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Multiplex Real-Time PCR for Rapid Staphylococcal Cassette Chromosome *mec* Typing[▽]

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Rapid identification and typing of methicillin (meticillin)-resistant *Staphylococcus aureus* (MRSA) is important for understanding the molecular epidemiology and evolution of MRSA and offers many advantages for controlling transmission in both health care and community settings. We developed a rapid molecular beacon real-time PCR (MB-PCR) assay for staphylococcal cassette chromosome *mec* (SCC*mec*) typing. The design of this system is based on the established definition of SCC*mec* types, namely, the combination of the *mec* class complex with the *ccr* allotype. The assay consists of two multiplex panels, the combination of which results in two targets (*mec* class, *ccr*) for each SCC*mec* type. MB-PCR panel I targets *mecA*, *ccrB2*, *mecI*, and the Δ*mecR1*-IS*1272* junction (*mec* class B); it can definitively identify SCC*mec* types II and IV. MB-PCR panel II detects *ccrC*, *ccrB1*, *ccrB3*, *ccrB4*, and the Δ*mecR1*-IS*431* junction (*mec* class C2) and is therefore capable of identifying SCC*mec* types I, III, V, and VI in combination with panel I. The method can also detect the recently described novel SCC*mec* type VIII (*ccrAB4* with *mec* class A). Our assay demonstrated 100% concordance when applied to 162 MRSA strains previously characterized by traditional SCC*mec* typing schemes. Four geographically and temporally diverse *S. aureus* collections were also successfully classified by our assay, along with 1,683 clinical isolates comprising both hospital- and community-associated MRSA and methicillin-susceptible *S. aureus* strains. As many as 96 isolates can be classified easily within 3 to 4 h, including DNA isolation, PCR cycling, and analysis. The assay is rapid, robust, sensitive, and cost-effective, allowing for high-throughput SCC*mec* typing of MRSA isolates.

Staphylococcus aureus is a commensal and opportunistic pathogen of significant clinical and veterinary importance, responsible for a wide array of different infections (12, 46). Antibiotic resistance in *S. aureus* poses further challenges, with the global prevalence of methicillin (meticillin)-resistant *Staphylococcus aureus* (MRSA) increasing unabatedly since the isolation of the first strain in 1961 (34). MRSA constitutes a major cause of nosocomial disease worldwide but has also become prevalent in community settings in recent years, further increasing the potential for endemic and zoonotic transmission (6, 54).

MRSA is defined by the presence of a large, heterogeneous genetic element known as "staphylococcal cassette chromosome *mec*" (SCC*mec*), which harbors the gene (*mecA*) coding for methicillin resistance (30, 35). This element is also found in several other staphylococcal species, and it is believed that methicillin-resistant coagulase-negative staphylococci (MR-CoNS) constitute a reservoir for SCC*mec* acquisition by *S. aureus* (36). The SCC*mec* elements discovered thus far are characterized by the presence of (i) terminal inverted and direct repeats, (ii) site-specific cassette chromosome recombinases (*ccr*), (iii) a *mecA* gene complex (*mec*), and (iv) a specific chromosomal insertion site within a conserved open reading frame (*orfX*) of unknown function (5, 29–31, 35, 49, 58, 83). For purposes of molecular epidemiologic analysis, different SCC*mec* "types" are recognized by binary combinations of the *mec* complex and the *ccr* allotype (Fig. 1; see also Table 2), described in detail at http://www.staphylococcus.net (Juntendo University). SCC*mec* types can be further classified (subtyped) by differences in regions other than *ccr* and *mec*, designated "joining" (J) regions: J1 (between *ccr* and the right-flanking chromosomal region), J2 (between *mec* and *ccr*), and J3 (between *mec* and *orfX*).

For *S. aureus*, five *ccr* allotypes (*ccrAB1* to *ccrAB4* and *ccrC*) and five classes of *mec* complex (A, B, C1, C2, and E), corresponding to eight SCC*mec* prototypes (I to VIII), have been described to date (5, 29–31, 35, 44, 50, 58, 83). Following the initial description of the SCC*mec* element (30), SCC*mec* types I, II, and III were retrospectively identified in nosocomial MRSA strains isolated in 1961 (NCTC10442), 1982 (N315), and 1985 (85/2082), respectively (29). Subsequently, two different candidates for SCC*mec* IV were proposed by Oliveira et al. (61) (HDE288) and Ma et al. (50) (CA05, 8/6-3P); the former has since been reclassified as SCC*mec* type VI (58). Ito and colleagues later described a strain (WIS) bearing a novel *ccr* element (*ccrC*) and *mec* class (C2); this type was accordingly named SCC*mec* V (31). More recently, various proposals for SCC*mec* type VII have appeared in the literature (5, 11,

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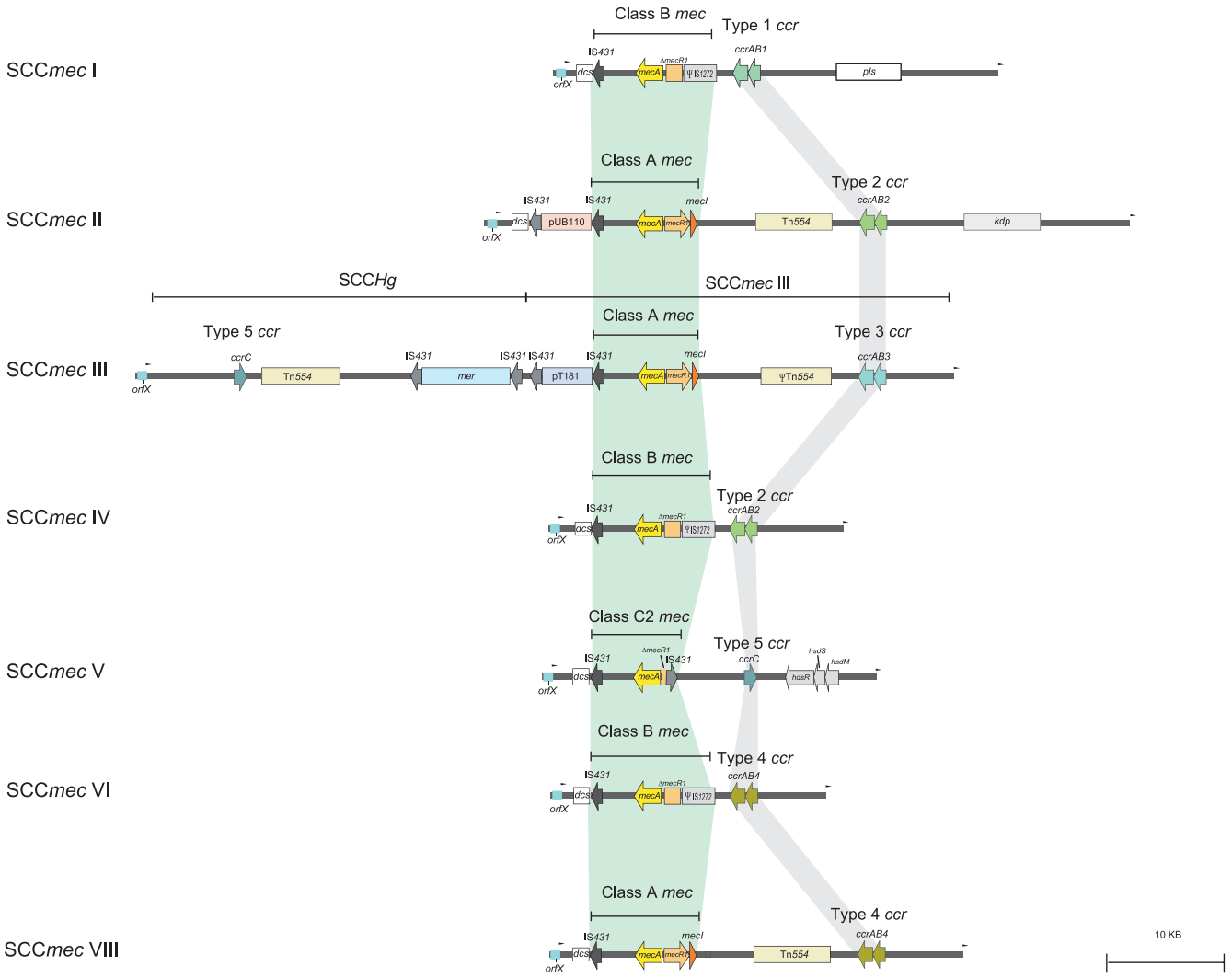

FIG. 1. Schematic representation of SCC*mec* types I to VI and recently described type VIII in MRSA prototype strains. SCC*mec* structures are illustrated according to nucleotide sequences deposited in GenBank under the following accession numbers (with strains and SCC*mec* types given in parentheses): AB033763 (NCTC10442, SCC*mec* I), D86934 (N315, SCC*mec* II), AB037671 (85/2082, SCC*mec* III), AB063172 (CA05, SCC*mec* IV), AB121219 (WIS, SCC*mec* V), AF411935 (HDE288, SCC*mec* VI), and FJ390057 (C10682, SCC*mec* VIII). The *ccr* and *mec* class complexes grey and utilized for SCC*mec* type classification are shaded and shown against a grey and green background, respectively; elements outside of these areas constitute "joining" regions (J1, J2, and J3, from right to left).

24); however, the International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC) has determined that the combination of *ccrC* and *mec* class C1 (5) should be recognized as the official prototype. Finally, the novel SCC*mec* type VIII (*ccrAB4* with *mec* class A), in an epidemic Canadian strain, has been described recently by Zhang et al. (83).

Understanding the molecular epidemiology and evolution of MRSA offers many advantages for controlling transmission in both health care and community settings, making rapid identification and typing of MRSA strains an important issue. Along with multilocus sequence typing (MLST), *spa* typing, and pulsed-field gel electrophoresis, SCC*mec* typing is considered an essential technique for the analysis of MRSA (12, 16). A uniform nomenclature has been proposed (15) in which the multilocus sequence type (ST) and SCC*mec* type are juxtaposed, e.g., ST5-MRSA-II (New York/Japan clone) versus ST5-MRSA-IV (Pediatric clone). SCC*mec* typing is also of potential clinical and epidemiologic interest; SCC*mec* types I, II, and III, for example, are usually associated with traditional nosocomial strains (hospital-acquired MRSA), whereas types IV, V, and VI are generally characteristic of recently emerging "community" strains (community-acquired MRSA [CA-MRSA]) (10, 29, 31, 79). Lastly, the distribution of SCC*mec* elements in MR-CoNS is likewise of interest, particularly with regard to the horizontal transmission of SCC*mec* elements across species boundaries (81, 82).

Various SCC*mec* typing schemes have been proposed over the past 8 years, most of them based on conventional multiplex PCR (7, 40, 47, 53, 57, 84). The majority of these involve electrophoretic analysis of multiple bands (often greater than 1 kb), thus necessitating DNA isolation methods incompatible with rapid, high-throughput typing. In this study, we describe a

novel procedure for rapid and simple characterization of SCC*mec* types using multiplex real-time PCR with molecular beacons. Molecular beacons are short oligonucleotide probes possessing a hairpin stem-loop structure, which exhibit fluorescence only when the loop region anneals to a complementary target (77). The presence of the hairpin significantly enhances specificity (76), allowing multiple targets to be distinguished in a single reaction. Real-time PCR assays also offer advantages in that they are highly sensitive, require minimal postamplification analysis, and minimize the possibility of laboratory contamination. The procedure described here uses a two-tiered approach based on the established classification of SCC*mec* types, i.e., by detecting binary combinations of the *mec* class and the *ccr* allotype, along with detection of the *mecA* gene as a positive control.

MATERIALS AND METHODS

Bacterial strains. Thirteen prototype MRSA strains were used for the derivation and optimization of the real-time PCR SCC*mec* typing scheme: NCTC10442 (SCC*mec* type I) (29), N315 (SCC*mec* type II) (30), BK351 (SCC*mec* type IIA) (40), JCSC3063 (SCC*mec* type IIb) (40), 85/2082 (SCC*mec* type III) (29), CA05 (SCC*mec* type IVa) (50), 8/6-3P (SCC*mec* type IVb) (50), JCSC4788 (SCC*mec* type IVc) (37), JCSC4469 (SCC*mec* type IVd) (49), AR43/3330.1 (SCC*mec* type IVE) (68), HAR22 (SCC*mec* type IVh) (52), WIS (SCC*mec* type V) (31), and HDE288 (SCC*mec* type VI) (58). All strains either were kindly provided by T. Ito (Juntendo University, Tokyo, Japan) or came from our own collections (Public Health Research Institute [PHRI], Instituto de Tecnologia Química e Biológica, Rockefeller University, Mount Sinai Hospital). Prototype strains for SCC*mec* types VII (JCSC6082) (5) and VIII (C10682) (83) were not available at the time this study was conducted. Additional strains used for validation, specificity, and applicability are described below.

Molecular beacons and primers. Molecular beacon and oligonucleotide primer sequences are listed in Table 1. Molecular beacon probes were designed according to guidelines presented at http://www.molecular-beacons.org/; oligonucleotide primers were designed by Primer3 online software (65). Potential cross-reactivity among beacons and primers was tested using the Autodimer online tool (78). Molecular beacon probes were obtained from Biosearch Technologies (Novato, CA) and purified by high-performance liquid chromatography as described elsewhere (80); oligonucleotide primers were obtained from Integrated DNA Technologies (Coralville, IA). Single-probe (simplex) reactions were conducted with each primer-probe set to evaluate specificity and sensitivity for individual targets. Two panels consisting of four molecular beacon targets each were then designed based on the established classification of SCC*mec* types by *mec* class and *ccr* allotype; a minimum of two targets were chosen for each SCC*mec* type, not including *mecA* (see Table 2).

The following considerations were incorporated into the design of individual targets. All *S. aureus* type 1 to 4 *ccrB* and *ccrC* gene sequences available in NCBI GenBank prior to 2009 were downloaded and aligned with ClustalW2 (43) in order to identify divergent and conserved regions. For strains possessing the *ccrAB* allotype, *ccrB* was used as a typing target, since it is more discriminatory than *ccrA* for purposes of SCC*mec* type assignment (59). The *ccrC* target was designed to detect the *ccrC* alleles found in SCC*mec* V (*ccrC1*) (accession number AB121219) and SCC*mec* III strains containing SCC*Hg* (formerly "SCC*mercury*") (*ccrC3*) (AB037671); of the *ccrC* alleles described to date, this target will also detect *ccrC2* (AY894416), *ccrC6* (EF190467), and *ccrC7* (EF190468), but it may not detect *ccrC4* (U10927), *ccrC5* (AP006716), *ccrC8* (AB353125), or *ccrC9* (NC_007350), due to the presence of one or more mismatches.

For strains possessing a class A *mec* complex (IS*431mec-mecA-mecR1-mecI*), a region in which both *mecI* (intact) and Δ*mecI* (truncated) were conserved was selected for target design. For strains possessing a class B *mec* complex (IS*431mec-mecA*-Δ*mecR1*-IS*1272*), the Δ*mecR1*-IS*1272* junction region was targeted, since it has been reported that the junction (IS*1272J*) between Δ*mecR1* and IS*1272* is conserved in class B *mec* complex sequences (39), whereas IS*1272* alone may occur in other regions of the chromosome (75). Similarly, for the class C2 *mec* complex (IS*431mec-mecA*-Δ*mecR1*-IS*431*), found in SCC*mec* V and V_T (8, 31, 74), the region overlapping Δ*mecR1*-IS*431* was chosen as a target. Finally, a universally conserved region of *mecA* was selected as an internal control for SCC*mec*, as well as to identify examples of SCC elements lacking *mecA*. Additional primers and molecular beacons used in this study included (i) a primer-probe set targeting the J1 region of SCC*mec* subtype IVa and (ii) a primer-probe set specific for the protein A (*spa*) gene of *S. aureus* (70), used to differentiate *S. aureus* from CoNS and to confirm MB-PCR results for methicillin-susceptible *S. aureus* (MSSA) strains.

DNA template isolation. Strains were streaked onto nutrient agar and grown overnight at 37°C. A loopful of bacterial growth was suspended in 100 μl of sterile distilled water containing 20 μg/ml lysostaphin (Sigma-Aldrich, St. Louis, MO), incubated at room temperature for 5 min, and then boiled for 10 min. After centrifugation at 10,000 × *g* for 5 min, the supernatant was diluted 40-fold into sterile water, and 1 μl of the diluted supernatant was used as a template in a 10-μl PCR mixture. For high-throughput applications, the DNA isolation procedure was scaled up using 96-well raised PCR plates as follows. One hundred microliters of the 20-μg/ml lysostaphin mixture was dispensed into each well, and as many as 96 strains were inoculated using sterile pipette tips. The plate was sealed using PCR strip caps overlaid by an aluminum plate sealer; then it was incubated and boiled as described above. Centrifugation was performed at 10,000 × *g* for 5 min in a centrifuge with microtiter plate adapters. Then the supernatant was diluted 40-fold as described above into a new 96-well plate. Diluted DNA lysates were used directly for PCR or stored at −20°C.

PCR amplification. Two multiplex real-time PCR panels were used in this assay (molecular beacon real-time PCR [MB-PCR] panels I and II). Each MB-PCR panel includes four sets of molecular beacons (four dyes), which are accommodated by most commercial real-time PCR instruments. The combination of the two panels results in a minimum of two specific targets for each SCC*mec* prototype (Table 2); for the purposes of this study, detection of both targets (*mec* class and *ccr* allotype) is considered "definitive" identification. MB-PCR panel I consists of primers and beacons targeting *mecA* (all SCC*mec* types), *ccrB2* (SCC*mec* types II and IV), *mecI* (SCC*mec* types II, III, and VIII), and IS*1272J* (SCC*mec* types I, IV, and VI) and is therefore capable of definitively identifying SCC*mec* types II and IV in a single reaction, as well as presumptively identifying the remaining SCC*mec* prototypes. MB-PCR panel II is designed to identify *ccrC* (SCC*Hg* [SCC*mec* type III], SCC*mec* V), *ccrB1/3* (SCC*mec* type I or III), *ccrB4* (SCC*mec* types VI and VIII), and the *mec*C2 complex (SCC*mec* type V), thereby definitively identifying SCC*mec* type V and confirming SCC*mec* types I, III, VI, and VIII. In MB-PCR panel II, *ccrB1* and *ccrB3* are detected by different primer sets but share the same molecular beacon (*ccrB1/3*); this hybrid target is a perfect match for *ccrB1* but exhibits a single-nucleotide mismatch relative to *ccrB3* (Table 1).

All amplifications were performed using the Mx4000 multiplex quantitative PCR system (Stratagene, La Jolla, CA). For both MB-PCR panels, each 10-μl reaction mixture consisted of 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), 4 mM $MgCl_2$, 250 μM each deoxynucleoside triphosphate, and 0.4 μM each primer (Table 1) in 1× PCR buffer (Applied Biosystems). Optimized molecular beacon probe concentrations for each panel were as follows: for panel I, 0.2 μM for *ccrB2*, 0.1 μM for *mecA* and IS*1272J*, and 0.05 μM for *mecI*; for panel II, 0.1 μM for *ccrC* and *ccrB4* and 0.2 μM for *ccrB1/3* and *mec*C2. Optimal cycling conditions included an initial denaturation step of 95°C for 10 min, followed by 40 cycles of 95°C for 30 s (denaturation), 50°C for 30 s (annealing), and 72°C for 30 s (extension). The entire PCR can be performed in less than 2 h on the Mx4000 instrument.

MB-PCR sensitivity. The analytical sensitivity of MB-PCR panels I and II was estimated by performing limiting dilution experiments using both purified genomic DNA and bacterial cultures. Six prototype strains (NCTC10442, N315, 85/2082, CA05, WIS, and HDE288) were grown overnight in Trypticase soy broth at 37°C with shaking and were used for both of the following procedures. To estimate sensitivity based on the genomic DNA copy number, 1 ml of each culture was subjected to DNA isolation using a Promega (Madison, WI) Wizard genomic DNA purification kit. Each eluate was diluted serially in sterile double-distilled water (ddH_2O), and the DNA concentration was measured using the Quant-iT PicoGreen dsDNA assay kit from Invitrogen (Molecular Probes, Eugene, OR). One microliter of each dilution was then used for PCR as described above, and limits of detection for each MB-PCR target were calculated using a previously described approach (23). To estimate sensitivity based on bacterial CFU, the remainder of the overnight cultures were pelleted by centrifugation and resuspended in sterile saline to an optical density corresponding to an A_{600} of 1.0. Each suspension was then used to prepare serial 10-fold dilutions using sterile ddH_2O. DNA template isolation was performed on 100 μl of each dilution using the boiling lysis procedure described above, and 1 μl of each lysate was used for PCR. The number of CFU per suspension (CFU per milliliter) was estimated by standard plating procedures and was then used to calculate limits of detection for the individual targets in both MB-PCR panels.

TABLE 1. Molecular beacons and primers used in this study

Primer/probe[a]	Target	Sequence (5′–3′)[b]	Size of amplicon(bp)	5′ fluorescent dye[c]	3′ quencher[c]
MB-PCR panel I					
mecA-F	*mecA*	GCAATACAATCGCACATACATT	148		
mecA-R		CCTGTTTGAGGGTGGATAGC			
mecA-MB		cgcga GATGGCAAAGATATTCAACTAACTA tcgcg		FAM	BHQ-1
ccrB2-F	Type 2 *ccr*	CTCATGTTACARATACTTGCG	107		
ccrB2-R		CCTTGATAATAGCCTTCTTGG			
ccrB2-MB		cgcgat TCTCGACAATGTTATTACGTTCG atcgcg		HEX	DABCYL
mecI-F	Class A	CGTTATAAGTGTACGAATGGTTTTTG	126		
mecI-R	*mec*	TCATCTGCAGAATGGGAAGTT			
mecI-MB		cgcga ACTCCAGTCCTTTTGCATTTGTA tcgcg		CAL Fluor Red 610	BHQ-2
IS1272J-F	Class B	GAAGCTTTGGGCGATAAAGA	98		
IS1272J-R	*mec*	GCACTGTCTCGTTTAGACCAATC			
IS1272J-MB		cgcga AGCCATTGCACATGAGTTAA tcgcg		Quasar 670	BHQ-2
MB-PCR panel II					
ccrC-F	Type 5 *ccr*	TCCAGTCTATAAAGGSTATGTCAG	124		
ccrC-R		ACTTATAATGGCTTCATGCTTACC			
ccrC-MB		cgcgat GCTGTTCAGCGAAGAAGTGGTAA atcgcg		FAM	DABCYL
ccrB1-F	Type 1 and	ACCACAAACACACTTAAAGATG	150		
ccrB1-R	3 *ccr*	CAATTTCAAGTATTTGGTCCATAAC			
ccrB3-F		AACACAACGAACACATTGAAAG	130		
ccrB3-R		CGTATTTCTCAATCACATCAGC			
ccrB1/3-MB[d]		cgcgat AAAGGCTCAAAGGTATGTTCTGC atcgcg		HEX	DABCYL
ccrB4-F	Type 4 *ccr*	CGAAGTATAGACACTGGAGCGATA	134		
ccrB4-R		GCGACTCTCTTGGCGTTTA			
ccrB4-MB		cgcga GACGAAAAGGACTCAATGAGG tcgcg		CAL Fluor Red 610	BHQ-2
mecC2-F	Class C2	TCAGTTCATTGCTCACGATATG	174		
mecC2-R	*mec*	GCCAACGGCTACAGTGATAA			
mecC2-MB		cgcga ACCAAAAGGAGTCTTCTGTATG tcgcg		Quasar 670	BHQ-2
SCC*mec* IVa subtyping					
IVa-J1-F	IVa-J1	CAGGATATTTTTCAAACTCCTTCA	131		
IVa-J1-R	region	TGGAGGACCAAGGATATTCG			
IVa-J1-MB		cgcga CATTAGTGTCCATGTCCGTT tcgcg		CAL Fluor Red 610	BHQ-2
spa (*S. aureus*)[e]					
spa-F	Protein A	CATTACTTATATCTGGTGGCG	98		
spa-R		GTTAGGCATATTTAAGACTTG			
spa-MB		cgcga TTGTTGAGCTTCATCGTGTTG tcgcg		Quasar 670	BHQ-2

[a] F, forward primer; R, reverse primer; MB, molecular beacon.
[b] Complementary arm sequences (hairpins) in each molecular beacon are shown in lowercase.
[c] FAM, fluorescein; HEX, hexachlorofluorescein; BHQ, Black Hole Quencher; DABCYL, 4-(4′-dimethylaminophenylazo)benzoic acid.
[d] The mismatch site between *ccrB1* and *ccrB3* is underlined; a 150-bp amplicon is generated with the *ccrB1* primers, versus a 130-bp amplicon with the *ccrB3* primers.
[e] *S. aureus*-specific primer-probe set (70) used to distinguish *S. aureus* from CoNS and to confirm MB-PCR results for MSSA.

TABLE 2. SCC*mec* classification by MB-PCR panels and targets used in this study

SCC*mec* type	*ccr* type and *mec* class[a]	Typing result by:							
		MB-PCR panel I				MB-PCR panel II			
		mecA	*ccrB2*	*mecI*	IS*1272J*	*ccrC*	*ccrB1/3*	*ccrB4*	*mec*C2
I	1B	+			+		+		
II	2A	+	+	+					
III	3A	+		+		(+)[b]	+		
IV	2B	+	+		+				
V	5C2	+				+			+
VI	4B	+			+			+	
VIII[c]	4A	+		+				+	

[a] According to the nomenclature system proposed by Chongtrakool et al. (9).
[b] (+), *ccrC* is also present in SCC*mec* III strains containing SCC*Hg*.
[c] Novel SCC*mec* type recently described by Zhang et al. (83).

MB-PCR specificity. The analytical specificities of all primer-probe sets in both MB-PCR panels were tested using a variety of nonstaphylococcal species (50 strains), selected for phylogenetic relatedness as well as nosocomial importance. Gram-positive bacterial strains included *Enterococcus* spp. ($n = 12$), *Streptococcus* spp. ($n = 8$), and *Macrococcus* spp. ($n = 2$). Gram-negative bacterial strains included *Enterobacter* spp. ($n = 4$), *Klebsiella* spp. ($n = 4$), *Acinetobacter* spp. ($n = 4$), *Pseudomonas* spp. ($n = 4$), *Serratia* spp. ($n = 4$), and *Escherichia coli* ($n = 4$). Four fungal strains (*Candida albicans*) were also included. All bacterial strains were obtained from the American Type Culture Collection (ATCC) or else from our own collections; *C. albicans* strains were generously provided by D. Perlin (PHRI).

Validation and application of MB-PCR for SCC*mec* typing. The MB-PCR scheme was initially optimized using the 13 prototype MRSA strains listed above. Then it was validated using a well-studied MRSA collection (59 strains, including the 13 prototypes) consisting of reference strains previously characterized with respect to genetic background and SCC*mec* type and chosen to maximize geographical and temporal diversity (Table 3). All strains were from our collections (Instituto de Tecnologia Química e Biológica, Rockefeller University, PHRI), except for the strains from T. Ito mentioned above and five MRSA carriage isolates from swine ($n = 3$) and swine workers ($n = 2$), generously provided by T. Smith of the University of Iowa (71). An additional 68 strains from our own

TABLE 3. Representative collection of MRSA strains used for MB-PCR validation

Isolate ID[a]	Geographic origin	Yr of isolation	Genotypic background				SCC*mec* type (previous)	MB-PCR SCC*mec* typing result[b]									Reference
			CC	ST	Allelic profile (MLST)	*spa*-type (Ridom)	*spa* repeat pattern (eGenomics)		*mecA* (control)	*ccrB2* (type 2 *ccr*)	*mecI* (class A *mec*)	IS*1272J* (class B *mec*)	*ccrC* (type 5 *ccr*)	*ccrB1*/3 (type 1/3 *ccr*)	*ccrB4* (type 4 *ccr*)	*mec*C2 (class C2 *mec*)	SCC*mec* (this study)	
NCTC10442	United Kingdom	1961	8	250	3-3-1-1-4-4-16	t008	YHGFMBQBLO	I	+	−	−	+	−	+	−	−	I	29
HAR21	Finland	2002	5	5	1-4-1-4-12-1-10	t001	TO2MBMDMGMK	I	+	−	−	+	−	+	−	−	I	59
HAR40	Belgium	1995	5	228	1-4-1-4-12-24-29	t001	TO2MBMDMGMK	I	+	−	−	+	−	+	−	−	I	59
E2125	Denmark	1964	8	247	3-3-1-12-4-4-16	t051	YHFGFMBQBLO	I	+	−	−	+	−	+	−	−	I	61
COL	United Kingdom	1960	8	250	3-3-1-1-4-4-16	t051	YHFGFMBQBLO	I	+	−	−	+	−	+	−	−	I	61
UK13136	United Kingdom	1965	8	250	3-3-1-1-4-4-16	t2481	YHFGFMBBLO	I	+	−	−	+	−	+	−	−	I	61
BK793	Egypt	1961	8	250	3-3-1-1-4-4-16	t008	YHGFMBQBLO	I	+	−	−	+	+	+	−	−	I	61
HPV107	Portugal	1992	8	247	3-3-1-12-4-4-16	t051	YHFGFMBQBLO	IA	+	−	−	+	−	+	−	−	I	61
BK1953	United States	1995	8	247	3-3-1-12-4-4-16	t051	YHFGFMBQBLO	IA	+	−	−	+	−	+	−	−	I	61
PER88	Spain	1992	8	247	3-3-1-12-4-4-16	t051	YHFGFMBQBLO	IA	+	−	−	+	−	+	−	−	I	61
PER184	Spain	1991	8	247	3-3-1-12-4-4-16	t121	YHFMBQBLO	IA	+	−	−	+	−	+	−	−	I	61
PER 34	Spain	1989	8	250	3-3-1-1-4-4-16	t051	YHFGFMBQBLO	IA	+	−	−	+	−	+	−	−	I	61
PL72	Poland	1991	5	5	1-4-1-4-12-1-10	t053	TJMBMDMGMB	I-var	+	−	−	+	−	+	−	−	I	59
POL3	Poland	1992	5	5	1-4-1-4-12-1-10	t053	TJMBMDMGMB	I-var	+	−	−	+	−	+	−	−	I	59
N315	Japan	1982	5	5	1-4-1-4-12-1-10	t002	TJMBMDMGMK	II	+	+	+	−	−	−	−	−	II	30
Mu3	Japan	1997	5	5	1-4-1-4-12-1-10	t002	TJMBMDMGMK	II	+	+	+	−	−	−	−	−	II	25
Mu50	Japan	1997	5	5	1-4-1-4-12-1-10	t002	TJMBMDMGMK	II	+	+	+	−	−	−	−	−	II	41
BK2464	United States	1996	5	5	1-4-1-4-12-1-10	t002	TJMBMDMGMK	II	+	+	+	−	−	−	−	−	II	57
MRSA252	United Kingdom	1997	30	36	2-2-2-2-3-3-2	t018	WGKAKAO MQQQ	II	+	+	+	−	−	−	−	−	II	27
BK351	Ireland	1991	8	8	3-3-1-1-4-4-3	t190	YMBQBLO	IIA	+	+	+	−	−	−	−	−	II	40
JCSC3063	Japan	2001–2002	91[c]	89	1-26-28-18-18-33-50	t375	Y2EJCMBPB	IIb	+	+	+	−	−	−	−	−	II	26
RN7170	Canada	1989	5	5	1-4-1-4-12-1-10	t002	TJMBMDMGMK	IIE	+	+	+	−	−	−	−	−	II	40
85/2082	New Zealand	1985	8	239	2-3-1-1-4-4-3	t037	WGKAOMQ	III	+	−	+	−	+	+	−	−	III	29
ANS46	Australia	1982	8	239	2-3-1-1-4-4-3	t037	WGKAOMQ	III	+	−	+	−	+	+	−	−	III	61
R35	United States	1987	8	239	2-3-1-1-4-4-3	t037	WGKAOMQ	III	+	−	+	−	+	+	−	−	III	61
LHH1	United States	1994	8	239	2-3-1-1-4-4-3	t037	WGKAOMQ	III	+	−	+	−	+	+	−	−	III	61
BK2421	United States	1996	8	239	2-3-1-1-4-4-3	t037	WGKAOMQ	III	+	−	+	−	+	+	−	−	III	61
HSJ216	Portugal	1997	8	239	2-3-1-1-4-4-3	t037	WGKAOMQ	III	+	−	+	−	+	+	−	−	III	61
HU106	Hungary	1996	8	239	2-3-1-1-4-4-3	t538	WGKAOKAOK AOMQ	III	+	−	+	−	+	+	−	−	III	61
HUSA304	Hungary	1993	8	239	2-3-1-1-4-4-3	[d]	WKAKAOKAOMQ	III	+	−	+	−	+	+	−	−	III	61
HU25	Brazil	1993	8	239	2-3-1-1-4-4-3	t138	XKAOMQ	IIIA	+	−	+	−	+	+	−	−	III	61
HDG2	Portugal	1993	8	239	2-3-1-1-4-4-3	t421	WGKAOM	IIIB	+	−	+	−	−	+	−	−	III	61
DEN907	Denmark	2001	8	239	2-3-1-1-4-4-3	t037	WGKAOMQ	NT[e]	+	−	+	−	−	+	−	−	III	59
CA05	United States	1999	45	256	10-14-8-6-10-37-2	t040	A2AKEMBKB	IVa	+	+	−	+	−	−	−	−	IV	50
MW2	United States	1998	1	1	1-1-1-1-1-1-1	t128	UJJFKBPE	IVa	+	+	−	+	−	−	−	−	IV	4
HSA74	Portugal	1993	5	5	1-4-1-4-12-1-10	t002	TJMBMDMGMK	IVa	+	+	−	+	−	−	−	−	IV	58
FFP311	Portugal	1996	5	5	1-4-1-4-12-1-10	t311	TJMBDMGMK	IVa	+	+	−	+	−	−	−	−	IV	58
FPR3757	United States	2003	8	8	3-3-1-1-4-4-3	t008	YHGFMBQBLO	IVa	+	+	−	+	−	−	−	−	IV	13
8/6-3P	United States	1996	8	770	1-3-1-1-4-4-3	t008	YHGFMBQBLO	IVb	+	+	−	+	−	−	−	−	IV	50
JCSC4788	Japan	2001	8	451	3-4-1-42-4-4-3	t1852	YGFMFMBQBLO	IVc	+	+	−	+	−	−	−	−	IV	37
ARG9	Argentina	1996	5	5	1-4-1-4-12-1-10	t002	TJMBMDMGMK	IVc	+	+	−	+	−	−	−	−	IV	58
Q2314	United States	1996	5	5	1-4-1-4-12-1-10	t088	TJMBMDMGGMK	IVc	+	+	−	+	−	−	−	−	IV	1
JCSC4469	Japan	1982	5	5	1-4-1-4-12-1-10	t002	TJMBMDMGMK	IVd	+	+	−	+	−	−	−	−	IV	49
AR43/3330.1	Ireland	1988–2002	8	8	3-3-1-1-4-4-3	t190	YMBQBLO	IVE	+	+	−	+	−	−	+	−	IV	68
HAR22	Finland	2002	22	22	7-6-1-5-8-8-6	t022	TJEJNF2MNF2MO MOKR	IVh	+	+	−	+	−	−	−	−	IV	52
HAR36	Greece	2002	8	254	3-12-1-1-4-4-3	t009	YGFMBQBLQ BLPO	IVh	+	+	−	+	+	−	−	−	IV	52
GRE120	Greece	1993	8	8	3-3-1-1-4-4-3	t036	YGFMBQBLPO	IV	+	+	−	+	−	−	−	−	IV	2
BARG II 17	United States	1996	8	8	3-3-1-1-4-4-3	t064	YHGCMBQBLO	IV	+	+	−	+	−	−	−	−	IV	61
WBG10049	Australia	1999	30	30	2-2-2-2-6-3-2	t019	XKAKAOMQ	IV	+	+	−	+	−	−	−	−	IV	64
COB3	Colombia	1996	5	5	1-4-1-4-12-1-10	t1107	TMDMGK	IV_{NT}	+	+	−	+	−	−	−	−	IV	61
WIS	Australia		45	45	10-14-8-6-10-37-2	t123	A2AKBEKBKB	V	+	−	−	−	+	−	−	+	V	31

HT0826	France	2003	152	377	46-75-49-50-13-68-60	t355	UJ2GMKKPNSG	V	+	–	–	+	–	–	+	V	53
SW31.1	United States	2008	398	398	3-35-19-2-20-26-39	t034	XKAOAOBQ	V	+	–	–	+	–	–	+	V	71
SW62.1	United States	2008	398	398	3-35-19-2-20-26-39	t034	XKAOAOBQ	V	+	–	–	+	–	–	+	V	71
SW181.1	United States	2008	398	398	3-35-19-2-20-26-39	t034	XKAOAOBQ	V	+	–	–	+	–	–	+	V	71
HU01010T	United States	2008	398	398	3-35-19-2-20-26-39	t034	XKAOAOBQ	V	+	–	–	+	–	–	+	V	71
HU01011N	United States	2008	398	398	3-35-19-2-20-26-39	t034	XKAOAOBQ	V	+	–	–	+	–	–	+	V	71
HDE288	Portugal	1996	5	5	1-4-1-4-12-1-10	t311	TJMBDMGMK	VI	+	–	–	+	–	+	–	VI	58
IPO92	Portugal	2001	5	5	1-4-1-4-12-1-10	t311	TJMBDMGMK	VI	+	–	–	+	–	+	–	VI	58

[a] Prototype strains used to optimize the MB-PCR assay are in boldface.
[b] +, detected; –, not detected.
[c] ST89 is currently assigned to CC509 by eBURST, version 3 (17).
[d] The spa repeat pattern WKAKAOKAOMQ is not recognized by the Ridom SpaServer as of this writing.
[e] NT, nontypeable.

collections, previously SCC*mec* typed by conventional multiplex PCR (40), were also tested using our MB-PCR assay, and the results were compared with those of Kondo et al. (40) (Table 4). A blinded collection of clinical MRSA strains from Canada ($n = 35$), selected to include diverse SCC*mec* types and subtypes previously characterized by traditional SCC*mec* typing methods (29, 31, 56, 57) and antibiotic susceptibility testing, was also tested (Table 4).

The applicability of the MB-PCR scheme was investigated further by performing SCC*mec* typing on a variety of clinical *S. aureus* isolates (Table 4), including (i) 66 MRSA and 135 MSSA strains (range, NRS1 to NRS407) from the collection of the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA), representing both geographically and temporally diverse isolates for which the genetic background has been determined by *spa* typing and MLST (see below); (ii) a well-characterized collection of historical MRSA isolates ($n = 270$) from a 1996 study (Bacterial Antibiotic Resistance Group [BARG] study) encompassing 12 New York City (NYC) hospitals (62); (iii) a large collection ($n = 639$) of isolates from community-associated MRSA infections, collected from 2004 to 2006 in outpatient settings throughout the NYC area; and (iv) a similarly sized collection ($n = 573$) of carriage and invasive isolates collected from 2007 to 2008 in several NYC area hospitals.

Lastly, our MB-PCR assay was tentatively used to type 71 staphylococcal strains representing the following species: *Staphylococcus aureus* subsp. *anaerobius* ($n = 1$), *S. capitis* ($n = 6$), *S. epidermidis* ($n = 25$), *S. gallinarum* ($n = 1$), *S. haemolyticus* ($n = 7$), *S. hominis* ($n = 4$), *S. lentus* ($n = 2$), *S. lugdunensis* ($n = 4$), *S. pseudintermedius* ($n = 2$), *S. saprophyticus* ($n = 5$), *S. sciuri* ($n = 2$), *S. simulans* ($n = 4$), *S. succinus* ($n = 1$), *S. xylosus* ($n = 5$), and *S. warneri* ($n = 2$) (Table 5). Staphylococcal strains were obtained from ATCC, NARSA, and our own collections; one *S. lentus* strain was provided by T. Smith of the University of Iowa.

Molecular typing. All *S. aureus* isolates used in the study were characterized genotypically by *spa* typing using eGenomics software, as described previously (51, 67); Ridom *spa* types were subsequently assigned using the *spa* typing website (http://www.spaserver.ridom.de/) developed by Ridom GmbH and curated by SeqNet.org (http://www.SeqNet.org/) (22). MLST was performed on all NARSA strains (data not shown) as described previously (14); MLST data for all other strains in Table 3 were obtained directly from the literature. For all strains in Table 4, the genotypic background is given in terms of the MLST "clonal complex" (CC), as determined by eBURST analysis (17); except for the NARSA strains, all CCs in Table 4 were inferred by *spa* repeat pattern analysis (51, 73).

DNA sequencing. During the development of this assay, four isolates with the combination of *ccrAB4* and the class A *mec* complex were found. The entire SCC*mec* region of one isolate (BK20781) was amplified using four sets of primers ranging from the right chromosomal junction to *orfX* (Table 6) and was then sequenced by primer walking.

Nucleotide sequence accession number. The sequence of the SCC*mec* region of BK20781 was submitted to GenBank as accession number FJ670542.

RESULTS

MB-PCR targets. In order to design the real-time PCR SCC*mec* typing platform, an extensive sequence similarity search was conducted using NCBI BLAST (3), followed by comprehensive alignment of all the *S. aureus* SCC*mec* sequences available in GenBank prior to 2009. For each SCC*mec* type available during the design phase (types I to VI), two loci were selected for target design, based on the combination of *mec* and *ccr* complex. A conserved region of *mecA* was selected as an internal control for methicillin resistance (Table 1). Two four-color multiplex PCR panels were then optimized and validated for the differentiation of SCC*mec* types I to VI.

Four targets (*mecA*, *ccrB2*, *mecI*, and IS*1272J*) were included in MB-PCR panel I, which was designed to identify methicillin resistance and definitively classify SCC*mec* types II (*mecA*, *ccrB2*, *mecI*) and IV (*mecA*, *ccrB2*, IS*1272J*) in a single reaction, with each type exhibiting three positive amplification curves (Fig. 2C and G). SCC*mec* types I and VI also possess the IS*1272J* target, resulting in two indistinguishable amplification curves (Fig. 2A and K), while SCC*mec* III possesses *mecI* (Fig. 2E) and SCC*mec* V is positive only for *mecA* (Fig.

TABLE 4. SCC*mec* MB-PCR results for MRSA and MSSA strains from various collections

Source or study	Geographic origin	Yr of isolation	Genotypic background[a]	MB-PCR result[b]				No. (%) of isolates
				mecA	*ccr* type	*mec* class	SCC*mec*	
Kondo et al., 2007 (40) (n = 68)[d]	Canada, Denmark, Egypt, Ireland, Switzerland, United Kingdom, United States	1960–1993	CC5	+	1	B	I	1 (1.5)
				+	2	A	II	22 (32.4)
				+	2	B	IV	2 (2.9)
			CC8	+	1	B	I	13 (19.1)
				+	1, C	B	I[c]	1 (1.5)
				+	2	A	II	5 (7.4)
				+	3	A	III	2 (2.9)
				+	3, C	A	III	6 (8.8)
				+	2	B	IV	2 (2.9)
			CC25	+	2	A	II	1 (1.5)
			CC30	+	2	A	II	3 (4.4)
				+	3, C	A	III	1 (1.5)
				+	–	–	NT	1 (1.5)
			CC45	+	2	A	II	1 (1.5)
			CC59	+	3	A	III	1 (1.5)
			CC88	+	2	A	II	1 (1.5)
			CC121	+	1	B	I	1 (1.5)
			Other	+	3	A	III	1 (1.5)
				+	3, C	A	III	1 (1.5)
				+	2	B	IV	1 (1.5)
				+	–	A	NT	1 (1.5)
Canada (n = 35)	Canada	2007–2009	CC5	+	2	A	II	5 (14.3)
				+	2	B	IV	5 (14.3)
				+	4	B	VI	1 (2.9)
			CC8	+	3, C	A	III	1 (2.9)
				+	2	B	IV	12 (34.3)
				+	C	C2	V	1 (2.9)
				+	4	A	VIII	1 (2.9)
			CC12	+	C	C2	V	1 (2.9)
			CC22	+	2	B	IV	1 (2.9)
			CC30	+	2	B	IV	1 (2.9)
				+	C	C2	V	1 (2.9)
			CC45	+	2	B	IV	2 (5.7)
			CC59	+	C	C2	V	2 (5.7)
			CC80	+	2	B	IV	1 (2.9)
NARSA MRSA (n = 66)[d]	Belgium, Brazil, France, Japan, Oman, South Korea, Switzerland, United Kingdom, United States	1935–2004	CC1	+	2	B	IV	5 (7.6)
			CC5	+	2	A	II	21 (31.8)
				+	2	B	IV	1 (1.5)
			CC8	+	1	B	I	11 (16.7)
				+	2	A	II	1 (1.5)
				+	3, C	A	III	4 (6.1)
				+	2	B	IV	14 (21.2)
			CC22	+	2	B	IV	1 (1.5)
			CC30	+	2	A	II	3 (4.5)
			CC45	+	2	A	II	2 (3.0)
			CC59	+	2	B	IV	1 (1.5)
			CC80	+	2	B	IV	1 (1.5)
			CC88	+	2	B	IV	1 (1.5)
NARSA MSSA (n = 135)			CC1	–	1[e]	–	–	2 (1.5)
				–	–	–	–	2 (1.5)
			CC5	–	–	–	–	14 (10.4)
			CC8	–	–	–	–	34 (25.2)
			CC15	–	–	–	–	5 (3.7)
			CC22	–	–	–	–	8 (5.9)
			CC30	–	–	–	–	28 (20.7)
			CC45	–	–	–	–	5 (3.7)
			CC59	–	–	–	–	1 (0.7)
			CC97	–	2	–	–	2 (1.5)
				–	–	–	–	1 (0.7)
			CC121	–	–	–	–	17 (12.6)
			Other[f]	–	–	–	–	16 (11.9)
BARG (n = 270)	United States (NYC)	1996	CC5	+	2	A	II	130 (48.1)
				+	2	B	IV	10 (3.7)
				+	2, 4	A	NT	1 (0.4)
			CC8	+	2	B	IV	44 (16.3)
				+	3, C	A	III	28 (10.4)
				+	3	A	III	1 (0.4)
				+	1	B	I	16 (5.9)
				+	–	–	NT	1 (0.4)
			CC30	+	2	A	II	20 (7.4)
				+	2, 4	A	NT	1 (0.4)
			CC45	+	2	A	II	4 (1.5)
			Other	+	2	A	II	2 (0.7)
				+	3	A	III	1 (0.4)

Continued on following page

TABLE 4—*Continued*

Source or study	Geographic origin	Yr of isolation	Genotypic background[a]	MB-PCR result[b]				No. (%) of isolates
				mecA	*ccr* type	*mec* class	SCC*mec*	
				+	2	B	IV	5 (1.9)
				+	−	−	NT	1 (0.4)
				−	−	−	MSSA	5 (1.9)
NYC outpatients (n = 639)	United States (NYC)	2004–2006	CC1	+	2	B	IV	23 (3.6)
			CC8	+	2	B	IV	480 (75.1)
				+	2	A	II	1 (0.2)
				+	4	A	VIII	2 (0.3)
			CC5	+	2	A	II	91 (14.2)
				+	2	B	IV	23 (3.6)
			CC30	+	2	A	II	4 (0.6)
				+	2	B	IV	2 (0.3)
			CC45	+	2	A	II	1 (0.2)
				+	2	B	IV	2 (0.3)
			CC59	+	2	B	IV	8 (1.3)
			CC80	+	2	B	IV	1 (0.2)
			CC88	+	2	B	IV	1 (0.2)
NYC inpatients (n = 573)	United States (NYC)	2007–2008	CC5	+	2	B	IV	111 (19.4)
				+	2	A	II	361 (63.0)
				+	−	−	NT	2 (0.3)
			CC8	+	2	B	IV	61 (10.6)
				+	2	A	II	1 (0.2)
				+	C	C2	V	1 (0.2)
				+	4	A	VIII	1 (0.2)
			CC22	+	2	B	IV	1 (0.2)
			CC30	+	2	A	II	13 (2.3)
			CC45	+	2	A	II	18 (3.1)
			CC59	+	2	B	IV	1 (0.2)
				+	−	−	NT	1 (0.2)
			CC398	+	−	−	NT	1 (0.2)

[a] Expressed in terms of the MLST CC; except for the NARSA strains, all CCs were inferred by *spa* repeat pattern analysis (51, 73).
[b] The *ccr* type and *mec* class are given according to the nomenclature proposed by Chongtrakool et al. (9). NT, nontypeable; +, *mecA* positive; −, not detected.
[c] The structure is similar to that of BK793; this isolate possesses an SCC*Hg* region, as reported previously (40).
[d] Not including strains already listed in Table 3.
[e] To prevent misclassification, singleplex PCRs using *ccrB1*- and *ccrB3*-specific primers in combination with the *ccrB1/3* beacon were conducted separately to confirm the multiplex MB-PCR results.
[f] "Other" NARSA MSSA strains include STs 10, 12, 25, 109, 395, 398, 707, 846, and 890.

2I); classification of these prototypes requires the second panel.

MB-PCR panel II targets *ccrC*, *ccrB1/3*, *ccrB4*, and the *mec*C2 complex and can identify SCC*mec* types I, III, V, and VI in combination with the results from MB-PCR panel I. As described in Materials and Methods, *ccrB1* and *ccrB3* utilize the same molecular beacon (*ccrB1/3*); this is a compromise measure adopted in order to accommodate two targets for each prototype by using only four dyes per panel. The amplification curve of *ccrB3* exhibits approximately half the magnitude of the amplification curve of *ccrB1* (Fig. 2B and F), due to the presence of a single-nucleotide mismatch between the *ccrB3* target and the *ccrB1/3* beacon (Table 1). SCC*mec* type V displays two amplification curves (*ccrC* and *mec*C2), while SCC*mec* types I (*ccrB1*) and VI (*ccrB4*) each exhibit only one curve (Fig. 2B and L), in MB-PCR panel II. The *ccrC* target is also present in SCC*mec* III elements that harbor SCC*Hg* (Fig. 2F) and by itself is not indicative of SCC*mec* type III or V.

Sensitivity of MB-PCR. The sensitivity of the MB-PCR platform was evaluated utilizing the following six SCC*mec* reference strains: NCTC10442 (type I), N315 (type II), 85/2082 (type III), CA05 (type IV), WIS (type V), and HDE288 (type VI). Limits of detection based on DNA copy numbers were obtained using purified genomic DNA as described in Materials and Methods, and the following lower limits were reliably detected within 40 cycles: 10 to 25 copies per PCR for *mecA*, IS*1272J*, and *mecI*; and 25 to 40 copies per PCR for *ccrB2*, *ccrB1/3, ccrB4, ccrC*, and *mec*C2. Limits of detection based on bacterial CFU were also estimated using the rapid DNA boiling lysis procedure described above; the assay was capable of reproducibly detecting as few as 0.5×10^3 CFU per PCR for *mecA* and *mecI*; 1.5×10^3 CFU for IS*1272J*, *ccrB1/3*, and *ccrB2*; and 4×10^3 CFU for *ccrB4*, *ccrC*, and *mec*C2.

Specificity of MB-PCR. As shown in Fig. 2, all molecular beacon probe-primer sets displayed good specificity for their respective SCC*mec* targets; no cross-reactivity was observed between the various *ccrB* and *ccrC* beacons, or among class A, B, and C2 *mec* complex beacons. Additionally, no amplification was observed for any of the molecular beacon targets with respect to the 50 nonstaphylococcal strains described in Materials and Methods, in agreement with the BLAST search results obtained during the assay design phase.

Validation of the MB-PCR assay. In order to validate the MB-PCR assay, a geographically, temporally, and genotypically diverse MRSA collection (n = 59) that had been characterized previously was tested using both panels (Table 3). We obtained 100% concordance for all isolates of SCC*mec* types I to VI, except for a type III strain (DEN907) previously described as nontypeable for *ccrAB* by conventional PCR and not assigned to a specific SCC*mec* type (57, 59). This strain was subsequently reanalyzed by *ccrB* sequence typing and was shown to cluster with *ccrAB3* (59, 60), in agreement with our

TABLE 5. MB-PCR SCC*mec* typing results for staphylococcal isolates (n = 71)

Species	MB-PCR result[a]				No. of isolates
	mecA	*ccr* type[b]	*mec* class	SCC*mec*	
S. epidermidis	+	3, C	A	III	6
	+	3	A	III	4
	+	2	B	IV	4
	+	2	A	II	1
	+	2, 3, 4	A	NT	2
	+	2, 4	A	NT	1
	+	−	−	NT	1
	−	2, 4	−	Unk	1
	−	2, 4, C	−	Unk	1
	−	−	−	−	4
S. haemolyticus	+	C	C2	V	4
	+	2	B	IV	1
	+	−	−	NT	1
	−	4	−	Unk	1
S. capitis	+	2	A	II	4
	+	3, 4, C	A	NT	1
	−	−	−	−	1
S. hominis	+	3, 4, C	A	NT	1
	+	3, 4	A	NT	3
S. saprophyticus	−	C	−	Unk	1
	−	−	B	Unk	1
	−	−	−	−	3
S. lugdunensis	−	3	−	Unk	1
	−	−	−	−	3
S. simulans	−	4	−	Unk	2
	−	−	−	−	2
S. lentus	+	−	A	NT	1
	−	3	−	Unk	1
S. xylosus	−	−	−	−	5
S. pseudintermedius	−	−	−	−	2
S. sciuri	−	−	−	−	2
S. warneri	−	−	−	−	2
S. gallinarum	−	−	−	−	1
S. succinus	−	−	−	−	1
S. aureus subsp. *anaerobius*	−	−	−	−	1

[a] The *ccr* type and *mec* class are given according to the nomenclature proposed by Chongtrakool et al. (9). NT, nontypeable SCC*mec*; Unk, unknown SCC structure; +, *mecA* positive; −, not detected.

[b] To prevent misclassification, singleplex PCRs using *ccrB1*- and *ccrB3*-specific primers in combination with the *ccrB1/3* beacon were conducted separately to confirm multiplex MB-PCR results.

MB-PCR result of SCC*mec* III (Table 3). Several mosaic/composite staphylococcal cassettes with more than one *ccr* locus (not including SCC*Hg*) were also identified. For example, strains BK793 (SCC*mec* I) and HAR36 (SCC*mec* IV) both possess *ccrC* in addition to their "native" *ccr* elements; similarly, strain AR43/3330.1 (SCC*mec* IVE) harbors *ccrAB4* in addition to *ccrB2*.

The MB-PCR assay was validated further using 68 MRSA isolates previously characterized by the SCC*mec* typing strategy described by Kondo et al. (40). Once again, our MB-PCR displayed 100% concordance with previously reported results; however, two strains classified as nontypeable in the study of Kondo et al. could not be successfully classified by our method, either (Table 4). Additionally, 35 blinded MRSA isolates from a Canadian laboratory (Table 4) displayed complete agreement with previous results obtained using various traditional SCC*mec* typing methods (29, 31, 56, 57), except for 1 isolate deemed nontypeable by conventional PCR. This isolate (BK23684) was positive by our MB-PCR method for both *mecI* and *ccrB4*, with a structure similar to that described recently for strain C10682 (GenBank accession number FJ390057), an epidemic Canadian clone (CMRSA-9) that has been classified by the IWG-SCC as SCC*mec* VIII (83).

Application of the multiplex PCR strategy. Several additional *S. aureus* collections (1,683 strains) were also tested in order to investigate the high-throughput applicability of the MB-PCR assay for SCC*mec* typing. Aggregate results are shown in Table 4, grouped according to source/study, genotypic background, and SCC*mec* type, and representing both historical and contemporary MRSA and MSSA isolates from nosocomial as well as outpatient settings. As shown in Table 4, our MB-PCR method was able to successfully type nearly all of the MRSA isolates from each of these collections, except for 10 strains classified as nontypeable. Several nontypeable MRSA strains were positive only for *mecA*, suggesting the possibility of novel SCC*mec* elements or structures. Addition-

TABLE 6. Primers used for long-range PCR and DNA sequencing

Procedure	Primer	Nucleotide sequence (5′–3′)	Region amplified	Size	Reference
Long-range PCR for BK20781	Left-F	ATTCGGTACACGCAATGAAA	Right chromosome junction to *ccrB4*	8.5 kb	This study
	B4-R1	CTTTATGATTCAATGCGTTC			
	A4-F1	TATTGTGTTGCTATCGCTTG	*ccrA4* to Tn*554*	9.5 kb	This study
	Tn554-R	CTAAAAACCAATTCCGACAG			
	Tn554-F	CGGAAAAATACCAAATCAAG	Tn*554* to *mecA*	11.5 kb	This study
	MA-R	AGTTTTTCGAGTCCCTTTTT			
	MA-F	AACAAATGGATCAAAATTGG	*mecA* to left chromosome junction	7.3 kb	This study
	CR2	AAACGACATGAAAATCACCAT			30
Long-range PCR for BK793	ccrC-2	ACTTATAATGGCTTCATGCTTACC	*ccrC* to *orfX*	10 kb	This study
	Xsau325	GGATCAAACGGCCTGCACA			28
Partial *ccrC* amplification and sequencing for BK793 and HAR36	ccrC-F2	CGAAATGGTRTTAAGTTGGAAA	Within *ccrC*	587 bp	This study
	ccrC-R1	TGGCTTCATGYTTWCCTTTG			
Long-range PCR for AR43/3330.1	B4-F4	CGGCAAAGGAACAAACCTAC	*ccrB4* to SCC*mec* J1	9.5 kb	This study
	J IVc-R	ATAGATTCTACTGCAAGTCC			52
Partial *ccrAB4* sequencing for AR43/3330.1, BK2391, and BK2539	A4-F1	TATTGTGTTGCTATCGCTTG	*ccrA4* to *ccrB4*	2.7 kb	This study
	B4-R3	GTGCTAGGGAGCACTTCGTC			

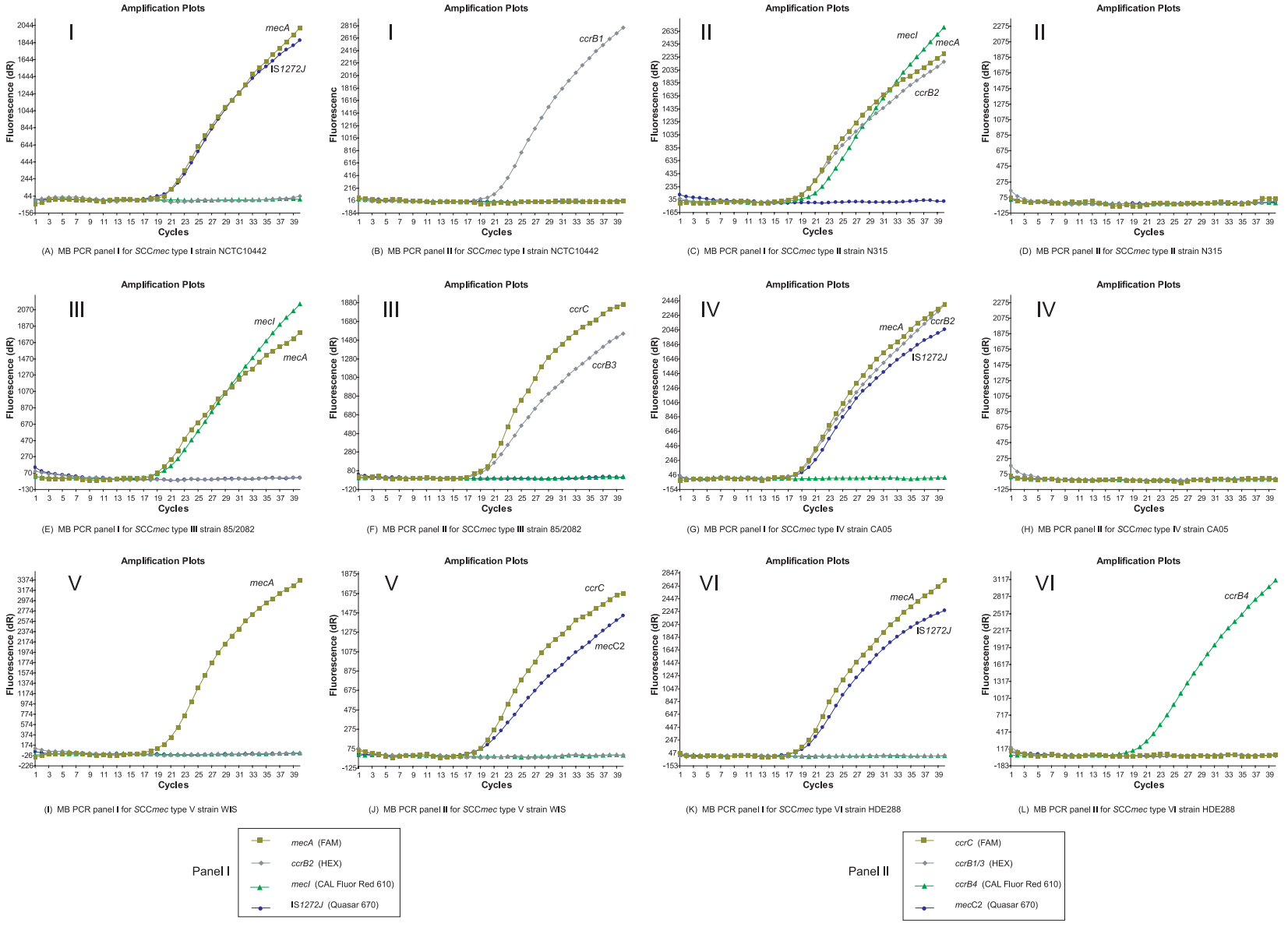

FIG. 2. Stratagene Mx4000 real-time PCR amplification curves for SCC*mec* type I to VI prototype strains (NCTC10442, N315, 85/2082, CA05, WIS, and HDE288). (Left) MB-PCR panel I results; (right) MB-PCR panel II results. Each MB-PCR panel consists of four molecular beacon probes labeled with different fluorescent dyes, as indicated in the keys at the bottom.

ally, two isolates from the BARG study with different genetic backgrounds, BK2391 (CC30) and BK2539 (CC5), possessed two *ccr* loci (*ccrB2*, *ccrB4*) in combination with the class A *mec* complex, a structure similar to that reported recently for two ST8-MRSA-II isolates from Ireland (M06/0075 and M06/0179) (69). Finally, five isolates from the same study, previously described by *mecA*-Tn*554* hybridization typing as "NH:NH" or "NH:unique" (NH stands for "no hybridization") (62), were confirmed as MSSA by our assay (Table 4).

In striking contrast to the BARG collection, which was obtained from 12 NYC area hospitals in 1996, no examples of SCC*mec* type I or III were found among a total of 1,212 MRSA isolates collected from the same region during 2004 to 2008. The majority of the latter isolates were either SCC*mec* II (40.4%) or SCC*mec* IV (58.9%), with the relative prevalence of each type differing according to setting; among nosocomial isolates, SCC*mec* type II was observed at a higher frequency (68.6%), whereas SCC*mec* IV was the predominant type among outpatient isolates (84.5%). Only one SCC*mec* V isolate was discovered in this collection, while no strains bearing SCC*mec* VI were observed. In contrast, three additional SCC*mec* VIII isolates (BK20015, BK20781, and BK25150) were discovered, two of which were found among outpatient CA-MRSA isolates. The entire SCC*mec* region from one of these strains (BK20781) has been sequenced and submitted to GenBank (accession number FJ670542).

An additional 71 staphylococcal strains, representing 15 different species, were also tested by the MB-PCR assay (Table 5); all were negative for the presence of *S. aureus* DNA by use of the *spa* molecular beacon, except for *S. aureus* subsp. *anaerobius*. Among 34 *mecA*-positive, coagulase-negative strains (MR-CoNS), 24 were successfully assigned SCC*mec* types; the rest were considered nontypeable, since they either possessed more than one *ccr* allotype or else were *mecA* positive with no detectable *ccr* or *mec* complex sequence (Table 5). Conversely, several strains positive for *ccr*, and one strain positive for IS*1272J* (*mec* complex B), were observed among *mecA*-negative strains. The *ccrB4* allotype was detected in five CoNS species (13 strains), usually accompanied by other types of *ccr* locus (e.g., *ccrB2*, *ccrB3*).

In contrast, no SCC elements were detected among 135 diverse MSSA strains from the NARSA collection mentioned above, except for 2 ST97 strains positive for *ccrB2* and 2 ST1 strains harboring *ccrB1*, as described previously for the SCC*far* element in MSSA476 (27) (Table 4). All MSSA isolates exhibited positive amplification using the *spa* molecular beacon.

DISCUSSION

SCC*mec* typing is one of the most important molecular tools for exploring the epidemiology and evolution of MRSA (12, 16). Knowledge about the nature of MRSA clones disseminating globally is necessary for implementing strategies to control the transmission of MRSA within nosocomial as well as community settings (6). Consequently, rapid characterization of MRSA strains by SCC*mec* typing is an important issue. A number of multiplex PCR-based SCC*mec* typing methods have been developed over the past several years (7, 40, 47, 53, 57, 84) and are reviewed extensively elsewhere (12). Several of these methods depend on targets located within J regions, in order to characterize both prototypes and subtypes simultaneously; however, since J regions are likely to be less conserved than *ccr* and *mec* complexes, these methods risk sacrificing discriminatory power for convenience. The method described by Zhang and colleagues (84), for example, uses a single J-region target to identify SCC*mec* III and has recently been reported to misclassify SCC*mec* V isolates as type III (33). Another disadvantage shared by these methods is that they are based on electrophoretic analysis of multiple bands, which can be time-consuming, laborious, and potentially subjective. Furthermore, reproducible amplification of all target bands can depend on template quality, thereby compromising high-throughput studies in which DNA isolation is often a rate-limiting step.

Various multiplex schemes not based on conventional PCR have also been reported. A real-time PCR assay utilizing a mixture of TaqMan and minor groove binding probes, and targeting the *ccrB* regions of SCC*mec* types I to IV, was published in 2004 by François et al. (18). More recently, a novel approach utilizing *ccr*-specific padlock probes and tag microarray analysis for identification of SCC*mec* types I to VI has been described (42). A shortcoming of both methods is that they detect only one locus (*ccr*) and ignore the *mec* complex altogether, which may result in misclassification, given the growing number of reports describing composite SCC*mec* elements with multiple *ccr* loci, *ccr* loci not linked to the *mecA* gene, and novel combinations of the *mec* complex and *ccr* in SCC*mec* elements (5, 69, 83). SCC*mec* typing approaches based on DNA sequencing have also been described (45, 59, 60). However, these methods are not suitable for rapid typing and may not work for strains with multiple *ccr* elements (e.g., *ccrAB2* plus *ccrAB4*) or particular *ccr* allotypes (e.g., *ccrC*). Finally, an approach utilizing single-nucleotide polymorphisms has been described, in which a minimum set of 22 markers can be used to identify all presently known SCC*mec* types and subtypes (72). Although no specific detection procedure is described, the authors propose that the single-nucleotide polymorphism marker sets be used to guide the design of real-time PCR or oligoarray hybridization assays. While such an approach would undoubtedly be comprehensive and may be of interest for evolutionary studies, it is inherently impractical for clinical applications or routine genotyping.

SCC*mec* types are defined by binary combinations of the various *mec* class complexes and *ccr* allotypes described to date (Fig. 1). Accordingly, a novel method of nomenclature has been proposed by Chongtrakool et al. (9), in which a given SCC*mec* type is denoted by the *ccr* allotype (Arabic numeral) and *mec* complex (capital letter), as shown in Table 2. It is therefore recommended by the IWG-SCC that SCC*mec* elements be initially assigned using this combination of *mec* class and *ccr* type, followed by differences in J regions for individual subtypes (32). In this study, we developed an MB-PCR platform for rapid typing of SCC*mec* prototypes in MRSA strains based on the recommended system of nomenclature. Our MB-PCR assay targets all five known *ccr* allotypes (*ccrAB1* to *ccrAB4* and *ccrC*), along with the three principal *mec* classes (A, B, and C2) identified thus far in *S. aureus*; the emphasis is on identification of SCC*mec* prototypes, and no attempt is made to subtype.

Our MB-PCR assay demonstrated 100% concordance with

previous characterizations of two MRSA reference collections ($n = 127$), as well as with conventional SCC*mec* typing of a blinded panel of isolates ($n = 35$). In this study, our assay identified only 10 (0.6%) nontypeable isolates out of 1,818 geographically and temporally diverse *S. aureus* strains. Moreover, we successfully identified a previously nontypeable strain (DEN907) as SCC*mec* III, in agreement with previous results based on *ccrB* sequence typing (59). The assay is also capable of readily identifying novel or composite SCC*mec* elements by virtue of the discrete target design. In this study, for instance, we discovered four strains possessing a novel combination of *ccrB4* and the class A *mec* complex, one of which was shown to be nearly identical (>99.9%) by DNA sequencing to a Canadian strain (GenBank accession number FJ390057) that has recently been classified as SCC*mec* type VIII (83). Interestingly, whereas the initial description of SCC*mec* VIII is based on a nosocomial CMRSA-9 strain (C10628) (83), two of the strains described above (BK20015 and BK20781) represent outpatient CA-MRSA isolates from the NYC area. To our knowledge, this is the first report of SCC*mec* VIII outside of Canada.

As described in Results, additional *ccrC* and *ccrB4* elements were also found in several historic *S. aureus* strains (AR43/3330.1, HAR36, BK793, BK2391, and BK2539). An insertion of at least 8 kb between the *dcs* and *orfX* loci in strain BK793 has been reported previously (61). Long-range PCR and sequencing using primers spanning *ccrC* and *orfX* (Table 6) were therefore performed, confirming the presence of a *ccrC5*-like sequence similar to that in *S. haemolyticus* strain JCSC1435 (GenBank accession number AP006716) and located approximately 10 kb upstream of *orfX*. Similarly, strain HAR36 is characterized as SCC*mec* IV but possesses a J3 region locus associated with SCC*mec* type III (SCC*Hg*) (53); *ccrC* universal primers (Table 6) were therefore designed and used to confirm the presence of a *ccrC3* element in this strain by DNA sequencing. Long-range PCR and sequencing of a *ccrB4* element detected in prototype SCC*mec* IVE strain AR43/3330.1 likewise demonstrated close identity with *S. epidermidis* strain ATCC 12228 (data not shown), and two class A *mec* strains from the 1996 BARG study (BK2391 and BK2539) were also found to possess both *ccrB4* and *ccrB2*. Such examples of multiple or composite SCC*mec* elements argue strongly against the reliability of using a single locus (e.g., *ccrC* or *ccrB4*) for assignment of SCC*mec* types (18, 42, 83), due to the heterogeneity and mobility of SCC elements (20, 63). For instance, novel SCC*mec* types involving combinations of *ccrC* with class B, E, and C1 *mec* complexes (5B, 5B1, 5E, and 5C1, respectively) have been reported (5, 55), but several existing SCC*mec* typing methods may misclassify these as SCC*mec* type V, based on the use of *ccrC* alone for typing (42, 47).

Our real-time MB-PCR platform offers several advantages over existing methods. Since the specificity of the assay is dependent on both oligonucleotide primer and molecular beacon design, the discriminatory power of target identification exceeds that afforded by conventional PCR. Although the assay is subject to target abrogation resulting from sequence heterogeneity or mosaic structure, the results are not sensitive to changes in fragment size as a result of insertions or deletions. Furthermore, the requirement for template quality is minimal, allowing for rapid typing of large numbers of isolates using a simple boiling lysis method. All PCR amplicons in our multiplex platform are ≤174 bp long (Table 1), thereby contributing directly to increased amplification efficiency and sensitivity of detection using minimal reaction volumes, as well as facilitating analysis of low-quality/fragmented DNA. The sensitivity afforded by our method may also allow for SCC*mec* typing directly from blood cultures (38), which is of potential interest in light of recent reports describing differences in clinical outcome between *S. aureus* bacteremias associated with particular SCC*mec* types (19).

The procedure described in this study is also particularly well-suited for large-scale MRSA analyses. Given the relaxed requirement for template quality, DNA isolation need not be a rate-limiting step, and strains can be typed easily in batches of 96 using the high-throughput method mentioned above. Data interpretation is also relatively straightforward compared to electrophoretic analysis of banding patterns, since it involves simple "yes/no" interrogation of each individual target (Fig. 2). In our experience, a single panel (MB-PCR panel I or II) can be performed on 96 strains in approximately 3 to 4 h, including DNA isolation, PCR cycling, and data analysis. Moreover, where routine typing of contemporary isolates is involved, a single panel may be sufficient for definitive SCC*mec* assignment of most strains. In our geographic region, for example, SCC*mec* types II and IV account for nearly all nosocomial and community MRSA isolates (Table 4), such that the results of MB-PCR panel I are usually definitive.

Our assay can also be used to investigate SCC elements in other staphylococcal species, as demonstrated by analysis of 71 strains from 15 different species (Table 5). Our results suggest that SCC structure is very diverse among CoNS, with various strains possessing more than one *ccr* allotype, or else possessing SCC elements without *mecA*, in agreement with previous studies (21, 66). The *ccrB4* allotype in particular appears to have been transmitted from *S. epidermidis* to *S. aureus* on more than one occasion, and *ccrB4* elements derived from *S. epidermidis* strain ATCC 12228 have been reported in MRSA strains of various genetic backgrounds (69, 83). Given the apparent prevalence of the *ccrB4* allotype among CoNS species, it is reasonable to expect that further transmission will occur in the future, leading to novel SCC*mec* variants or prototypes in MRSA (20).

Several limitations are associated with our assay. First, despite a concerted effort to design all molecular beacon targets using conserved regions, the universality of any given target cannot be guaranteed, due to the heterogeneity of SCC*mec* elements. For example, the class B *mec* complex target (IS*1272J*) selected for this study was not detected within the class B *mec* complexes from two *S. haemolyticus* strains, due to a previously published deletion (39); although no such deletion has been reported in MRSA, the potential for inability to detect class B *mec* in variant strains exists. Second, since a hybrid beacon is used to detect both *ccrB1* and *ccrB3*, the potential exists for misclassification of isolates possessing *mec* class A in combination with *ccrAB1*, or *mec* class B in combination with *ccrAB3*. However, since there have been no reports to date of *S. aureus* containing SCC*mec* elements possessing (i) both *ccrAB1* and *ccrAB3*, (ii) *ccrAB1* with class A *mec*, or (iii) *ccrAB3* with class B *mec*, the *ccrB1/3* beacon in conjunction with IS*1272J* (class B *mec*) or *mecI* (class A *mec*) can theoret-

ically be used to distinguish SCC*mec* types I and III, respectively. Although no such combinations have been observed in *S. aureus*, an example of the former observed in a *Staphylococcus cohnii* strain has been described previously (66). Third, as described in Materials and Methods, the *ccrC* molecular beacon may not reliably detect certain variants of this allotype; among these, however, only *ccrC4* and *ccrC8* have been described in *S. aureus* (48, 74). Finally, our assay is not capable of detecting the recently described *mec* class C1 element associated with SCC*mec* type VII (5).

An additional limitation of our assay is the inability to detect SCC*mec* subtypes. In this study our goal was to design a multiplex MB-PCR platform for rapid typing of SCC*mec* prototypes, and J-region targets used for subtyping were not included. However, depending on the needs of potential users, subtyping probes can be designed and substituted in either of the two MB-PCR panels. As an example, a primer-probe set specific to the J1 region of SCC*mec* IVa was designed (Table 1) and substituted for *mecI* in panel I, in order to further characterize SCC*mec* IV isolates from a large-scale CA-MRSA study. Only SCC*mec* IV isolates bearing the IVa-specific J1-region target were amplified using this approach (data not shown). Similar probes can be designed for other SCC*mec* subtypes of interest, as has been done using conventional PCR; alternatively, dedicated subtyping panels can be created using combinations of different J-region targets.

As the availability of real-time PCR instruments with 5-dye capability becomes commonplace, it will be possible to expand each panel to include an additional target. For example, a *ccrB3*-specific target can be added to panel I, thereby allowing for the definitive identification of SCC*mec* types II, III, and IV in a single reaction and eliminating the need to use a hybrid target for the identification of *ccrB1* and *ccrB3*. Similarly, an additional target can be added to panel II in order to detect the *mec*C1 complex mentioned above (5), thereby allowing the assay to definitively classify SCC*mec* type VII as well. With projected developments in real-time PCR spectrofluorometric thermal cyclers, more than seven fluorophore targets may be detected in a single reaction.

Finally, although the initial investment for the full complement of molecular beacons is significant (approximately $400 per beacon, including purification), it is offset by the reduced requirements for template quality, reaction volume, and time. A detailed cost analysis of all reagents and consumables (not including labor) indicates that the cost of running both panels is approximately USD 2.6 per sample. Moreover, as described above, one panel may suffice for routine typing, in which case the cost per sample is only USD 1.3, significantly less than that reported for conventional PCR (USD 2.4 to 4.9) (47). Consequently, for laboratories with access to real-time PCR instrumentation, our assay represents an economically feasible approach for high-throughput SCC*mec* typing, especially considering the additional savings in labor and processing time.

We have developed a multiplex MB-PCR platform for rapid, high-throughput SCC*mec* typing of MRSA. This novel assay is faster, more robust, and significantly more sensitive than previously published schemes and is based on unambiguous classification of SCC*mec* types according to established nomenclature. Nevertheless, as molecular characterization of MRSA and MR-CoNS continues to widen in scope, it is likely that numerous SCC elements and/or novel SCC*mec* types will be discovered. Therefore, the typing system described here should be seen as reflecting the current situation and must necessarily be developed further to accommodate novel developments.

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