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Novel Multiplex PCR Assay for Characterization and Concomitant Subtyping of Staphylococcal Cassette Chromosome *mec* Types I to V in Methicillin-Resistant *Staphylococcus aureus*

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Staphylococcal cassette chromosome *mec* (SCC*mec*) typing is essential for understanding the molecular epidemiology of methicillin-resistant *Staphylococcus aureus* (MRSA). SCC*mec* elements are currently classified into types I to V based on the nature of the *mec* and *ccr* gene complexes, and are further classified into subtypes according to their junkyard region DNA segments. Previously described traditional SCC*mec* PCR typing schemes require multiple primer sets and PCR experiments, while a previously published multiplex PCR assay is limited in its ability to detect recently discovered types and subtypes such as SCC*mec* type V and subtypes IVa, b, c, and d. We designed new sets of SCC*mec* type- and subtype-unique and specific primers and developed a novel multiplex PCR assay allowing for concomitant detection of the methicillin resistance (*mecA* gene) (also serving as an internal control) to facilitate detection and classification of all currently described SCC*mec* types and subtypes I, II, III, IVa, b, c, d, and V. Our assay demonstrated 100% sensitivity and specificity in accurately characterizing 54 MRSA strains belonging to the various known SCC*mec* types and subtypes, when compared with previously described typing methods. Further application of our assay in 453 randomly selected local clinical isolates confirmed its feasibility and practicality. This novel assay offers a rapid, simple, and feasible method for SCC*mec* typing of MRSA, and may serve as a useful tool for clinicians and epidemiologists in their efforts to prevent and control infections caused by this organism.

Methicillin, the first semisynthetic penicillin to be developed, was introduced in 1959 to overcome the problem of penicillin-resistant *Staphylococcus aureus* due to β-lactamase (penicillinase) production (17). However, methicillin-resistant *S. aureus* (MRSA) strains were identified soon after its introduction (2, 14). MRSA strains have acquired and integrated into their genome a 21- to 67-kb mobile genetic element, termed the staphylococcal cassette chromosome *mec* (SCC*mec*), which harbors the methicillin resistance (*mecA*) gene and other antibiotic resistance determinants (10, 12, 19). Since then, strains of MRSA have spread and become established as major nosocomial pathogens worldwide (1, 5, 8, 26, 32). Recently, these organisms have evolved and emerged as a major cause of community-acquired infections (16, 31). These newly emerging community-acquired MRSA strains possess novel, small, mobile SCC*mec* type IV or V genetic elements which contain the *mecA* gene with or without additional antibiotic resistance genes and are more easily transferred to other strains of *S. aureus* than larger SCC*mec* (types I, II, and III) elements (22, 31). The emerging spread of these community-acquired MRSA strains poses a significant threat to public health (16, 31).

SCC*mec* is a mobile genetic element characterized by the presence of terminal inverted and direct repeats, two essential genetic components (the *mec* gene complex and the *ccr* gene complex), and the junkyard (J) regions (10, 12, 19). The *mec* gene complex is composed of IS*431mec*, *mecA*, and intact or truncated sets of regulatory genes, *mecR1* and *mecI*. The *ccr* gene complex encodes the recombinases (*ccr*) that mediate the integration of SCC*mec* into and its excision from the recipient chromosome and are, therefore, responsible for its mobility. The rest of the SCC*mec* element is comprised of J regions (J1, J2, and J3) that are located between and around the *mec* and *ccr* complexes and contain various genes or pseudogenes whose does not appear to be essential or useful for the bacterial cell, although notable exceptions include plasmid- or transposon-mediated resistance genes for non-β-lactam antibiotics or heavy metals (13). To date, there are three classes (A, B, and C) of *mec* complex and four allotypes (types 1, 2, 3, and 5) of *ccr* complex. Different combinations of these complex classes and allotypes generate various SCC*mec* types (Table 1). SCC*mec* elements are currently classified into types I, II, III, IV, and V based on the nature of the *mec* and *ccr* gene complexes, and are further classified into subtypes according to differences in their J region DNA (10, 12, 19).

A thorough understanding of the molecular epidemiology and evolution of MRSA is required to help detect, track, control and prevent human disease due to this organism. Full characterization of MRSA requires definition of not only the putative bacterial genetic background but also of the complex and heterologous SCC*mec* elements. SCC*mec* typing is an important molecular tool and its importance in community clonal

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TABLE 1. Current SCC*mec* types and type IV subtypes

SCC*mec* type[a]	*mec* complex[b]	*ccr* complex[c]	Original strain	GenBank no. (reference)
I	Class B	Type 1	NCTC10442	AB033763 (10)
II	Class A	Type 2	N315	D86934 (10)
III	Class A	Type 3	85/2082	AB37671 (10)
IVa	Class B	Type 2	CA05	AB063172 (19)
IVb	Class B	Type 2	8/6-3P	AB063173 (19)
IVc	Class B	Type 2	MR108	AB096217 (13)
IVd	Class B	Type 2	JCSC4469	AB097677
V	Class C	Type 5	WIS [WBG8318]-JCSC3624	AB12121 (12)

[a] Subtypes of SCC*mec* IV differ based on the junkyard (J) region DNA.
[b] Class A *mec:* IS*431-mecA-mecR1-mecI*; class B *mec:* IS*431-mecA*-Δ*mecR1*-IS*1272*; class C *mec:* IS*431-mecA*-Δ*mecR1*-IS*431.*
[c] Type 1 *ccr*: *ccrB1-ccrA1*; type 2 *ccr*: *ccrB2-ccrA2*; type 3 *ccr*: *ccrB3-ccrA3*; type 5 *ccr*: *ccrC.*

outbreaks is increasingly being recognized (16, 22, 31). The new MRSA nomenclature scheme recently set by the International Union of Microbiology Societies incorporates SCC*mec* typing information in conjunction with that provided by multilocus sequence typing (7, 28, 29). Previously described SCC*mec* traditional PCR typing schemes have required the use of many (20 to 30) primer sets and multiple individual PCR experiments (23). The only previously described multiplex PCR assay for SCC*mec* typing (24) is more difficult to interpret and is limited in its ability to detect SCC*mec* subtypes IVa, b, c, and d plus the newly described type V, these groups being implicated in currently emerging community MRSA outbreaks (12, 31).

Here we report a recently developed novel multiplex PCR assay capable of detecting and classifying all currently described SCC*mec* types plus the major subtypes, with simultaneous discrimination of MRSA from methicillin-susceptible *S. aureus* (MSSA).

MATERIALS AND METHODS

Bacterial strains and isolates. The SCC*mec* typing standard MRSA control strains, including 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 obtained from K. Hiramatsu and T. Ito at the Juntendo University in Tokyo, Japan (10, 12, 19, 23). Additional SCC*mec* reference strains, including type I (COL and PER34) and type III (ANS46), were kindly provided by H. de Lencastre, Rockefeller University, New York (24). The Canadian epidemic MRSA reference strains, CMRSA-1 to 6, and strain N02-590 were provided by M. Mulvey, National Microbiology Laboratory, Health Canada, Winnipeg, Canada (30).

Our local strains of MRSA belonging to various SCC*mec* types were obtained from Calgary Laboratory Services (CLS), Calgary, Alberta, Canada, and which had previously underwent complete phenotypic and genotypic analyses at the Centre for Antimicrobial Resistance, Calgary, Alberta, Canada (Table 3). Clinical MRSA isolates used for assessing the applicability and utility of our multiplex PCR (M-PCR) assay were randomly selected from the CLS frozen clinical isolate stock collected over the August 1999 to November 2004 time period. Additional historical clinical MRSA strains were recovered from five tertiary acute-care teaching hospitals located in 4 cities in 3 provinces of the Canadian Prairies (Winnipeg, Manitoba; Saskatoon, Saskatchewan; Calgary, Alberta; and Edmonton, Alberta) during the 1989 to 1994 period (6).

Identification and phenotypic susceptibility testing of staphylococcal isolates. The staphylococcal isolates were identified morphologically and biochemically by standard laboratory procedures (21). The coagulase plasma test (Remel, Lenexa, KS) was performed on organisms exhibiting typical staphylococcal colony morphology to allow for discrimination of *S. aureus* from coagulase-negative staphylococci. Screening for methicillin and other antibiotic resistance phenotypes was done by VITEK (bioMerieux, Inc. Durham, NC) along with the NCCLS oxacillin agar screen, while confirmation of methicillin resistance was achieved using an in-house assay for the *mecA* gene (9).

Sequence alignment and primer design. New sets of SCC*mec* type- and subtype-unique and specific primers, as well as the novel specific primers for *mecA* gene, and for typing *mec* and *ccr* gene complexes (Table 2) were designed based on the comprehensive analyses and alignments of the *S. aureus* and MRSA genomes and SCC*mec* sequences currently available in the GenBank database (National Center for Biotechnology Information; updated as of December, 2004). Gene targets, strains and accession numbers for each primer pair are as follows: type I, ORF E008 of strain NCTC10442 (AB033763); type II, kdpE of strain N315 (D86934); type III, ORF CZ049 of strain 85/2082 (AB37671); type IVa, ORF CQ002 of strain CA05 (AB063172); type IVb, ORF CM001 of strain 8/6-3P (AB063173); type IVc, ORF CR002 of strain MR108 (AB096217); type IVd, ORF CG001 of strain JCSC4469 (AB097677); type V, ORF V011 of strain JCSC3624 (AB12121); *mecA*, *mecA* gene of strain NCTC8325 (X52593); *mecI*, of strain N315; IS*1272* and *mecR1*, *R* of strain CA05; *ccrC*, of strain JSCS 3624. The *ccrAB* primers are as previously described (10). The oligonucleotide primers used in this study were synthesized and purchased from QIAGEN Operon (QIAGEN Inc., Alameda, CA).

DNA extraction. Frozen bacteria were subcultured twice onto 5% sheep blood Columbia agar plates (PML Microbiologicals, Wilsonville, OR) prior to DNA extraction. For rapid DNA extraction, one to five bacterial colonies were suspended in 50 μl of sterile distilled water and heated at 99°C for 10 min. After centrifugation at 30,000 × *g* for 1 min, 2 μl of the supernatant was used as template in a 25-μl PCR (33).

PCR amplification. Our SCC*mec* M-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 the *mecA* gene (Table 2). The M-PCR assay used for characterization of *mec* gene and *ccr* gene complexes, respectively, contained 4 primers each (mecI-F, mecI-R, IS1272-F and mecR1-R for *mec* gene M-PCR, and ccrAB-β2, ccrAB-α2, ccrAB-α3, and ccrAB-α4 for *ccr* gene M-PCR) (Table 2). The single target amplification PCR was used to determine type 5 ccr using ccrC-F and ccrC-R primers (Table 2). These primers and their respective concentrations used in the PCR are listed in Table 2. All PCR assays were performed directly from bacterial suspensions obtained after the rapid DNA extraction method. An aliquot of 2 μl of this 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(dATP, dUTP, dGTP, and dCTP) (Invitrogen Inc., Carlsbad, CA), various concentrations of the respective primers (Table 2), and 1.0 unit of Platinum *Taq* DNA polymerase (Invitrogen Inc., Carlsbad, CA).

The amplification was performed in a GeneAmp PCR system 9700 or 9600 Thermal Cycler (Applied Biosystems, Foster City, CA) beginning with an initial denaturation step at 94°C for 5 min followed by 10 cycles of 94°C for 45 seconds, 65°C for 45 seconds, and 72°C for 1.5 min and another 25 cycles of 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 1.5 min, ending with a final extension step at 72°C for 10 min and followed by a hold at 4°C. 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°C for 5 min followed by 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min, ending with a final extension step at 72°C for 10 min. For comparative purposes, SCC*mec* typing using Oliveira's method was performed using primer and PCR conditions described previously (24). All PCR assay runs incorporated a reagent control (without template DNA). The PCR amplicons were visualized using a UV light box after electrophoresis on a 2% agarose gel containing 0.5 μg/ml ethidium bromide.

Limiting dilution experiments for estimation of M-PCR sensitivity. The sensitivity of amplification of various pairs of primers by M-PCR was estimated by limiting dilution experiments. Briefly, bacterial cultures from overnight growth at 37°C on 5% sheep blood agar plates were suspended in sterile saline to a density corresponding to a 1.0 McFarland turbidity standard. These suspensions were then used to prepare serial 10-fold dilutions using sterile double distilled water. DNA extraction, using the rapid method described previously, was performed on 50 μl of each dilution. The standard M-PCR assay was performed to determine its sensitivity. The lower limits of detection (or minimal numbers of CFU detectable) of the target genes by M-PCR were then calculated based on correlation of the 1.0 McFarland standard to 3×10^8 CFU/ml.

Validation and application of SCC*mec* typing method. Our M-PCR assay was first optimized in the standard control strains and then validated with other control strains, and simultaneously compared with the traditional SCC*mec* typing methods including *mec* and *ccr* gene complex typing (described above) and a previously described multiplex PCR assay-Oliveira's method (24). To assess the

TABLE 2. Primers used in this study

Primer	Oligonucleotide sequence (5′-3′)	Concn (μM)	Amplicon size (bp)	Specificity	Reference
Type I-F	GCTTTAAAGAGTGTCGTTACAGG	0.048	613	SCC*mec* I	This study
Type I-R	GTTCTCTCATAGTATGACGTCC				
Type II-F	CGTTGAAGATGATGAAGCG	0.032	398	SCC*mec* II	This study
Type II-R	CGAAATCAATGGTTAATGGACC				
Type III-F	CCATATTGTGTACGATGCG	0.04	280	SCC*mec* III	This study
Type III-R	CCTTAGTTGTCGTAACAGATCG				
Type IVa-F	GCCTTATTCGAAGAAACCG	0.104	776	SCC*mec* IVa	This study
Type IVa-R	CTACTCTTCTGAAAAGCGTCG				
Type IVb-F	TCTGGAATTACTTCAGCTGC	0.092	493	SCC*mec* IVb	This study
Type IVb-R	AAACAATATTGCTCTCCCTC				
Type IVc-F	ACAATATTTGTATTATCGGAGAGC	0.078	200	SCC*mec* IVc	This study
Type IVc-R	TTGGTATGAGGTATTGCTGG				
Type IVd-F5	CTCAAAATACGGACCCCAATACA	0.28	881	SCC*mec* IVd	This study
Type IVd-R6	TGCTCCAGTAATTGCTAAAG				
Type V-F	GAACATTGTTACTTAAATGAGCG	0.06	325	SCC*mec* V	This study
Type V-R	TGAAAGTTGTACCCTTGACACC				
MecA147-F	GTG AAG ATA TAC CAA GTG ATT	0.046	147	*mecA*	This study
MecA147-R	ATG CGC TAT AGA TTG AAA GGA T				
mecI-F	CCCTTTTTATACAATCTCGTT	0.08	146	Class A *mec*	This study
mecI-R	ATATCATCTGCAGAATGGG				
IS1272-F	TATTTTTGGGTTTCACTCGG	0.08	1,305	Class B *mec*	This study
mecR1-R	CTCCACGTTAATTCCATTAATACC				
ccrAB-β2	ATTGCCTTGATAATAGCCITCT	0.08			10
ccrAB-α2	AACCTATATCATCAATCAGTACGT	0.08	700	Type 1 *ccr*	10
ccrAB-α3	TAAAGGCATCAATGCACAAACACT	0.08	1,000	Type 2 *ccr*	10
ccrAB-α4	AGCTCAAAAGCAAGCAATAGAAT	0.08	1,600	Type 3 *ccr*	10
ccrC-F	ATGAATTCAAAGAGCATGGC	0.08	336	Type 5 *ccr*	This study
ccrC-R	GATTTAGAATTGTCGTGATTGC				

applicability and utility of our SCC*mec* typing assay, 453 randomly selected local clinical isolates from our MRSA clinical isolate frozen stock collection for the 1989 to 2004 time period were tested. To verify our assay's ability to differentiate MRSA from MSSA, comparison of our assay with standard phenotypic susceptibility testing (VITEK) and the conventional *mecA* gene PCR test (above) was conducted in 150 randomly selected local clinical MSSA isolates, in addition to the above 453 clinical MRSA isolates.

RESULTS

Identification and selection of unique and specific loci and primer design for SCC*mec* types and subtypes. To design the SCC*mec* type- and subtype-unique and specific primers, an extensive BLAST sequence similarity search was conducted and was followed by comprehensive analyses and alignments of the *S. aureus* and MRSA genomes and SCC*mec* sequences currently available in the GenBank database. These loci consisted of open reading frames (ORFs) or sequence fragments, including ORF E008 of strain NCTC10442 (AB033763), kdpE of strain N315 (D86934), ORF CZ049 of strain 85/2082 (AB37671), ORF CQ002 of strain CA05 (AB063172), ORF CM001 of strain 8/6-3P (AB063173), ORF CR002 of strain MR108 (AB096217), ORF CG001 of strain JCSC4469 (AB097677), and ORF V011 of strain JCSC3624 (AB12121), and were found to be unique and specific for SCC*mec* types and subtypes I, II, III, IVa, IVb, IVc, IVd, and V, respectively (refer to Materials and Methods for details of gene targets, strains, and accession numbers). The corresponding SCC*mec* type- and subtype-unique and specific primers were designed (Table 2) and their uniqueness and specificity were further confirmed with a GenBank database BLAST search. Utilization of these primers in our novel M-PCR assay allowed us to specifically detect the currently described SCC*mec* types and subtypes of MRSA strains and clinical isolates.

New M-PCR for typing and subtyping SCC*mec* types I to V, and simultaneous detection of methicillin resistance (*mecA* gene). We developed a new and simple single M-PCR assay to determine (classify) SCC*mec* types and subtypes I, II, III, IVa, IVb, IVc, IVd, and V, and simultaneously discriminate MRSA from MSSA. The M-PCR assay targeted the unique and specific loci of SCC*mec* types and subtypes I, II, III, IVa, IVb, IVc, IVd, and V, with concomitant *mecA* gene detection, the last

serving as a determinant of methicillin resistance but also serving as an internal positive control for the assay.

To ensure the individual primer pairs were adequate for the amplification of all nine loci (gene fragments), the single-target PCR protocol with each individual primer pair was conducted prior to the M-PCR optimization, using eight SCC*mec* standard control strains: 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) (Tables 1 and 3). Each individual PCR amplification reaction yielded the fragment of the expected size, i.e., 613, 398, 280, 776, 493, 200, 881, 325, and 147 bp for the unique and specific loci of SCC*mec* types and subtypes I, II, III, IVa, IVb, IVc, IVd, and V, and *mecA* gene in their corresponding strains, respectively. The optimized M-PCR condition as described above was obtained through assaying different primer concentrations and other PCR components. Amplification in a single M-PCR produced distinct bands corresponding to their respective molecular sizes that were easily recognizable in agarose gels stained with ethidium bromide (Fig. 1).

Sensitivity of M-PCR. The sensitivity of our M-PCR assay was examined in 8 SCC*mec* standard control strains for 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). This assay was capable of detecting, with reproducibility, a band in ethidium bromide-stained gels at dilutions corresponding to 6×10^4 CFU per PCR for all eight type- and subtype-specific genes. However, the sensitivity for the internal control *mecA* gene varied slightly depending on the strains examined, being 6×10^5 CFU per PCR for the strains NCTC10442 (type I), JCSC4469 (type IVd) and WIS (type V), and 6×10^4 CFU per PCR for all other type or subtype strains [N315 (type II), 85/2082 (type III), CA05 (type IVa), 8/6-3P (type IVb), and MR108 (type IVc)]. This sensitivity is quite compatible with the single target PCR assay (1×10^4 to 6×10^5) (data not shown), suggesting that our M-PCR assay is sufficiently robust.

Validation of M-PCR assay. To validate the M-PCR assay, we simultaneously compared our assay with the traditional PCR SCC*mec* typing scheme including *mec* and *ccr* gene complex typing and a previously described M-PCR assay (24). Validation of our assay was performed by testing a total of 54 well-characterized MRSA strains with known SCC*mec* types including type I ($n = 3$), type II ($n = 14$), type III ($n = 9$), type IVa ($n = 18$), type IVb ($n = 4$), type IVc ($n = 3$), type IVd ($n = 2$), type V ($n = 1$). We found a 100% concordance in typing SCC*mec* types I-IV between the PCR results of our M-PCR, traditional SCC*mec* typing method, and Oliveira's assay (Table 3) except for one type V strain. However, in the WIS strain (type V), both our assay and the traditional SCC*mec* typing method correctly identified this strain as SCC*mec* type V, but Oliveira's M-PCR falsely categorized the strain as SCC*mec* type III (Table 3). In addition, our assay had more discriminatory power and was able to further classify type IV strains into subtypes IVa, b, c, and d (Table 3).

To address our assay's ability in differentiating MRSA from MSSA, we tested 150 randomly selected local clinical MSSA isolates, in addition to the above 54 MRSA control strains and the 453 clinical MRSA isolates (below) and found a *mecA* gene band (147 bp) in all MRSA isolates but not in any MSSA isolates, hence being 100% concordant with phenotypic susceptibility (VITEK) and conventional *mecA* gene PCR test results.

Applicability and accuracy of M-PCR. To assess the applicability and accuracy of the M-PCR assay, we further applied our SCC*mec* typing assay to test a total of 453 local clinical MRSA isolates randomly selected from our clinical stock collection for the 16-year period from 1989 to 2004. Among them, 235 (51.88%), 122 (26.93%), 74 (16.34%), 5 (1.1%), and 4 (0.88%) isolates belonged to SCC*mec* types and subtypes II, III, IVa, IVb, and IVc, but no SCC*mec* types and subtypes I, IVd, or V were found among the isolates tested. However, there were 13 (2.87%) isolates that were not typeable using our assay, with five (1.10%) isolates having multiple bands and eight (1.77%) isolates with amplification of only the *mecA* gene.

These not-typeable isolates were further characterized using the traditional PCR SCC*mec* typing method and Oliveira's M-PCR assay. In five multiple-band isolates, one isolate presenting two bands of 200 bp and 280 bp (corresponding to types IVc and III by our new assay) was also not-typeable by the traditional PCR but was found to be type III by Oliveira's M-PCR, while the other four isolates with bands of 398 bp and either 613 bp or 200 bp (corresponding to types II and either type I or IVc by our new assay) were typed as types II in both other assays (Table 4), and may represent undescribed new variant subtypes of SCC*mec* type II. However, among the other eight isolates with amplification of only the *mecA* gene, only one isolate (*mecA*-band 8) was determined to be type IV by both the traditional PCR SCC*mec* typing method and Oliveira's M-PCR assay, while the remaining (7 isolates) had incongruent typing results among the two other typing methods (Table 4), potentially representing new types or variant subtypes.

DISCUSSION

SCC*mec* typing is one of the most important molecular tools available for understanding the epidemiology and clonal strain relatedness of MRSA, particularly with the emerging outbreaks of community-acquired MRSA occurring on a worldwide basis. However, due to the very complex and diverse structure of the SCC*mec* element, SCC*mec* typing is usually achieved by DNA sequence analysis (21 to 67 kb) (10, 11, 25), Southern blot analysis using three or more restriction enzymes and several key probes specific for each SCC*mec* type (25), and by PCR. Previously described traditional PCR SCC*mec* typing schemes target the individual regions of the classes of the *mec* complex (*IS431-mecA*, *IS1272-mecA*, and *mecI-mecRI*), the allotypes of the *ccr* complex (*ccrA1*, *ccrA2*, *ccrA3*, *ccrB1*, *ccrB2*, *ccrB3*, and *ccrC*), and individual subtypes of the J regions, and therefore require the use of many primer sets and PCR experiments (12, 23). These methods are laborious, time-consuming and expensive, resulting in limited utility for clinical and surveillance purposes.

Oliveira and de Lencastre developed a multiplex PCR strategy for *mec* element type assignment and defined types of SCC*mec* based on genes located within the J-regions of SCC*mec* elements as follows: locus A, located downstream of the *pls* gene and is specific for SCC*mec* type-1; locus B, internal

TABLE 3. Comparison of our assay with the traditional PCR and an M-PCR SCC*mec* typing method

Strain[a]	Traditional PCR typing[b]			Oliveira's M-PCR[c]	Our novel assay
	mec complex type	*ccr* complex type	SCC*mec* type		
NCTC10442	B	1	I	I	I
COL	B	1	I	I	I
PER34	B	1	I	I	I
N315	A	2	II	II	II
CMRSA-2	A	2	II	II	II
MRSA-80	A	2	II	II	II
CLS-5153	A	2	II	II	II
CLS-5371	A	2	II	II	II
CLS-440	A	2	II	II	II
CLS-72251	A	2	II	II	II
CLS-69500	A	2	II	II	II
CLS-68961	A	2	II	II	II
CLS-6146	A	2	II	II	II
CLS-4021	A	2	II	II	II
CLS-2516	A	2	II	II	II
CLS-52692	A	2	II	II	II
CLS-19095	A	2	II	II	II
85/2082	A	3	III	IIIB	III
ANS46	A	3	III	III	III
CMRSA-3	A	3	III	IIIA	III
CMRSA-6	A	3	III	III	III
CLS-5861	A	3	III	III	III
CLS-1777	A	3	III	III	III
H163	A	3	III	III	III
H478	A	3	III	III	III
H527	A	3	III	III	III
CA05	B	2	IV	IV	IVa
N02-590	B	2	IV	IV	IVa
CLS-2207	B	2	IV	IV	IVa
CLS-3860	B	2	IV	IV	IVa
CLS-2772	B	2	IV	IV	IVa
CLS-1236	B	2	IV	IV	IVa
CLS-884	B	2	IV	IV	IVa
CLS-2772	B	2	IV	IV	IVa
CLS-4550	B	2	IV	IV	IVa
CLS-2245	B	2	IV	IV	IVa
CLS-5897	B	2	IV	IV	IVa
CLS-847	B	2	IV	IV	IVa
CLS-846	B	2	IV	IV	IVa
CLS-2525	B	2	IV	IV	IVa
CLS-3497	B	2	IV	IV	IVa
CLS-5401	B	2	IV	IV	IVa
CLS-5381	B	2	IV	IV	IVa
CLS-284	B	2	IV	IV	IVa
8/6-3P	B	2	IV	IV	IVb
CLS-4584	B	2	IV	IV	IVb
CLS-5827	B	2	IV	IV	IVb
CLS-6572	B	2	IV	IV	IVb
MR108	B	2	IV	IV	IVc
CLS-1040	B	2	IV	IV	IVc
H434	B	2	IV	IV	IVc
JCSC 4469	B	2	IV	IV	IVd
CMRSA-5	B	2	IV	IV	IVd
JCSC 3624 WIS [WBG8318]	C2	5	V	III	V

[a] The SCC*mec* typing standard MRSA control strains were: type 1 (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); additional SCC*mec* reference strains were type 1 (COL and PER34) and type III (ANS46); The Canadian epidemic MRSA reference strains were CMRSA-1 to 6 and strain N02-590. Our local SCC*mec* type control strains are designated CLS and H.

[b] Traditional PCR SCC*mec* typing methods (10, 12, 19, 23).

[c] Oliveira's multiplex PCR assay (24).

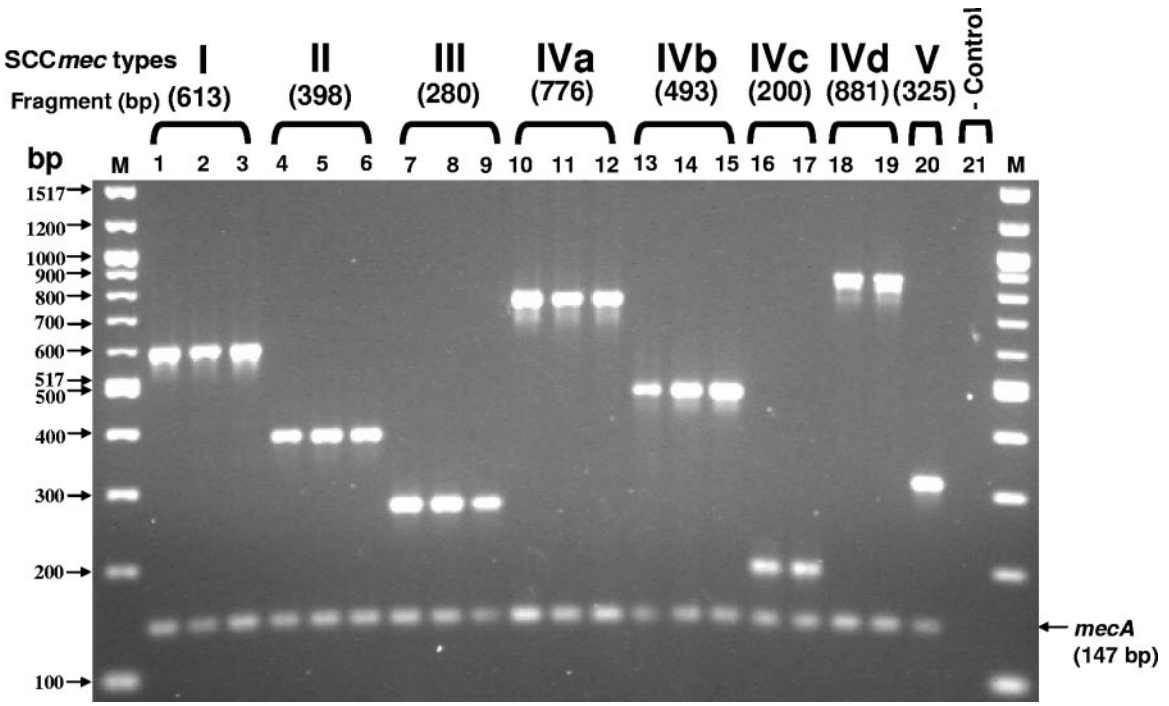

FIG. 1. New multiplex PCR assay identifies SCC*mec* types and subtypes I, II, III, IVa, IVb, IVc, IVd, and V, and simultaneously detects the methicillin resistance (*mecA* gene). Type I, lanes 1 to 3 (strains NCTC10442, COL, and PER34, respectively); type II, lanes 4 to 6 (strains N315, CLS-5153, and CLS-440, respectively); type III, lanes 7 to 9 (strains 85/2082, ANS46, and CMRSA-3, respectively); type IVa, lanes 10 to 12 (strains CA05, N02-590, and CLS-2207, respectively); type IVb, lanes 13 to 15 (strains 8/6-3P, CLS-4584, and CLS-5827, respectively); type IVc, lanes 16 and 17 (strains MR108 and CLS-1040, respectively); type IVd, lanes 18 and 19 (strains JCSC4469 and CMRSA-5, respectively); type V, lane 20 (strain WIS [WBG8318]-JCSC3624); lane 21, negative control; and lanes M, molecular size markers, 100-bp DNA ladder (BioLabs). Refer to Table 3 for details of each strain.

to the kdp operon, which is specific for SCC*mec* type II; locus C, internal to the *mecI* gene present in SCC*mec* types II and III; locus D, internal to the dcs region present in type I, II, and IV; locus E, located in the region between integrated plasmid pI*258* and transposon Tn*554*, specific for SCC*mec* type III; locus F, which is also specific for SCC*mec* type-III located in the region between Tn*554* and *orfX*; locus G, the left junction between IS*431* and pUB110; and locus H, the left junction between IS*431* and pT181 (24). This is the only single-step multiplex PCR assay published to date, but it too has its limitations. Since it is much simpler and easier to perform than the traditional (nonmultiplex) PCR assays for SCC*mec* typing,

TABLE 4. Comparison of *SCCmec* typing results for traditional PCR and Oliviera's multiplex PCR assays for isolates not typeable by our multiplex PCR assay

Isolate[a]	Our novel assay[b]		Traditional PCR typing[c]	Oliveira's M-PCR[d]
	Specific PCR product(s) (bp)	Corresponding to:		
Multiband 1	200 + 280	Type IVc + III	Not typeable	Type III
Multiband 2	398 + 613	Type II + I	Type II	Type II
Multiband 3	398 + 613	Type II + I	Type II	Type II
Multiband 4	398 + 200	Type II + IVc	Type II	Type II
Multiband 5	398 + 200	Type II + IVc	Type II	Type II
mecA band 1	147	*mecA* gene	Not typeable	Type IV
mecA band 2	147	*mecA* gene	Not typeable	Not typeable
mecA band 3	147	*mecA* gene	Type IV	Type I
mecA band 4	147	*mecA* gene	Type IV	Not typeable
mecA band 5	147	*mecA* gene	Type II	Type IV
mecA band 6	147	*mecA* gene	Type II	Type IV
mecA band 7	147	*mecA* gene	Type I	Not typeable
mecA band 8	147	*mecA* gene	Type IV	Type IV

[a] Not-typeable isolates (multiple bands or single *mecA* gene band) using our new assay.
[b] Our new multiplex PCR assay.
[c] Traditional PCR SCC*mec* typing methods (10, 12, 19, 23).
[d] Oliveira's multiplex PCR assay (24).

it has been increasingly used in favor of the traditional method. As a result, different SCC*mec* types are named according to the standard SCC*mec* type definition first established by Hiramatsu's group (T. Ito, X. Ma, Y. Kondo, P. Changtrakool, S. Traklsomboon, C. Tiensasitorn, M. Jamklang, T. Chavalit, J. Song, and K. Hiramastsu, Abstr. 44th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 115, 2004).

In addition to hampered interpretation due to the presence of multiple bands for each SCC*mec* type (because of non-type-specific targets) and difficulties in assay optimization, Oliveira's assay (24) has limitations in detecting the newly described SCC*mec* type V, misclassifying them as type III (Table 3), while failing to discriminate type IV into subtypes a, b, c, and d (24). Since the newer SCC*mec* types IV and V have recently been associated with community-acquired infection (12, 31), detecting type V and discriminating type IV into subtypes IVa, b, c, and d may play an important role in the understanding of the epidemiology and ultimate prevention and control of currently emerging community MRSA clonal outbreaks. Therefore, a more robust and simpler SCC*mec* typing assay is urgently required.

We designed eight new sets of SCC*mec* type- and subtype-unique and specific primers and 1 new set of methicillin resistance (*mecA* gene-based) primers based on comprehensive analyses and alignments of the MSSA and MRSA genomes and SCC*mec* sequences, and have successfully developed a novel multiplex PCR SCC*mec* typing assay (in a single multiplex PCR with a single band for each type or subtype) capable of classifying MRSA isolates into SCC*mec* types and subtypes I, II, III, IVa, IVb, IVc, IVd, and V, according to the current updated SCC*mec* typing system, while simultaneously being able to discriminate MRSA from MSSA.

Simultaneous comparison of our new assay with the traditional PCR SCC*mec* typing method (including *mec* and *ccr* gene complex typing) and Oliveira's assay demonstrated 100% sensitivity and specificity when testing a large number of control strains. Further application of our assay in randomly selected local clinical isolates confirmed its feasibility and practicality. This novel assay offers a rapid, simple, and feasible method for SCC*mec* typing of MRSA, and may serve as a useful tool for clinicians and epidemiologists in their efforts to prevent and control infections caused by these organisms. It has been used in our regional MRSA surveillance program and helped identify and confirm the emergence of an outbreak of community-acquired MRSA infection in Calgary, Canada in 2004, with resultant expedient implementation of prevention and control measures (J. Conly, M. Gilbert, K. Zhang, D. Gregson, S. Elsayed, M. Mulvey, K. Laupland, L. Louie, H. Rabin, B. Baylis, and P. Boiteau, Abstr. AMMI Canada-CACMID 2005 Annual Conference, Abstr. and Presentation G5, 2005; M. Gilbert, J. Suishansian, J. MacDonald, D. Gregson, S. Elsayed, K. Zhang, K. Laupland, M. Louie, T. Louie, D. Nielsen, G. Keays, A. Honish, D. Gravel, M. Mulvey, J. Gillespie, J. Conly, Abstr. AMMI Canada-CACMID 2005 Annu. Conf., abstr. and presentation G2, 2005).

It has only recently been shown that some methicillin-susceptible staphylococci, including MSSA and methicillin-susceptible coagulase-negative staphylococci, could harbor SCC elements that contain the essential features of SCC*mec* but lack the *mecA* gene (4, 15, 18, 20). These SCC elements serve as a vehicle of transfer for various genetic markers including genes mediating antibiotic resistance or virulence. The potential role of SCC for mediating gene movement in staphylococci is awaiting further investigation. Hence, our multiplex assay (incorporating a concomitant *mecA* gene into specific SCC*mec* typing system) may play a critical role in this regard.

Both the traditional PCR SCC*mec* typing scheme and Oliveira's multiplex PCR technique are PCR methods targeting unique loci. Not-typeable MRSA isolates are encounted when using the traditional PCR SCC*mec* typing scheme and Oliveira's multiplex PCR technique but the nontypeability rate is variable. Ito et al. used their traditional PCR typing method to type 617 MRSA isolates from Asian countries and found 5 (0.81%) strains were not-typeable (T. Ito, X. Ma, Y. Kondo, P. Changtrakool, S. Traklsomboon, C. Tiensasitorn, M. Jamklang, T. Chavalit, J. Song, and K. Hiramastsu, Abstr. 44th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 115, 2004).

Perez-Roth et al. (27) found 11 not-typeable clones out of 375 isolates (2.93%) (due to unmatching patterns) when typing MRSA clinical isolates during a 5-year period (1998 to 2002) in a Spanish hospital, and Chung et al. (3) found 4 out of 113 isolates (3.54%) were not-typeable when typing MRSA strains recovered at a Florida hospital, when both groups of investigators used Oliveira's assay. We used our newly developed assay to type 453 local clinical randomly selected isolates and found 13 (2.87%) not-typeable isolates. Except for one isolate (*mecA*-band 8), the remaining 12 isolates (Table 4) are potentially new types or subtypes. The explanation for these observations, as quoted by others, may be related to the presence of new structural types and subtypes or structural rearrangements and recombination of the *mec* element (3, 27). Further investigations, including sequencing the *mec* element, are needed in order to characterize these currently not-typeable isolates.

Our newly described assay was designed to target the SCC*mec* type- and subtype-unique and specific gene loci, based on the currently available sequence data of the MRSA and MSSA genomes and variable SCC*mec* type and subtype sequences in the GenBank database. This new assay is more robust and has greater simplicity in its ability to classify all known types in a single PCR compared to currently available typing methodologies. However, we acknowledge the potential future limitations of our assay because of the possibility that as more SCC*mec* elements from newly emerging clinical isolates are sequenced, new SCC*mec* types and subtypes will be discovered, necessitating a reconfiguration of our assay to allow for the detection of these newer types.

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