
IIE	(II.3)	2A	Same as that of type IV.2 SCC*mec* of 8/6-3P	Tn*554* positive, short J2 region	pUB110 (−)	AJ810120	
	II.4.1.1	2A	RN06 in J1 region of type II.4 SCC*mec*	Tn*554* and its flanking region	pUB110 (+)	AB261975	RN7170
III	III.1.1.1	3A	Z004 in J1 region of type III.1 SCC*mec*	ΨTn*554* and its flanking region	pT181 (+)	AB037671	85/2082
IIIA	(III.1.1.2)	3A		ΨTn*554* and its flanking region	pT181 (−)	AF422651–AF422696	
IVa	IV.1.1.1	2B	CQ002 in J1 region of type IV.1 SCC*mec*	NT	pUB110 (−)	AB063172	CA05
IVb	IV.2.1.1	2B	M001 in J1 region of type IV.2 SCC*mec*	NT	pUB110 (−)	AB063173	8/6-3P
IVc	IV.3.1.1	2B	CR008 in J1 region of type IV.3 SCC*mec*	NT	pUB110 (−)	AB096217	81/108
IVE	(IV.3.1.4)	2B	Same as that of 81/108	NT	pUB110 (−)	AJ810121	
IVF	(IV.2.1.4)	2B	Same as that of 8/6-3P	NT	pUB110 (−)	23	
IVd	IV.4.1.1	2B	CD002 in J1 region of type IV.4 SCC*mec*	NT	pUB110 (−)	AB097677	JCSC4469
IVA	(IV.n.n.2)	2B	ND	ND	pUB110 (+)	1	
IVg	(IV.5)	2B	J1 region specific to IVg	NT	pUB110 (−)	DQ106887	
V	V.1	5C	V024 in J1 region of type V SCC*mec*	NT	NT	AB121219	WIS
IV	V1	4B	ND	NT	NT	AF411935	HDE288

[a] Types of SCC*mec* elements that are not used in this study are given in parentheses.
[b] NT, not tested in this study.
[c] ND, not described in the paper.
[d] BK351, isolated in Ireland, was provided by B. N. Kreiswirth.

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form of SCC into which a *mec* gene complex integrated. Moreover, several different J1 regions have been identified in type II and type IV SCC*mec* elements (7, 15, 17, 20, 23). The presence or absence of integrated plasmids encoding drug resistance genes in the J3 regions of SCC*mec* elements can also be used as markers to classify SCC*mec* elements further in epidemiological studies (1, 19).

In this study, we describe a convenient and reliable method for SCC*mec* typing based on a set of multiplex PCRs (M-PCRs). In this system, M-PCRs 1 and 2 are used for SCC*mec* type assignment; M-PCR 3 or M-PCR 4 is used for J1 region difference-based subtyping, and M-PCRs 5 and 6 are used for the identification of integrated copies of transposons (Tn*554* or ΨTn*554*) and plasmids (pUB110 or pT181).

MATERIALS AND METHODS

Bacterial strains. Twelve MRSA strains were used as standard strains for SCC*mec* typing (Table 1). In addition, 99 MRSA strains were used to validate the multiplex PCR, including 78 strains isolated in the United States, Canada, Ireland, England, and Egypt from 1960 through 1993 (including 8 strains with no record of their origin) provided by B. N. Kreiswirth (note that these strains were included in the study by Pfaller et al. [21]); 10 strains isolated in France in 1996, provided by J. Etienne; and 11 strains isolated in the United States and Australia, as reported previously by Okuma et al. (18).

Nomenclature of SCC*mec* elements. In this paper, we use our proposed nomenclature for SCC*mec* elements (2). As shown in Table 1, the SCC*mec* element type (defined by the combination of *ccr* and *mec* allotypes) is indicated with roman numerals, while the SCC*mec* element subtype (defined by differences in the junkyard regions) is indicated with Arabic numbers separated by a period, where each number indicates the structure of the J regions (J1, J2, and J3, respectively) according to the chronological priority of the description. It should be noted that the description of the J regions has been modified from our original proposal according to the suggestion of Kunyan Zhang (Calgary University, Canada).

M-PCRs. Chromosomal DNA was extracted from MRSA strains by using the small-scale phenol extraction method and was used as a template (8).

The primer pairs used for PCR experiments are listed in Table 2. M-PCR 1 for *ccr* type assignment contained two primers to identify *mecA* and eight primers used for the identification of five *ccr* genes: four primers including a common forward primer (common to *ccrB1-3*) and three reverse primers specific for *ccrA1*, *ccrA2*, and *ccrA3* for identifying *ccr1-3* based on the differences in *ccrA* genes; two primers for identifying *ccr4*; and two primers for identifying *ccr5*. M-PCR 2 for *mec* class assignment contained four primers to identify the gene lineages of *mecA-mecI* (class A *mec*), *mecA*-IS*1272* (class B *mec*), and *mecA*-IS*431*(class C *mec*). M-PCR 3 contained five primer pairs: one pair for identifying specific ORF in the J1 region of type I SCC*mec* elements and four pairs for identifying specific ORFs in the J1 regions of four subtypes of type IV SCC*mec*

TABLE 2. Primers used in this experiment

Primer for PCR	Nucleotide sequence (5′→3′)	Constructed on:	Location of primer: Start position(s) in reference	Location of primer: Stop position(s) in reference	Location of primer: Reference SCC*mec* or SCC sequence(s)[a]	Gene(s) or gene allele(s) detected (primer pair)	Expected size of product (bp)
M-PCR 1 (for amplification of *ccr* gene complex type with *mecA*)							
mA1	TGCTATCCACCCTCAAACAGG	*mecA*	45813	45833	Type II.1	*mecA* (mA1-mA2)	286
mA2	AACGTTGTAACCACCCCAAGA	*mecA*	46098	46078	Type II.1		
α1	AACCTATATCATCAATCAGTACGT	*ccrA1*	24845	24868	Type I.1	*ccrA1-ccrB* (α1-βc)	695
α2	TAAAGGCATCAATGCACAAACACT	*ccrA2*	26325	26348	Type II.1	*ccrA2-ccrB* (α2-βc)	937
α3	AGCTCAAAAGCAAGCAATAGAAT	*ccrA3*	5486	5508	Type III.1	*ccrA3-ccrB* (α3-βc)	1,791
βc	ATTGCCTTGATAATAGCCITCT	*ccrB1*, *ccrB2*, *ccrB3*	25539, 27261, 7276	25518, 27240, 7255	Type I.1, II.1, III.1		
α4.2	GTATCAATGCACCAGAACTT	*ccrA4*	8745	8764	Type VI	*ccrA4-ccrB4* (α4.2-β4.2)	1,287
β4.2	TTGCGACTCTCTTGGCGTTT	*ccrB4*	10031	10012	Type VI		
γR	CCTTTATAGACTGGATTATTCAAAATAT	*ccrC*	60319, 16838	60346, 16811	SCC*mercury*, type V	*ccrC* (γR-γF)	518
γF	CGTCTATTACAAGATGTTAAGGATAAT	*ccrC*	60836, 16321	60810, 16347	SCC*mercury*, type V		
M-PCR 2 (for amplification of *mec* gene complex class							
mI6	CATAACTTCCCATTCTGCAGATG	*mecI*	42866	42888	Type II.1	*mecA-mecI* (mA7-mI6)	1,963
IS7	ATGCTTAATGATAGCATCCGAATG	IS*1272*	28624	28647	Type I.1	*mecA*-IS*1272* upstream of *mecA* (mA7-IS7)	2,827
IS2(iS-2)	TGAGGTTATTCAGATATTTCGATGT	IS*431*	8772	8748	Type V	*mecA*-IS*431* upstream of *mecA* (mA7-IS2 [iS-2])	804
mA7	ATATACCAAACCCGACAACTACA	*mecA*	44830, 31450, 7969	44808, 31428, 7991	Type I.1, II.1, V		
M-PCR 3 (for amplification of ORFs in J1 region of type I and type IV SCC*mec*)							
1a3	TTTAGGAGGTAATCTCCTTGATG	E007	5278	5300	Type I.1	E007 in type I.1 SCC*mec* (1a3-la4)	154
1a4	TTTTGCGTTTGCATCTCTACC	E007	5431	5411	Type I.1		
4al	TTTGAATGCCCTCCATGAATAAAAT	CQ002	4726	4750	Type IV.1	CQ02 in type IV.1 (IVa) SCC*mec* (4al-4a3)	458
4a3	AGAAAAGATAGAAGTTCGAAAGA	CQ002	5183	5161	Type IV.1		
4b3	AACCAACAGTGGTTACAGCTT	M001	2457	2477	Type IV.2	M001 in type IV.2 (IVb) SCC*mec* (4b3-4b4)	726
4b4	CGGATTTTAGACTCATCACCAT	M001	3182	3161	Type IV.2		
4c4	AGGAAATCGATGTCATTATAA	CR008	8260	8240	Type IV.3	CR008 in type IV.3 (IVc) SCC*mec* (4c4-4c5)	259
4c5	ATCCATTTCTCAGGAGTTAG	CR008	8002	8021	Type IV.3		
4d3	AATTCACCCGTACCTGAGAA	CD002	2390	2409	Type IV.4	CD002 in type IV.4 (IVd) SCC*mec* (4d3-4d4)	1,242
4d4	AGAATGTGGTTATAAGATAGCTA	CD002	3631	3609	Type IV.4		
M-PCR 4 (for amplification of ORFs in J1 region of type II, type III, and type V SCC*mec*)							

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kdpB1	GATTACTTCAGAACCAGGTCAT	*kdpB*	12436	12415	Type II.1	*kdpB* in type II.1 (IIa) SCC*mec* (kdpB1-kdpB2)	287
kdpB2	TAAACTGTGTCACACGATCCAT	*kdpB*	12150	12171	Type II.1		
2b3	GCTCTAAAAGTTGGATATGCG	S01	1497	1517	Type II.2	SA01 in type II.2 (IIb) SCC*mec* (2b3-2b4)	1,518
2b4	TGGATTGAATCGACTAGAATCG	S01	3014	2993	Type II.2		
4b3	AACCAACAGTGGTTACAGCTT	IIE3, M001	2457, 2156	2477, 2176	Type IV.2, II.3 (IIE)	IIE03 in type II.3 (IIE) SCC*mec* or M001 in type IV.2 (IVb) SCC*mec* (4b3-4b4)	726
4b4	CGGATTTTAGACTCATCACCAT	IIE3, M001	3182, 2881	3161, 2860	Type IV.2, type II.3 (IIE)		
II4-3	GTACCGCTGAATATTGATAGTGAT	RN06	11848	11871	Type II.4	RN06 in type II.4 SCC*mec* (II4-3-II4-1)	2,003
II4-1	ACTCTAATCCTAATCACCGAAC	RN06	13850	13829	Type II.4		
3a1	ATGGCTTCAGCATCAATGAG	Z004	3333	3352	Type III.1	Z004 in type III.1 SCC*mec* (3a1-3a2)	503
3a2	ATATCCTTCAAGCGCGTTTC	Z004	3835	3816	Type III.1		
5a1	ACCTACAGCCATTGCATTATG	V024	26564	26584	Type V	V024 in type V SCC*mec* (5a1-a2)	1,159
5a2	TGTATACATTTCGCCACTAGCT	V024	27722	27701	Type V		
M-PCR 5 (for amplification of gene alleles located in J2 region of SCC*mec* elements)							
ermA1	TGAAACAATTTGTAACTATTGA	*ermA*	35078	35099	Type II.1	*ermA*-CN030 or CZ021 in J2 region of type II.1 (IIa) or type III.1 SCC*mec* (ermA1-mN5)	2,756
cad4	ATTGCGATTCTTTCCGATATGG	*cadB*	16107	16128	Type III.1	*cadB*-CN030 or CZ021 in J2 region of type II.1 (IIa) or type III.1 SCC*mec* (cad4-mN5)	1,540
mN5	TTGCTTCGGGACTTACCTCTAGT	CN030, CZ021	37833, 17646	37811, 17624	Type II.1, type III.1		
M-PCR 6 (for amplification of gene alleles located in J3 region of SCC*mec* elements)							
ant1	CAGACCAATCAACATGGCACC	*ant(4)′*	50764	50744	Type II.1	*mecA*-*ant(4′)* in pUB110 (mA1-ant1)	4,952
pT181-2	AGGTTTATTGTCACTACAATTGA	*tetK*	32869	32847	Type III.1	*mecA*-*tetK* in pT181 (mA1-pT181-2)	7,406
mA1	TGCTATCCACCCTCAAACAGG	*mecA*	45813, 25464	45833, 25484	Type II.1, type III.1		
Primer sets for PCR used for identification of genes or gene alleles							
1a1	ATTCCATATGAAACTAAACGCGT	*pls*	11864	11841	Type I.1	*pls* (CE010) in type I SCC*mec* (1a1-1a2)	1,065
1a2	TAGTGAACCAAATAATGTGCCATT	*pls*	10800	10823	Type I.1		
merA2	TCTTCACAGCCTGTGCATGTCATGCCT	*merA*	39874	39900	SCC*mercury*	*mer* operon (merA2-merG)	1,546
merG	TGATACCGCGAATGAATCAAAGGT	CZ046	41419	41396	SCC*mercury*		

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TABLE 2—*Continued*

Primer for PCR	Nucleotide sequence (5′→3′)	Constructed on:	Location of primer: Start position(s) in reference	Location of primer: Stop position(s) in reference	Location of primer: Reference SCC*mec* or SCC sequence(s)[a]	Gene(s) or gene allele(s) detected (primer pair)	Expected size of product (bp)
mN21	TCATCTTTAACTACGATGGTGT	CZ055	47848	47869	SCC*mercury*	J region in SCC*mercury* (mN21-mN22)	577
mN22	ACTACAGCCATCTTCAGATAGA	CZ056	48424	48403	SCC*mercury*		
Primer sets for amplification of type II.4 SCC*mec* carried by RN7170							
cR1	AAGAATTGAACCAACGCATGA	*orfX*	57847	57827	Type II.1	*orfX-mecA* (cR1-mA3)	11,756[b]
mA3	AACGTTACAAGATATGAAGTGGTAAATGGTA	*mecA*	46093	46123	Type II.1		
mA2	AACGTTGTAACCACCCCAAG	*mecA*	46098	46078	Type II.1	*mecA-ermA* (mA2-ermA1)	11,020[b]
ermA1	TGAAACAATTTGTAACTATTGA	*ermA*	35078	35099	Type II.1		
ermA3	TGGGTAAACCGTGAATATCGTGT	*ermA*	35214	35192	Type II.1	*ermA-ccr* gene complex (ermA3-2AJ1)	9,937[b]
2AJ1	ATTAGCCGATTTGGTAATTGAA	Noncoding region between RN01 and RN02	4270	4249	Type II.4		
βc	ATTGCCTTGATAATAGCCITCT	*ccrB*	2287	2308	Type II.4	*ccr* gene complex chromosomal region flanking SCC*mec* (βc-cL4)	15,000[c]
cL4	CAGTCGCATCAAATGTCTCTAATG	Chromosomal region flanking to SCC*mec*	3218	3241	Type II.1		

[a] Accession numbers deposited in DDBJ/EMBL/GenBank database used as reference sequences for SCC*mec* elements and SCC*mercury* are as follows: type I.1 SCC*mec*, AB033763; type II.1 SCC*mec*, D86934; type II.2 SCC*mec*, AB127982; type II.3 (type IIE) SCC*mec*, AJ810120; type II.4 SCC*mec*, AB261975; type III.1 SCC*mec* and SCC*mercury*, AB037671; type IV.1 SCC*mec*, AB063172; type IV.2 SCC*mec*, AB063173; type IV.3 SCC*mec*, AB096217; type IV.4 SCC*mec*, AB097677; type V SCC*mec*, AB121219; type VI SCC*mec*, AF411935.

[b] The sizes of DNA fragments estimated from the nucleotide sequence of the type II.1 SCC*mec* element are given. The sizes of DNA fragments amplified from chromosomal DNA of RN7170 were judged to be the same as those from chromosomal DNA of N315 by agarose gel electrophoresis.

[c] The combination of the primer pair βc and cL4 should amplify the DNA fragment of 24 kb from chromosomal DNA of N315.

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elements. M-PCR 4 contained six primer pairs: four pairs for identifying specific ORFs in J1 regions of four subtypes of type II SCC*mec* elements, one pair for identifying specific ORFs in the J1 region of type III SCC*mec* elements, and one pair for identifying specific ORFs in the J1 region of type V SCC*mec* elements. M-PCR 5 contained three primers: one primer specific to the J2 regions of type II and type III SCC*mec* elements and two primers specific to *ermA* and *cadB*, respectively, to identify the J2 regions of type II or type III SCC*mec* elements. M-PCR 6 contained three primers: one primer specific for *mecA* and two primers specific for *ant(4′)* in plasmid pUB110 and *tetK* in plasmid pT181, respectively, to identify integrated plasmids in the J3 regions.

For M-PCR 1, reaction mixtures contained 10 ng chromosomal DNA, oligonucleotide primers (0.1 μM), 200 μM each deoxynucleotide triphosphates, Ex *Taq* buffer, and 2.5 U Ex *Taq* polymerase (Takara Bio Inc., Kyoto, Japan) in a final volume of 50 μl. The concentration of $MgCl_2$ was 3.2 mM. A Takara PCR thermal cycler was used for amplification with an initial denaturation step (94°C, 2 min); 30 cycles of denaturation (94°C, 2 min), annealing (57°C, 1 min), and extension (72°C, 2 min); and a final elongation step at 72°C for 2 min. For M-PCRs 2 to 5, the reaction mixtures were the same as those for M-PCR 1 except that the concentration of $MgCl_2$ was 2 mM and the annealing temperature was raised to 60°C for 1 min to avoid the generation of nonspecific DNA fragments. For M-PCR 6, we performed long-range PCR using the Expand High Fidelity PCR system according to the manufacturer's recommendations (Roche Diagnostics Co., Indianapolis, IN). Briefly, reactions were performed using a final reaction mixture volume of 50 μl, which contained 10 ng template DNA, oligonucleotide primers (0.3 μM), 200 μl each deoxynucleotide triphosphate, 1× Expand High Fidelity buffer, 1.5 mM $MgCl_2$, and 2.6 U Expand High Fidelity PCR system enzyme mix. The PCR consisted of a denaturation step (94°C, 2 min); 10 cycles of denaturation (94°C, 15 s), annealing (50°C, 30 s), and extension (68°C, 8 min); 20 cycles of denaturation (94°C, 15 s), annealing (50°C, 30 s), and extension (68°C, 12 min); and a final elongation step (72°C, 7 min).

PCRs for the identification of SCC*mercury* and customary (nonmultiplexed) PCRs to identify *pls* were carried out with the primer pairs listed in Table 2 according to a previously described procedure (10). PCR products were visualized by agarose gel electrophoresis.

PCR-based identification and determination of part of the nucleotide sequence of the type II.4 SCC*mec* element. DNA fragments encompassing the entire SCC*mec* element of strain RN7170 were amplified by long-range PCR using the Expand High Fidelity PCR system under the same conditions as those used for M-PCR 6. The primer sets used for amplifying the DNA fragments are given in Table 2. The amplicon sizes estimated by agarose gel electrophoresis are as follows: the region from *orfX* to *mecA*, amplified with primers cR1 and mA3, was 11 kb; the region from *mecA* to *ermA* in Tn*554*, amplified with primers mA2 and ermA1, was 11 kb; the region from *ermA* in Tn*554* to *ccr*, amplified with primers ermA3 and 2AJ1, was 12 kb; and the region from *ccr* to the right-flanking chromosomal region, amplified with primers cβ and cL4, was 15 kb. The locations of the primers are indicated in Fig. 1. PCR products were purified with the QIAquick PCR purification kit (QIAGEN, Hilden, Germany), and nucleotide sequences of the DNA fragments from *ccr* to the right-flanking chromosomal region were determined by primer walking.

Nucleotide sequence accession number. The nucleotide sequence of the region containing J1 and the *ccr* gene complex of the type II.4 SCC*mec* of strain RN7170 has been deposited in the DDBJ/EMBL/GenBank database under accession no. AB261975.

RESULTS AND DISCUSSION

Development of two M-PCRs for *ccr* and *mec* components of SCC*mec* elements. M-PCR 1 was developed to identify *ccrC* and *ccr4* in addition to three *ccr* types that could be identified with previous systems (10). M-PCR 1 successfully amplified DNA fragments corresponding in size to each *ccr* gene as follows: type 1 *ccr*, 695 bp; type 2 *ccr*, 937 bp; type 3 *ccr*, 1,791 bp; type 4 *ccr*, 1,287 bp; and type 5 *ccr*, 518 bp (Table 2 and Fig. 2A). The 286-bp amplification product appearing in each lane represents *mecA*, the most essential gene in SCC*mec*, which is used as an internal amplification control. Two DNA fragments of 518 bp and 1,791 bp were amplified with chromosomal DNA of strain 85/2082, indicating that it carried both type 3 *ccr* and *ccrC*, consistent with the fact that this strain carries two SCC elements, a type III SCC*mec* (type 3 *ccr*) and an SCC*mercury* (*ccrC*) (Table 1).

M-PCR 2, developed for assigning the *mec* class, contained primer pairs for identifying *mecA*-*mecI* (class A), *mecA*-IS*1272* (class B), and *mecA*-IS*431* (class C), and it can replace the three reactions used in traditional methods. The sizes of the amplified DNA fragments matched the sizes expected for each class of *mec*: 1,963 or 1,797 bp for *mecA*-*mecI* (class A), 2,827 bp for *mecA*-IS*1272* (class B), and 804 bp for *mecA*-IS*431* (class C) (Table 2 and Fig. 2B). The size of the amplified DNA fragment obtained with strain 85/2082 was shorter than that obtained with strain N315, in agreement with the fact that *mecR1* of 85/2082 has a 166-bp deletion relative to that in N315 (10). This case seems to be exceptional, because we successfully amplified DNA fragments of sizes similar to those of N315 in 49 of 49 class A *mec* strains tested.

Development of two M-PCRs for J1 regions of SCC*mec* elements. M-PCRs 3 and 4 were developed to identify specific ORFs in the J1 region of each SCC*mec* element: M-PCR 3 for type I and type IV SCC*mec* elements carrying class B *mec* and M-PCR 4 for type II and type III SCC*mec* elements carrying class A *mec* and type V SCC*mec* elements carrying class C *mec*.

With M-PCR 3, the J1 regions of all five SCC*mec* type I or IV elements (type I.1, type IV.1, type IV.2, type IV.3, and type IV.4) (Table 1) were identified with primer pairs specific for each subtype. The sizes of the amplified DNA fragments matched those predicted for the J1 regions of the five SCC*mec* elements (type I.1, 154 bp; type IV.1, 458 bp; type IV.2, 726 bp; type IV.3, 259 bp; type IV.4, 1,242 bp) (Fig. 2C and Table 2).

In this study, we identified a new subtype of type II SCC*mec* elements carried by RN7170. By amplifying entire SCC*mec* region and determining the nucleotide sequence of the J1 region of the element, we designated it type II.4 SCC*mec*. Interestingly, the J1 region carried by type IIB, IIC, IID, and IIE SCC*mec* elements was the same as that of the type IV.2 SCC*mec* element (23). Therefore, we considered these SCC*mec* elements to be type II.3, type II elements carrying the third identified J1 region (although it was identified previously in the type IV.2 SCC*mec*). With M-PCR 4, the J1 regions of six SCC*mec* types, type II, III, or V (type II.1, type II.2, type II.3, type II.4, type III.1, and type V.1) (Table 1), were identified with primer pairs specific for each type. The sizes of the amplified DNA fragments matched those predicted for the J1 regions of all six SCC*mec* types (type II.1, 287 bp; type II.2, 1,518 bp; type II.3, 726 bp; type II.4, 2,003 bp; type III.1, 503 bp; type V.1, 1,159 bp) (Fig. 2D and Table 2).

Development of two M-PCRs to identify resistance plasmids/transposons integrated into SCC*mec* elements (J2 and J3 regions). M-PCR 5 was developed to identify transposon Tn*554* or ΨTn*554* integrated into type II and type III SCC*mec* elements by targeting ORFs located in the J2 region flanking these elements and resistance determinants carried by the transposons (*ermA* by Tn*554* and *cadB* by ΨTn*554*). As expected, M-PCR 5 amplified DNA fragments of 2,756 bp (Fig. 2E, lanes 1 and 2), corresponding to CN030-*ermA*, and 1,540 bp (lane 3), corresponding to CZ021-*cadB*.

M-PCR 6 was developed to identify differences in the J3 region based on the presence or absence of resistance plasmids pUB110 and pT181 by amplifying the J3 regions *mecA*-*ant(4′)*-1 for pUB110 and *mecA*-*tetK* for pT181. As expected,

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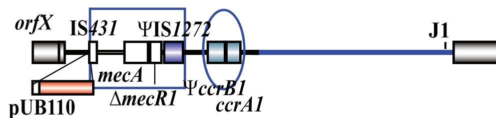
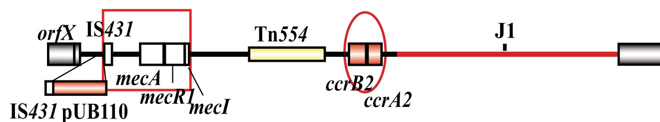
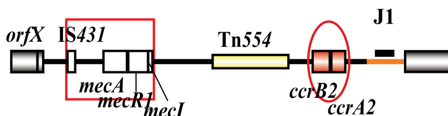
Type I.1



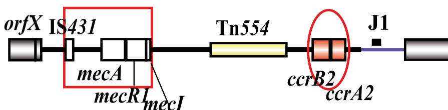
Type II.1



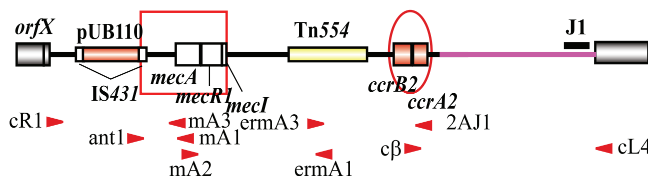
Type II.2

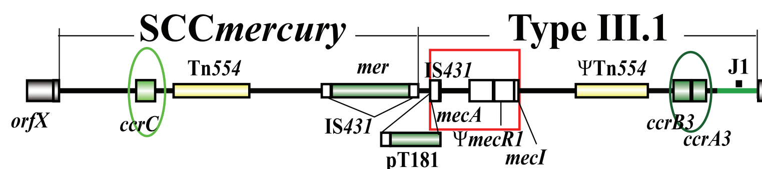


Type II.3

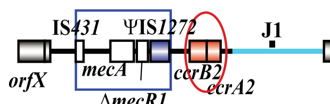


Type II.4

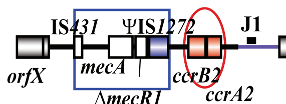




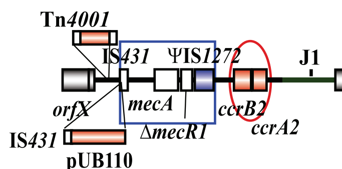
Type IV.1



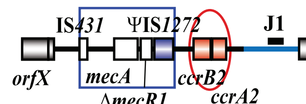
Type IV.2



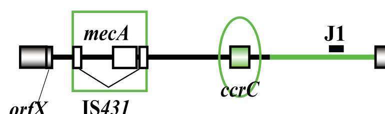
Type IV.3



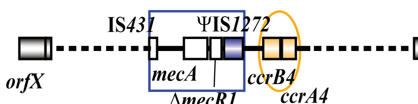
Type IV.4



Type V.1



Type VI



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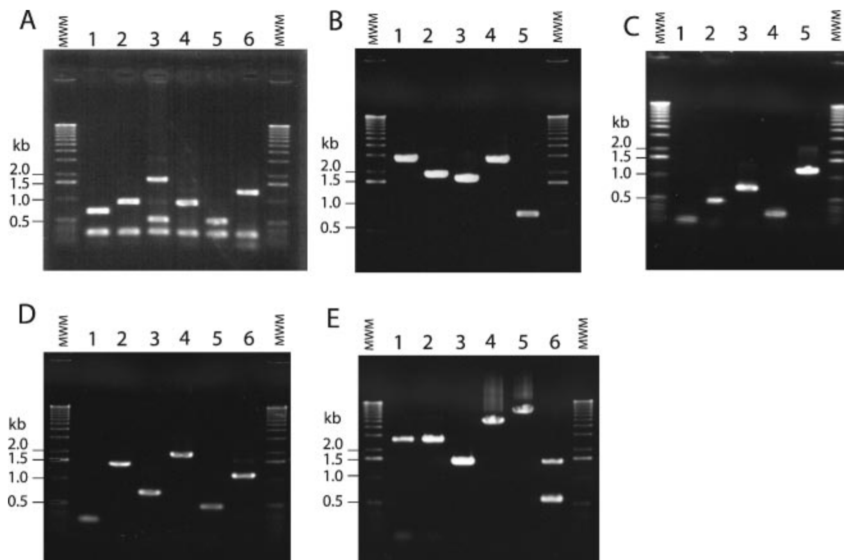

FIG. 2. Six multiplex PCRs for SCC*mec* type assignment. (A) M-PCR 1 for identification of *ccr* genes for the assignment of the type of *ccr* gene complex. Chromosomal DNAs from standard strains were used as templates. Lane 1, NCTC10442; lane 2, N315; lane 3, 85/2082; lane 4, CA05; lane 5, WIS; lane 6, HDE288. DNA fragments of the expected sizes for each *ccr* gene were amplified. The DNA fragment corresponding to *mecA* served as an internal control in each lane. (B) M-PCR 2 for identification of three gene alleles for assignment of the *mec* gene complex. Chromosomal DNAs from standard strains were used as templates. Lane 1, NCTC10442; lane 2, N315; lane 3, 85/2082; lane 4, CA05; lane 5, WIS. DNA fragments of the expected sizes for class A *mec* (lanes 2 and 3), class B *mec* (lanes 1 and 4), and class C *mec* (lane 5) were amplified. (C) M-PCR 3 for J1 region difference-based subtyping of type I and type IV SCC*mec* elements, which carry the class B *mec*. Chromosomal DNAs from standard strains were used as templates. Lane 1, NCTC10442; lane 2, CA05; lane 3, 8/6-3P; lane 4, 81/108; lane 5, JCSC4469. DNA fragments of the expected sizes for each J1 region were amplified. (D) M-PCR 4 for J1 region-based subtyping of type II and type III SCC*mec* elements, which carry the class A *mec*, and the type V SCC*mec*, which carries the class C *mec*. Chromosomal DNAs from standard strains were used as templates. Lane 1, N315; lane 2, JCSC3063; lane 3, BK351; lane 4, RN7170; lane 5, 85/2082; lane 6, WIS. (E) Identification of resistance determinants. Transposons (Tn*554* and ΨTn*554*) were assigned by M-PCR 5 (lanes 1 to 3), and plasmids (pUB110 and pT181) were assigned by M-PCR 6 (lanes 4 and 5). SCC*mercury* was assigned with an M-PCR with the four sets of primers listed in Table 2 (lane 6). Chromosomal DNAs from standard strains were used as templates. Lanes 1 and 4, N315; lane 2, BK645; lanes 3, 5, 6, and 85/2082. MWM, molecular weight marker.

the DNA fragments amplified by M-PCR 6 were 4,952 bp (Fig. 2E, lane 4) for *mecA-ant(4′)*-1 and 7,406 bp (Fig. 2E, lane 5) for *mecA-tetK*. It should be noted that with M-PCR 6, plasmids pUB110 and pT181 could be detected if they were located downstream of *mecA* but could not be detected if they were located distant from *mecA*. In contrast, in the M-PCR developed previously by Oliveira and Lencastre (20), these plasmids were identified with primer sets specific for each plasmid, regardless of their relative position to *mecA*, leaving the possibility that these plasmids are located outside the SCC*mec* element.

Validation of six multiplex PCRs. We evaluated our system of six M-PCRs by examining a total of 99 MRSA strains. The results obtained with the M-PCRs were identical to those obtained by traditional methods, which mostly used a set of two primers to identify each gene or different allotypes (7, 10, 18).

M-PCR 1 amplified a single DNA fragment belonging to one of five *ccr* types from the chromosomal DNAs of 83 of the 99 MRSA strains. In 10 strains, two DNA fragments were amplified, signifying that these strains carry two *ccr*s, while in 6 strains, no DNA fragments were amplified (Table 3). Among the 10 strains that carried two *ccr*s, 8 carried a type 3 *ccr* and *ccrC* and 2 carried a type 1 *ccr* and *ccrC*. Since *ccrC* is present in SCC elements carrying the mercury resistance operon (SCC*mercury*) or the capsule gene cluster (SCC*cap1*) (2, 16), we conducted an M-PCR experiment to identify the mercury resistance operon and J region in SCC*mercury* by using four primers (merA2, merG, mN21, and mN22) listed in Table 2. DNA fragments indicating the carriage of the mercury resistance operon were amplified from the chromosomal DNAs of all 10 strains, and DNA fragments indicating the carriage of the J region of SCC*mercury* were amplified from the chromosomal DNAs of 9 of them, signifying that these 9 strains carried SCC*mercury*. We tentatively regarded the remaining strain as a type 1 *ccr* strain, presuming that *ccrC* might be carried by other unknown mobile genetic elements, since no SCC*mec* element carrying the combination of *ccrC* (type 5 *ccr*) and class A *mec* or class B *mec* has been identified yet.

FIG. 1. Schematic structures of representative SCC*mec* elements based on the nucleotide sequences deposited in the EMBL/GenBank/DDBJ database under the accession numbers listed in Table 1. Circles indicate the *ccr* genes that can be identified by M-PCR 1. The *mec* gene complexes that can be identified by M-PCR 2 are indicated by squares. Black bars indicate the locations of the J1 region-specific primers used for M-PCRs 3 and 4. The locations of primers used for the amplification of the entire type II.4 SCC*mec* region of strain RN7170 are indicated by red arrowheads.

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TABLE 3. SCC*mec* types identified in 99 MRSA strains by PCR

SCC*mec* type	Subtype[a]	PCR result									No. of strains	Origin(s)[b]
		M-PCR 1		M-PCR 2 *mec* type	M-PCR 3, 4 J1 region type	M-PCR 5		M-PCR 6		PCR identifying SCC*mercury*[e]		
		ccr type	*ccrC*			Tn*554*	ΨTn*554*	pUB110	pT181			
I	1.n.1	1	−	B	1	ND[c]	ND	−	−	−	13	D, Eng, I, S, U, UN
	1.n.1		+		1	ND	ND	−	−	+ (1)	2	Eng, E
	1.n.2		−		1	ND	ND	+	−	−	1	US
	N.n.1		−		NT[d]	ND	ND	−	−	−	1	I
II	1.1.1	2	−	A	1	+	−	+	−	−	32	C, I, US, UN
	1.n.2		−		1	−	−	−	−	−	1	US
	1.1.2		−		1	+	−	−	−	−	1	C
	3.1.1		−		3	+	−	+	−	−	1	I
	4.1.1		−		4	+	−	+	−	−	2	C, US
III	1.1.1	3	+	A	1	−	+	−	+	+	8	C, Eng, I, US, UN
	1.1.2		−		1	−	+	−	−	−	3	US, UN
	1.2.1		−		1	+	−	−	−	−	1	US
IV	1.n.1	2	−	B	1	ND	ND	−	−	−	9	A, C, US
	2.n.1		−		2	ND	ND	−	−	−	3	US
	3.n.1		−		3	ND	ND	−	−	−	2	US
	3.n.2		−		3	ND	ND	+	−	−	10	F
	4.n.1		−		4	ND	ND	−	−	−	3	US
NT		−	−	A	−	+	−	+	−	−	2	UN
		+	−	NT	−	−	−	+	−	−	1	US
		−	−	NT	−	−	−	−	−	−	3	US

[a] The subtypes are shown as Arabic numerals separated by a period. The first number indicates the subtype of the J1 region, the second number indicates the subtype of the J2 region, and the third number indicates the subtype of the J3 region. Each number is separated by a period. If the subtype of the J2 region was not examined, it was indicated as n.
[b] Abbreviations: A, Australia; C, Canada; D, Denmark; E, Egypt; F, France; I, Ireland; Eng, England; S, Switzerland; U, Uganda; US, United States; UN, unknown.
[c] ND, not determined.
[d] NT, nontypeable.
[e] Numbers in parentheses indicate the number of positive strains if the number is not the same as the number of strains indicated in the next column.

With M-PCR 2, the *mec* gene complexes of 95 of 99 tested strains were judged to belong to either class A or class B *mec*, and those of 4 strains were unclassifiable (Table 3).

As such, SCC*mec* elements carried by 93 of the 99 tested strains were classified into one of the six known types of SCC*mec* elements (Table 3).

With M-PCRs 3 and 4, these SCC*mec* elements could be further classified based on differences in the J1 region. Overall, we were able to classify the J1 regions of SCC*mec* elements carried by 92 of 93 strains that had an identified SCC*mec* type (Table 3). Interestingly, no DNA fragment from the chromosomal DNAs of the six untypeable strains was amplified by M-PCR 3 and M-PCR 4, suggesting that these strains might carry new SCC*mec* elements.

We used M-PCR 5 to establish the presence or absence of Tn*554* and ΨTn*554* in the J2 regions of type II and type III SCC*mec* elements. Tn*554* was identified in 36 of 37 type II SCC*mec* elements and 1 of 12 type III SCC*mec* elements. ΨTn*554* was identified in 11 of 12 type III SCC*mec* elements (Table 3). One type III SCC*mec* strain carried Tn*554* instead of ΨTn*554* at the J2 region, as previously reported (9).

We used M-PCR 6 to determine the presence or absence of plasmids pUB110 and pT181. Plasmid pUB110 was carried by 1 of 17 type I SCC*mec* elements, 35 of 37 type II SCC*mec* elements, 10 of 27 type IV SCC*mec* elements, and 3 of the 6 untypeable strains. Remarkably, all 10 tested gentamicin-susceptible MRSA strains (isolated in France in 1996) carried a type IV.3 SCC*mec* with an integrated plasmid pUB110. No type III SCC*mec* harbored pUB110 downstream of *mecA*. In contrast, 8 of 12 type III SCC*mec* elements harbored plasmid pT181, which was not identified in type I, type II, and type IV SCC*mec* elements.

Atypical and unclassifiable SCC*mec* elements. We were not able to identify the *ccr* gene of six *mecA*-positive strains with M-PCR 1. Two of them carried class A *mec*, the J2 region of the type II SCC*mec* element, and the integrated plasmid pUB110, indicating that the gene lineage pUB110-IS*431*-*mecA*-*mecR1*-*mecI*-Tn*554*, which is usually located in the type II SCC*mec* element, was carried by these two strains. Further studies to determine the nucleotide sequences of the region between Tn*554* and the right-flanking chromosomal region will clarify whether these strains carry a deleted type II SCC*mec* or a novel region with new *ccr* genes. Three of them carried the *mecR1* gene, whereas the *mecI* gene was not detected by PCR testing for the respective genes (18). One strain carried neither *mecR1* nor *mecI*. The structures of those elements will be the subject of further investigation.

Comparison to previously reported M-PCRs. Our M-PCRs do not conflict with two previously reported M-PCRs based primarily on the identification of junkyard regions (20, 26). The M-PCR described previously by Oliveira and Lencastre (20) has the advantage that it identifies multiple loci simultaneously (e.g., the *mecA* gene, the *mecI* gene, the J1 region of type I and type II SCC*mec* elements, *ccrC*, the *dcs* [down-

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stream constant sequence] region, pT181, and pUB110). The M-PCR described previously by Zhang et al. (26) has the advantage that it identifies the J1 region of eight SCC*mec* elements simultaneously. M-PCRs 3 to 6, which were developed to identify specific ORFs in J regions, are based on the same concept as that of the two previously reported M-PCRs.

We first used the *pls* (plasmin-sensitive protein) gene to identify the J1 region of the type I.1 SCC*mec*, but that approach was changed since only 12 of 17 strains were positive for this gene (6). In addition, the *pls* gene was identified with the chromosomal DNA from a type III SCC*mec* strain, suggesting that it could not be a specific marker for the J1 region of type I SCC*mec* elements. Oliveira and Lencastre and Zhang et al. also designed primers specific to the J1 region of the type I SCC*mec* in regions other than the *pls* gene. The J region of 16 of 17 type I SCC*mec* strains was classified as subtype 1 with both the primers described previously by Oliveira et al. and our primers.

The identification of the J1 region of the type III SCC*mec* element was a bit confusing because we first reported the nucleotide sequence of the type III SCC*mec* element carried by strain 85/2082 as being the longest one, but this turned out to be a composite of SCC*mercury* and a type III SCC*mec* element (as indicated in Fig. 1, the length of the type III SCC*mec* element is different from that originally reported). Both Oliveira and Lencastre and Zhang et al. happened to design primers on the nucleotide sequence of SCC*mercury* for the identification of type III SCC*mec* elements. Therefore, the identification of locus E and locus F in the M-PCR described previously by Oliveira and Lencastre and the identification of the locus used by Zhang et al. indicate the likely presence of SCC*mercury* and are not specific for type III SCC*mec* elements.

The identification of the J1 regions of type IV SCC*mec* elements is similar with our and primers and those described previously by Zhang et al., except for a primer pair used for the identification of a type IV.3 SCC*mec* element designed by Zhang et al. on a locus outside the type IV.3 SCC*mec*, designated IE25923.

Prospects for assignment of SCC*mec* elements. The typing system designed here is not final and should be developed further, since it could not identify every known difference. For example, some differences in the J3 regions of the type IV SCC*mec* element, such as the carriage of Tn*4001* in the type IV SCC*mec* of strain 81/108, the different J3 region structures in type IVE and type IVF SCC*mec* elements reported previously by Shore et al. (23), and the presence of *dcs* in the type III SCC*mec* element reported previously by Chongtrakool et al. (2), were not identified with our system. Although the structure of the J1 region is rather specific to each type and correlates well with the SCC*mec* type, we want to emphasize that the identification of the J region did not correlate exactly with the type of SCC*mec* element. The type II.3 MRSA strain isolated in Ireland is a good example: if only the multiplex PCR identifying the J1 region had been conducted, it would have been classified as type IV.2.

It is not easy to conduct six M-PCRs for every case. We suggest that M-PCRs 1 and 2, for identifying *ccr* and *mec*, should be conducted first to assign types of SCC*mec* elements, and they might be enough in most of the cases for epidemiological purposes. In cases where further typing is required, we suggest to proceed with identification of the J1 region structure with M-PCR 3 or 4 or with M-PCRs developed by Zhang et al., since the structure of J1 region might reflect the structure of an SCC in which the *mec* gene complex was integrated. The remaining two M-PCRs, M-PCR 5 and M-PCR 6, should be conducted, if necessary, for additional typing.

Although it might be difficult to determine all SCC*mec* types carried by staphylococci, the determination of as many unknown SCC*mec* types as possible would be of help for epidemiological studies as well as for inferring the origins of MRSA strains. Further discussion is needed in order to form a consensus among staphylococcal researchers regarding how to define SCC*mec* element types and how to assign SCC*mec* elements to cope with the ever-increasing diversity of SCC*mec* elements.

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