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A new multiplex PCR-based reverse line-blot hybridization (mPCR/RLB) assay for rapid staphylococcal cassette chromosome *mec* (SCC*mec*) typing

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The aim of this study was to develop a new discriminatory method for MRSA SCC*mec* typing based on multiplex PCR-based reverse line-blot hybridization (mPCR/RLB) assay to enable rapid identification and classification of MRSA SCC*mec* types in a clinical laboratory. Forty-five primer sets and 49 probes were designed and tested in uniplex PCR (uPCR) and mPCR/RLB. Probes were compared *in silico* to 14 whole-genome sequences and 18 partial SCC*mec* gene sequences of *Staphylococcus aureus* and complete genome and partial SCC*mec* genes of seven non-MRSA strains, including meticillin-susceptible *S. aureus* and meticillin-resistant coagulase-negative staphylococci. The method was tested on a set of 42 well-characterized reference MRSA strains. It identified all five epidemiologically relevant SCC*mec* types and 26 subtypes, including established and new subtypes of SCC*mec* III, IV (eight subtypes each) and V (three subtypes). The discriminatory power of mPCR/RLB SCC*mec* typing was similar to that of MLST and *spa* typing (Simpson indices of diversity of 0.916, 0.926 and 0.882, respectively; differences not statistically significant). The application of mPCR/RLB hybridization assay to MRSA SCC*mec* typing can improve the specificity, discriminatory power and throughput of the typing procedure. The detection of up to 43 mPCR products in a single hybridization assay transforms MRSA SCC*mec* typing from passive epidemiological library typing into a potential tool for near-real-time infection control surveillance and tracking of MRSA transmission in hospitals.

INTRODUCTION

Morbidity and mortality from meticillin-resistant *Staphylococcus aureus* (MRSA) infections and the emerging threat of community-associated MRSA highlight the importance of timely surveillance (Aires de Sousa & de Lencastre, 2004; Ma *et al.*, 2002). Meticillin resistance is conferred by the *mecA* gene, which is carried on a mobile genetic element known as the staphylococcal cassette chromosome *mec* (SCC*mec*) (Ito & Hiramatsu, 1998; Ito *et al.*, 1999). The combination of *mec* complex class and cassette chromosome recombinase (*ccr*) genes determines the SCC*mec* type (Ito *et al.*, 2004; Kondo *et al.*, 2007). Currently, seven SCC*mec* types (I–VII) and a number of subtypes have been described, ranging in size from 20.9 to 66.9 kb (Ito *et al.*, 2001, 2004; Daum *et al.*, 2002; Oliveira *et al.*, 2006a; Takano *et al.*, 2008). SCC*mec* types I and IV–VII are usually resistant only to β-lactam antibiotics, while types II and III are often resistant to multiple classes of antibiotics due to additional resistance genes integrated into SCC*mec* (Deurenberg & Stobberingh, 2008). MRSA SCC*mec* types I–III have been linked with healthcare-associated infections (Ito *et al.*, 2001) whereas community-acquired MRSA infections are typically caused by SCC*mec* types IV, V or VII (Ito *et al.*, 2004; Daum *et al.*, 2002; Takano *et al.*, 2008). Evidence has been emerging of

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Abbreviations: mPCR/RLB, multiplex PCR-based reverse line-blot hybridization; MLST, multilocus sequence typing; MRSA, meticillin-resistant *S. aureus*; MSSA, meticillin-sensitive *S. aureus*; SCC*mec*, staphylococcal cassette chromosome *mec*; uPCR, uniplex PCR.

Table 1. Strains of 42 well-characterized meticillin-resistant *S. aureus* isolates used in the study

ID	Strain	*spa* type*	Clonal type†
1	FH53‡	t127	ST~1-SCC*mec*-I
2	COL§	t008	ST250-SCC*mec*-I
3	BK2464§	t002	ST5-SCC*mec*-II
4	E822485‡	t018	ST36-SCC*mec*-II
5	RPAH 18‡	t037	ST239-SCC*mec*-III
6	RPAH15‡	t037	ST239-SCC*mec*-III
7	HDG2§	t421	ST239-SCC*mec*-IIIB
8	14176-5710‡	t1959	ST~239-SCC*mec*-III
9	RDH81‡	t037	ST239-SCC*mec*-IIIA
10	AH1‡	t037	ST128-SCC*mec*-IIIA
11	AH13‡	t037	ST239-SCC*mec*-IIIA
12	HU25§	t138	ST239-SCC*mec*-IIIA
13	K704540‡	t037	ST~239-SCC*mec*-III
14	K711532‡	t037	ST~239-SCC*mec*-III
15	ANS46§	t037	ST239-SCC*mec*-III
16	PC8‡	t127	ST1-SCC*mec*-IV
17	FH43‡	t127	ST~1-SCC*mec*-IV
18	RPH 85‡	t127	ST~1-SCC*mec*-IV
19	RHH10‡	t127	ST~1-SCC*mec*-IV
20	B8-10‡	t711	ST~8-SCC*mec*-IV
21	PAH 58‡	t019	ST30-SCC*mec*-IV
22	E804531‡	t002	ST5-SCC*mec*-IV
23	CH97‡	T002	ST73-SCC*mec*-IV
24	RBH98‡	t202	ST93-SCC*mec*-IV
25	13792-4492‡	t202	ST~93-SCC*mec*-IV
26	IP01M1081‡	t216	ST59-SCC*mec*-IV
27	MW2§	t128	ST1-SCC*mec*-IV
28	CH16‡	t032	ST22-SCC*mec*-IV
29	CH69‡	t1963	ST~22-SCC*mec*-IV
30	DEN2988§	t008	ST8-SCC*mec*-IVA
31	F829549‡	t186	ST88-SCC*mec*-IV
32	PAH 1‡	t019	ST30-SCC*mec*-IV
33	IP01M2046‡	t1958	ST78-SCC*mec*-IV
34	SJOG 30‡	t127	ST1-SCC*mec*-IV
35	RHH58‡	T127	ST~1-SCC*mec*-IV
36	IMVS 67‡	t008	ST8-SCC*mec*-V
37	RPH 74‡	t123	ST45-SCC*mec*-V
38	J710566‡	t065	ST45-SCC*mec*-V
39	B827549‡	t1784	ST~1-SCC*mec*-new
40	SN39‡	t127	ST~1-SCC*mec*-new
41	RPH2‡	t190	ST8-SCC*mec*-new
42	C801535‡	t325	ST88-SCC*mec*-new

**spa* type described by Cai *et al.* (2007).

†ST ~, single nucleotide polymorphism (SNP) type as described previously using the computer program Minimum SNPs to compare with existing MLST data (Huygens *et al.*, 2004, 2006). SCC*mec*, staphylococcal cassette chromosome. ST, multilocus sequence type (MLST). Information provided by strain donors.

‡Provided by Dr Philip Giffard, Cooperative Research Centre for Diagnostics, Queensland University of Technology, Brisbane, Australia, and Dr Graeme Nimmo, Queensland Health Pathology Services (QHPS), Princess Alexandra Hospital, Brisbane, Australia, and tested in previous studies (Huygens *et al.*, 2004, 2006; Stephens *et al.*, 2006; Coombs *et al.*, 2004).

§Seven isolates, which had been used in previous studies (Oliveira *et al.*, 2006b), were provided by Professor Herminia de Lencastre, Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Oeiras, Portugal; *spa* types identified in this study were identical with those previously reported for these strains (Cai *et al.*, 2007).

potential associations between MRSA genotype and clinical outcomes (Lalani *et al.*, 2008).

SCC*mec* typing and subtyping provides an opportunity to detect and reduce the transmission of MRSA in healthcare settings (Ito *et al.*, 2004; Kondo *et al.*, 2007). Several multiplex PCR (mPCR)-based methods have been described to determine the structure of the SCC*mec* complex (Oliveira & de Lencastre, 2002; Zhang *et al.*, 2005; Kondo *et al.*, 2007; Milheirico *et al.*, 2007) but most require several reactions, produce long amplicons and are time consuming (Chongtrakool *et al.*, 2006; Kondo *et al.*, 2007), making them impractical for outbreak investigations or early-warning systems. Identification of the 46 major and minor SCC*mec* variants currently known relies on detection of the presence or absence of at least 22 of 34 binary targets (Stephens *et al.*, 2007). mPCR-based reverse line-blot hybridization assay (mPCR/RLB) can overcome the limitations of current methods by allowing rapid and simultaneous detection of a large number of different PCR products using membrane-bound sequence-specific probes (Kong & Gilbert, 2006). This technique has been successfully applied to molecular typing of *Streptococcus agalactiae* (Kong *et al.*, 2005), *Streptococcus pneumoniae* (Kong *et al.*, 2006) and *Staphylococcus aureus* (Cai *et al.*, 2007).

The aim of this study was to develop a new discriminatory method for MRSA SCC*mec* typing based on mPCR/RLB suitable for the rapid identification and classification of the MRSA isolates in a clinical laboratory.

METHODS

Bacterial isolates. Seven well-characterized reference strains [Cai *et al.*, 2007; kindly provided by Professor Herminia de Lencastre, Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa (UNL), Oeiras, Portugal] and 35 previously characterized clinical strains (provided by Drs Graeme Nimmo, Queensland Health Pathology Services, Princess Alexandra Hospital, Brisbane, Queensland, Australia, and Philip Giffard, Cooperative Research Centre for Diagnostics, Queensland University of Technology, Brisbane, Queensland, Australia) were used in this study (Table 1). Isolates were stored at –70 °C, until DNA extracts were prepared, as previously described (Cai *et al.*, 2007); the latter were stored at –20 °C until tested.

Primer and probe design. The mPCR primers (Kondo *et al.*, 2007) were modified to adjust T_m values to 60–65 °C and labelled with biotin. Immediate downstream or upstream sequences of the published sense and antisense primers were used to design antisense or sense mPCR/RLB probes as described previously (Kong & Gilbert, 2006). An inner primer was designed for each of the published sense and anti-sense primers with expected product size of around 100 bp. In total, 45 primer sets and 49 probes were assembled (some primer sets contained two probes). All primer sets were tested with each of the 42 reference strains using uniplex PCR (uPCR) and mPCR/RLB (Table 2).

uPCR and mPCR reactions. PCR amplifications were performed in a thermal cycler with HotStar *Taq* DNA polymerase (Qiagen). A 25 µl uPCR mixture was prepared follows: 2.5 µl 10× PCR buffer with a final $MgCl_2$ concentration of 1.5 mM, 0.2 mM deoxynucleoside triphosphates, 12.5 µM of each of the forward and reverse primers, 2 µl template DNA, 0.2 µl Qiagen Hotstar *Taq* polymerase (5 U μl^{-1}) and molecular biology grade H_2O (Eppendorf) was added to a total volume of 25 µl. Two mPCR mixes were prepared based on uPCR results for the 42 reference strains: the first contained 39 primer pairs (primer sets 1–18, 21–24 and 29–45, Table 2), which produced positive uPCR results for at least one strain tested, and the second contained six primer pairs (primer sets 19, 20, and 25–28, Table 2) that were uPCR negative for all the strains tested, but showed *in silico* matches with one or more SCC*mec* sequences in GenBank.

Each mPCR mix comprised 2 µl DNA template and 3 µl 10× PCR buffer with a final concentration of: 1.5 mM $MgCl_2$ (Qiagen), 0.2 mM dNTP, 1.5 U HotStar *Taq* DNA polymerase and 12.5 µM of each of the forward and reverse primers. Molecular biology grade H_2O (Eppendorf) was added to a total volume of 30 µl. PCR was performed according to the Qiagen HotStar *Taq* polymerase kit instructions: 95 °C for 15 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s, and 72 °C for 10 min. PCR products were stained with SYBR safe DNA gel stain and visualized in a 2 % agarose gel.

Reverse line blot (RLB) hybridization. The RLB hybridization assay was performed as described previously (Kong & Gilbert, 2006; Cai *et al.*, 2007). Briefly, all probes, synthesized with a 5′ amine modification at concentrations of 5–10 µM in 0.5 M $NaHCO_3$ (pH 8.4), were covalently linked to a Biodyne C hydrophilic nylon 6,6 (0.45 µm) membrane (PALL Gelman Laboratory), which was negatively activated by incubation in freshly prepared 16 % (w/v) 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) (Sigma). The membrane was placed in a miniblotter (MN45, Immunetics) on a support cushion. A volume of 20 µl of PCR products was added to 150 µl 2× SSPE (Amresco) in 0.1 % SDS (Sigma), denatured at 100 °C for 10 min and cooled immediately on ice. Hybridization was conducted by adding 150 µl of the diluted denatured PCR products to alternate slots on the miniblotter, which was then incubated at 60 °C for 60 min. The membrane was washed twice in 2× SSPE buffer in 0.5 % SDS at 60 °C for 10 min, incubated in peroxidase-labelled streptavidin conjugate (Roche) at 42 °C for 60 min, then washed twice in 2× SSPE buffer in 0.5 % SDS at 42 °C for 10 min and twice in 2× SSPE buffer at room temperature for 5 min. For detection, the membrane was incubated in 15 ml chemiluminescence blotting substrate ECL detection reagent (Amersham, GE Healthcare) for 2 min, covered with transparent plastic sheet in a film cartridge and exposed with chemiluminescence film (Amersham Hyperfilm ECL, GE Healthcare) for 4 min. RLB results were regarded as positive when a hybridization dot signal was clearly visible.

Quality control of mPCR/RLB results. To ensure reproducibility of mPCR/RLB results, positive and negative controls were run on each membrane. The positive control was a mixture of DNA extracts from MRSA strains COL, BK2464, E822485, ANS46, DEN2988, PAH1, IP01M2046, RHH58, RPH74, B827549 and RPH2, which together contain all 43 probe targets; the negative control was master mix only, without DNA. Any signal produced by a test isolate that was similar to or stronger than the corresponding signal of the positive control was identified as positive. A signal produced by the negative control was assumed to be due to contamination and the assay was repeated. In practice, rare equivocal results would be resolved by uPCR for the relevant target, with sequencing of the amplicon, if required, as 'gold standard', but for the purpose of this study, all mPCR/RLB results were confirmed with uPCR.

SCC*mec* gene and staphylococcal whole-genome sequence analysis. Thirty-five SCC*mec* gene sequences published in NCBI GenBank, including 20 known SCC*mec* type (I–VI) and subtype sequences and 14 *Staphylococcus aureus* genome and 1 meticillin-resistant *S. epidermidis* genome sequences were analysed as reference sequences and tested *in silico* using the mPCR/RLB assay. These sequences (with strain numbers; and DDBJ/EMBL/GenBank accession numbers) were as follows: (a) 18 SCC*mec* sequences: type I.1 SCC*mec* (NCTC10442; AB033763); type II.1 SCC*mec* (N315; D86934); type II.2 SCC*mec* (JCSC3063; AB127982); type II.B SCC*mec* (AR05; AJ810123); type II.3 (type IIE) SCC*mec* (AR13.1/3330.2; AJ810120); type II.4 SCC*mec* (RN7170; AB261975); type III.1 SCC*mec* (HU25; AF422696); type III.1 SCC*mec* and SCC*mercury* (85/2082; AB037671); type IV.1 SCC*mec* (CA05 [JCSC1968]; AB063172); type IV.2 SCC*mec* (JCSC1978 [8/6-3P] AB063173); type IV.3 SCC*mec* (81/108 [MR108]; AB096217); type IV.C SCC*mec* (2314; AY271717); type IV.4 SCC*mec* (JCSC4469; AB097677); type IV.E SCC*mec* (AR43/3330.1; AJ810121); type IV.g SCC*mec* (M03-68; DQ106887); type IV.h (PL72; AF411936); type V SCC*mec* (JCSC3624 [WIS]; AB121219); type VI SCC*mec* (HDE288; AF411935); and (b) 14 *S. aureus* genomes: COL (CP000046), JH1 (CP000736), JH9 (CP000703), MRSA252 (BX571856), MW2 (BA000033), Mu3 (AP009324), Mu50 (BA000017), N315 (BA000018), USA300 (CP000255), USA300_TCH1516 (CP000730), RF122 (AJ938182), MSSA476 (BX571857), NCTC8325 (CP000253), Newman (AP009351).

In addition, the mPCR/RLB system was tested *in silico* against whole-genome and partial SCC*mec* sequences of seven non-MRSA strains: five meticillin-susceptible *S. aureus* (MSSA) [AJ938182, BX571857, CP000253, AP009351, AM983545 (SSC*mec* type II)], plus one meticillin-resistant coagulase-negative *Staphylococcus* [*S. epidermidis* CP000029 (SCC*mec* type II)] and *Staphylococcus pseudintermedius* [AM904732 (SCC*mec* type III)].

GenBank SCC*mec* sequence searches and alignments and design of primers and probes were conducted using BioManager (Sydney Bioinformatics, https://www.angis.org.au/) and Sigma (http://www.sigma-genosys.com/calc/DNACalc.asp) browsers. Results of BLASTN alignments of designed probe sequences with all currently available SCC*mec* types (I–VI) and subtype sequences (35 GenBank reference sequences) including whole-genome and partial SCC*mec* sequences of non-MRSA staphylococci were applied for *in silico* prediction of RLB hybridization patterns. The uPCR and mPCR/RLB results were recorded as binary data, exported into BioNumerics software (version 4.6, Applied Maths). Dendrograms were generated using the categorical coefficient and clustering by UPGMA algorithm.

Statistical analysis. The discriminatory power was estimated by calculating Simpson's index of diversity (Hunter & Gaston, 1988) and the 95 % confidence intervals (CI) were determined as described previously (Grundmann *et al.*, 2001).

Table 2. Primers and probes used in mPCR/RLB for SCC*mec* genotyping

Primer/ probe set	Primers (b) and probes (p) for mPCR/RLB*	GenBank accession no.	Gene or ORF	Reference SCC*mec* or SCC sequence(s)	Probe location	Primer and probe sequence (5′-3′)†
1	mA1b	D86934	*mecA*	Type II.1		$_{45813}$TGCTATCCACCCTCAAACAGG$_{45833}$
	mecAAp	D86935	*mecA*		*mec* complex	$_{45860}$GGTGTGCTTACAAGTGCTAATAATTC$_{45835}$
	mecASp-1	D86936	*mecA*		*mec* complex	$_{46053}$GGTAAAGGTTGGCAAAAAGATA$_{46075}$
	mA2b	D86934	*mecA*	Type II.1		$_{46098}$AACGTTGTAACCACCCCAAGA$_{46078}$
2	1b	AB033763	*ccrA1*	Type I.1		$_{24838}$CAATCAAAACCTATATCATCAATCAGTACG$_{24867}$
	ccrA1Ap	AB033763	*ccrA1*		*ccr* complex	$_{24890}$TGGAAAGCTTTTCGAATTTGAT$_{24869}$
	1An	AB033763	*ccrA1*			$_{24944}$TCATCAATATAGGGGTACAACATGTT$_{24911}$
3	βc1Sn	AB033763	*ccrB1*			$_{25421}$CAGGCAAGTTAATGCTCCAG$_{25440}$
	ccrB1Sp	AB033763	*ccrB1*		*ccr* complex	$_{25497}$CGGACAACGTCAAAGAGC$_{25514}$
	βcb	AB033763	*ccrB1*, *ccrB2*, *ccrB3*	Type I.1, II.1, III.1		$_{25541}$ATTGCCTTGATAATAGCCITCT$_{25518}$
4	2b	D86934	*ccrA2*	Type II.1		$_{26325}$TAAAGGCATCAATGCACAAACACT$_{26348}$
	ccrA2Ap	D86934	*ccrA2*		*ccr* complex	$_{26378}$TCTTTGCACACTTCTAATACTTTATCTTCA$_{26349}$
	2An	D86934	*ccrA2*			$_{26432}$CGTTTGTCAATCGCACCT$_{26415}$
5	βc2Sn	D86934	*ccrB2*			$_{27168}$TGCGAGTTTCTCAGAATTCG$_{27187}$
	ccrB2Sp	D86934	*ccrB2*		*ccr* complex	$_{27226}$ACGAGACGTGCCCAAGAA$_{27243}$
	βcb	D86934	*ccrB1, ccrB2, ccrB3*	Type I.1, II.1, III.1		$_{27261}$ATTGCCTTGATAATAGCC$_{27244}$
6	A3b	AB037671	*ccrA3*	Type III.1		$_{5486}$AGCTCAAAAGCAAGCAATAGAAT$_{5508}$
	orfS04Ap	AB037671	*orfS04*		Adjacent to *ccr* complex	$_{5533}$TGAATATTGTGCTTTGGAGCTA$_{5512}$
	3An	AB037671	*orfS04*			$_{5583}$TTCGATTATCTGTTCTGCCTGA$_{5562}$
7	βc3Sn	AB037671	*ccrA3*			$_{7173}$TACAAATTCTAGCGAGTTTTTCAG$_{7196}$
	ccrA3Sp	AB037671	*ccrA3*		*ccr* complex	$_{7241}$ACCCGACGCGCTCAAGAA$_{7258}$
	βcb	AB037671	*ccrB1, ccrB2, ccrB3*	Type I.1, II.1, III.1		$_{7276}$ATTGCCTTGATAATAGCC$_{7259}$
8	4.2b	AF411935	*ccrA4*	Type VI		$_{8741}$AAAGGTATCAATGCACCAGAACTT$_{8764}$
	ccrA4Ap	AF411935	*ccrA4*		*ccr* complex	$_{8780}$ACAAGTGTCGAGCACTTTACTTTC$_{8765}$
	4.2An	AF411935	*ccrA4*			$_{8843}$TGAGTATTGTGTCATTCAATCGATTA$_{8818}$
9	β4.2Sn	AF411935	*ccrB4*			$_{9939}$TGAGGAACCCATTATTGCTG$_{9958}$
	ccrB4Sp	AF411935	*ccrB4*		*ccr* complex	$_{9985}$AAAGCTTTATGGGATAAAGTACAGTT$_{10010}$
	β4.2b	AF411935	*ccrB4*	Type VI		$_{10031}$TTGCGACTCTCTTGGCGTTT$_{10012}$
10	Rb	AB121219	*ccrC*	SCC*mercury*, type V		$_{16838}$GCCTTTATAGACTGGATTATTCAAAATA$_{16812}$
	ccrCSp	AB121219	*ccrC*		*ccr* complex	$_{16792}$TCAGTGTTTGGCGTGAAAT$_{16810}$
	Fasn	AB121219	*ccrC*			$_{16741}$ACGACAATTCTAAATCAAAAAGGA$_{16764}$
11	Ran	AB121219	*ccrC*			$_{16430}$TTGAATTCATGAATAATATTAAACACGT$_{16403}$
	ccrCAp	AB121219	*ccrC*		*ccr* complex	$_{16375}$ATTTCCAACTTAATACCATTTCGATT$_{16350}$
	Fb	AB121219	*ccrC*	SCC*mercury*, type V		$_{16318}$AAACGTCTATTACAAGATGTTAAGGATAAT$_{16347}$
12	mI6b	D86934	*mecI*	Type II.1		$_{42866}$CATAACTTCCCATTCTGCAGATG$_{42888}$
	mecR1Ap	D86934	*mecR1*		*mec* complex	$_{42924}$GAGTTAATATAATGGATAATAAAACGTATGAAAT$_{42891}$
	mA7An	D86934	*mecR1*			$_{42971}$ATGGCGAAAAAGCACAAC$_{42954}$
13	IS7b	AB033763	IS*1272*	Type I.1		$_{28624}$ATGCTTAATGATAGCATCCGAATG$_{28647}$

Table 2. cont.

Primer/ probe set	Primers (b) and probes (p) for mPCR/RLB*	GenBank accession no.	Gene or ORF	Reference SCC*mec* or SCC sequence(s)	Probe location	Primer and probe sequence (5′-3′)†
	orfS034Ap	AB033763	*orfS034*		Adjacent to *mec* complex	$_{28670}$TTTTGATTTTGTGATAGCCACA$_{28649}$
	mA7An-1	AB033763	*orfS035*			$_{28726}$ATAAAGCATCTACTTTTGGATTTACTCT$_{28699}$
14	IS7Sn	AB033763	*mecR1*			$_{31288}$CACCTTCTACACCTCCATATCA$_{31309}$
	mecASp-2	AB033763	*mecA*		*mec* complex	$_{31395}$AAAGATAAAAATTGTTCCACTTATTTTAATAG$_{31426}$
	mA7b	AB033763	*mecA*	Type I.1, II.1, V		$_{31450}$ATATACCAAACCCGACAACTACA$_{31428}$
15	IS2(iS-2)Sn	AB121219	IS*431*			$_{8670}$ACAGATCAGGCACCTTCAAC$_{8689}$
	IS431Sp	AB121219	IS*431*		Adjacent to *mec* complex	$_{8721}$TTTAAACTTAAACCTGACTGTCATTGT$_{8747}$
	IS2(iS-2)b	AB121219	IS*431*	Type V		$_{8772}$TGAGGTTATTCAGATATTTCGATGT$_{8748}$
16	1a3b	AB033763	E007	Type I.1		$_{5277}$TTTTAGGAGGTAATCTCCTTGATG$_{5300}$
	E007Ap	AB033763	E007		J1	$_{5328}$TTTATAACATTGTCCTCTTTACCTTCAG$_{5301}$
	E007Sp	AB033763	E007		J1	$_{5387}$AATAAGCATACATGGAGACGTTTT$_{5410}$
	1a4b	AB033763	E007	Type I.1		$_{5431}$TTTTGCGTTTGCATCTCTACC$_{5411}$
17	4alb	AB063172	CQ002	Type IV.1		$_{4726}$TTTGAATGCCCTCCATGAATAAAAT$_{4750}$
	CQ002Ap	AB063172	CQ002		J1	$_{4777}$CGGGTAATATGAGAATAAATAAAGAAG$_{4751}$
	4a1An	AB063172	CQ002			$_{4844}$GCGTATTATGATACTAAGCAAAGTATTAAAATAG$_{4811}$
18	4a3Sn	AB063172	CQ002			$_{5079}$TTGAAATCCTGGGAATCTATCACT$_{5102}$
	CQ002Sp	AB063172	CQ002		J1	$_{5133}$ACATGCTGTAGTCATTTTATACTGTTTG$_{5160}$
	4a3b	AB063172	CQ002	Type IV.1		$_{5183}$AGCATATAGAAAAGATAGAAGTTCGAAAGA$_{5161}$
19	4b3b	AB063173	M001	Type IV.2, II.3		$_{2457}$AACCAACAGTGGTTACAGCTT$_{2477}$
	M001Ap	AB063173	M001		J1	$_{2509}$AAATTTTAAACAATTCTAATTCAAATAAGTTG$_{2478}$
	4b3An	AB063173	M001			$_{2562}$ATCAAATTTTTTAGCAGATGTTGG$_{2539}$
20	4b4Sn	AB063173	M001			$_{3041}$ATAGTGAACGTACTGATAACAAATTATTAGAT$_{3072}$
	M001Sp	AB063173	M001		J1	$_{3131}$ATCATAAAATTACAGCTTACGATATACAAA$_{3160}$
	4b4b	AB063173	M001	Type IV.2, II.3		$_{3182}$CGGATTTTAGACTCATCACCAT$_{3161}$
21	4c4b	AB096217	CR008	Type IV.3		$_{8266}$AAGAAAAGGAAATCGATGTCATTATAA$_{8240}$
	CR008Ap	AB096217	CR008		J1	$_{8055}$TCCTACTCCTATAAAGAGGTTACAGAATTATT$_{8024}$
	CR008Sp	AB096217	CR008		J1	$_{8208}$CAGATAGTATGGTACATATCCTTTAGGATT$_{8237}$
	4c5b	AB096217	CR008	Type IV.3		$_{7998}$CTGCATCCATTTCTCAGGAGTTAG$_{8021}$
22	4d3b	AB097677	CD002	Type IV.4		$_{2390}$AATTCACCCGTACCTGAGAA$_{2409}$
	CD002Ap	AB097677	CD002		J1	$_{2440}$AGGATTTGGTGTATATAAAGGAGTGAAATA$_{2411}$
	4d3An	AB097677	CD002			$_{2496}$GCCTTTACACTAAGTACAGGGACATT$_{2471}$
23	4d4Sn	AB097677	CD002			$_{3497}$TCTCTTAGAATTTTTTTGATATATTCATCTGA$_{3528}$
	CD002Sp	AB097677	CD002		J1	$_{3575}$TGTACATCTCGTATGTTAGAAACATTATTTAAA$_{3607}$
	4d4b	AB097677	CD002	Type IV.4		$_{3635}$GTCCAGAATGTGGTTATAAGATAGCTA$_{3609}$
24	kdpB1b	D86934	*kdpB*	Type II.1		$_{12439}$TGAGATTACTTCAGAACCAGGTCAT$_{12415}$
	kdpBAp	D86934	*kdpB*		J1	$_{12190}$GGCTATAGGGATTGCAGGG$_{12172}$
	kdpBSp	D86934	*kdpB*		J1	$_{12388}$CAAACCAATCATTTTATCTAAAAATG$_{12413}$

Table 2. cont.

Primer/ probe set	Primers (b) and probes (p) for mPCR/RLB*	GenBank accession no.	Gene or ORF	Reference SCC*mec* or SCC sequence(s)	Probe location	Primer and probe sequence (5′-3′)†
	kdpB2b	D86934	*kdpB*	Type II.1		$_{12150}$TAAACTGTGTCACACGATCCAT$_{12171}$
25	2b3b	AB127982	S01	Type II.2		$_{1497}$GCTCTAAAAGTTGGATATGCG$_{1517}$
	S01Ap	AB127982	S01		J1	$_{1546}$AAAGTAATCTATATGATCCCATTTTTGA$_{1519}$
	2b3An	AB127982	S01			$_{1599}$TTAGTATTTGTTACAATGCCCACA$_{1576}$
26	2b4Sn	AB127982	S01			$_{2906}$ACAGTTAGAACATGTTAATAAAAACATCG$_{2934}$
	S01Sp	AB127982	S01		J1	$_{2963}$CAAATTAAGCAAACTTACTGAATATTTAGC$_{2992}$
	2b4b	AB127982	S01	Type II.2		$_{3014}$TGGATTGAATCGACTAGAATCG$_{2993}$
27	II4-3b	AB261975	RN06	Type II.4		$_{11846}$AAGTACCGCTGAATATTGATAGTGAT$_{11871}$
	RN06Ap	AB261975	RN06		J1	$_{11909}$CCTATTAATTGAGATATTGTATGATAATATCCTT$_{11876}$
	II4-3An	AB261975	RN06			$_{11975}$GGTTTTGTTTTTTCAATATAATACCTTG$_{11948}$
28	II4-1Sn	AB261975	RN06			$_{13715}$TTGAAATACATGTTTTTGAAGAATATATATTG$_{13746}$
	RN06Sp	AB261975	RN06		J1	$_{13797}$AGAAATTGATTTAATAAGTTCTTCTGAAAATA$_{13828}$
	II4-1b	AB261975	RN06	Type II.4		$_{13850}$TTTCTACTCTAATCCTAATCACCGAAC$_{13829}$
29	3a1b	AB037671	Z004	Type III.1		$_{3333}$ATGGCTTCAGCATCAATGAG$_{3352}$
	Z004Ap	AB037671	Z004		J1	$_{3381}$AGTGCATTGCTTAATGATTTAATGTACT$_{3354}$
	3a1An	AB037671	Z004			$_{3430}$GACACCCGTATACAACGATGAC$_{3409}$
30	3a3Sn	AB037671	Z004			$_{3734}$ACTGCAGCGGCTTTAGGA$_{3751}$
	Z004Sp	AB037671	Z004		J1	$_{3793}$GTTTAGATCATGGAATGGCACT$_{3814}$
	3a2b	AB037671	Z004	Type III.1		$_{3835}$ATATCCTTCAAGCGCGTTTC$_{3816}$
31	5a1b	AB121219	V024	Type V		$_{26564}$ACCTACAGCCATTGCATTATG$_{26584}$
	V024Ap	AB121219	V024		J1	$_{26621}$TCTTGATACATTTTAATATACTCACTAGTATTCGAT$_{26586}$
	5a1An	AB121219	V024			$_{26684}$GCGAATATAAATTCATATACTTTTTCATCA$_{26655}$
32	5a2Sn	AB121219	V024			$_{27617}$GGGATTTCATCATCTTTTTAATGA$_{27640}$
	V024Sp	AB121219	V024		J1	$_{27671}$AATTGATACTTATAAAATGTTAATAGCGCT$_{27700}$
	5a2b	AB121219	V024	Type V		$_{27722}$TGTATACATTTCGCCACTAGCT$_{27701}$
33	ermA1b	D86934	*ermA*	Type II.1		$_{35071}$AATTTAGTGAAACAATTTGTAACTATTGA$_{35099}$
	ermAAp	D86934	*ermA*		J2	$_{35126}$TATCGAAGGAACAATTTCTTTCTATT$_{35101}$
	ermA1An	D86934	*ermA*			$_{35182}$TAAAAACCAATTCCGACAGG$_{35163}$
34	mN5Sn	D86934	CN031			$_{37724}$CCCTCCCATATATATAACAAGTTTAGG$_{37750}$
	CN031Sp	D86934	CN031		J2	$_{37783}$ATCCACTAAGTGTACTGAAAAATACTTG$_{37810}$
	mN5b	D86934	CN031			$_{37833}$TTGCTTCGGGACTTACCTCTAGT$_{37811}$
35	cad4b	AB037671	*cadB*	Type III.1		$_{16107}$ATTGCGATTCTTTCCGATATGG$_{16128}$
	cadBAp	AB037671	*cadB*		J2	$_{16156}$CTATTTAATGCTACCAAAATAGTGGCTC$_{16129}$
	cad4An	AB037671	Z020-Z021 spacer			$_{16213}$TTCCGTAAATTGCACACATATAGTACTT$_{16186}$
36	mN5Sn	AB037671	Z023			$_{17537}$CCCTCCCATATATATAACAAGTTTAGGA$_{17564}$
	Z023Sp	AB037671	Z023		J2	$_{17594}$TGATCCACTAAGTGTACTGAAAAATACTTG$_{17623}$
	mN5b	AB037671	CN030, CZ021	Type II.1, III.1		$_{17646}$TTGCTTCGGGACTTACCTCTAGT$_{17624}$
37	mA1b	D86934	*mecA*	Type II.1, III.1		$_{45813}$TGCTATCCACCCTCAAACAGG$_{45833}$
	antAp	D86934	*mecA*		J3	$_{45859}$GTGTGCTTACAAGTGCTAATAATTCA$_{45834}$

Table 2. cont.

Primer/ probe set	Primers (b) and probes (p) for mPCR/RLB*	GenBank accession no.	Gene or ORF	Reference SCC*mec* or SCC sequence(s)	Probe location	Primer and probe sequence (5′-3′)†
	ant1An	D86934	*mecA*			$_{45914}$GGCATGAGTAACGAAGAATATAATAA$_{45889}$
38	mA1Sn	D86934	*ant*(4)′			$_{50668}$AGGCAAATGGCGTAATATTC$_{50687}$
	antSp	D86934	*ant*(4)′		J3	$_{50724}$CTGTACAGGTAGCAATGGCA$_{50743}$
	ant1b	D86934	*ant*(4)′	Type II.1		$_{50764}$GGTGCCATGTTGATTGGTCTG$_{50744}$
39	mA1Sn-1	AB037671	CZ034-CZ036 spacer			$_{32761}$AATAATAAGGATAGCCATGGCTACAA$_{32786}$
	CZ036Sp	AB037671	CZ034-CZ036 spacer		J3	$_{32815}$ATTTATAAATTCCAGAACTAGTTTACGATT$_{32844}$
	pT181-2b	AB037671	CZ034-CZ036 spacer	Type III.1		$_{32869}$GGAGGTTTATTGTCACTACAATTGA$_{32847}$
40	1a2b	AB033763	*pls*	Type I.1		$_{10800}$TAGTGAACCAAATAATGTGCCATT$_{10823}$
	CE009Ap	AB033763	CE009		Adjacent to J1	$_{10849}$CAGATACTGGTAATGATGCTCAA$_{10827}$
	1a1An	AB033763	CE009			$_{10904}$GACGCAGATAGAGATCATAATGAC$_{10881}$
41	1a2Sn	AB033763	*pls*			$_{11765}$TTGTTGTTGTTTTTTCACCATTTT$_{11788}$
	plsSp	AB033763	*pls*		J1	$_{11819}$CTGGTTCCATTGTTGGATCTAA$_{11840}$
	1a1b	AB033763	*pls*	Type I.1		$_{11864}$ATTCCATATGAAACTAAACGCGT$_{11841}$
42	merA2b	AB037671	*merA*	SCC*mercury*		$_{39874}$TCTTCACAGCCTGTGCATGTCATGCCT$_{39900}$
	merAAp	AB037671	*merA*		*mer* operon	$_{39928}$CTCAAAATTCATATAAAATACCCATTCA$_{39901}$
	merA2An	AB037671	*merT*			$_{39991}$AAATATGAGATAAATGTACAGAACTGTCTATC$_{39960}$
43	merGSn	AB037671	CZ046			$_{41319}$GACACCAGGTTGCCATAAAATA$_{41340}$
	CZ046Sp	AB037671	CZ046		*mer* operon	$_{41372}$ACTGGATTTCCTTGAATGTCTTGA$_{41395}$
	merGb	AB037671	CZ046	SCC*mercury*		$_{41419}$TGATACCGCGAATGAATCAAAGGT$_{41396}$
44	mN21b	AB037671	CZ055	SCC*mercury*		$_{47844}$TTCATCATCTTTAACTACGATGGTGT$_{47869}$
	CZ055Ap	AB037671	CZ055		J region in SCC*mercury*	$_{47891}$AATAAATCAGGTACTGGGGCTA$_{47870}$
	mN21An	AB037671	CZ055			$_{47938}$TTCAACAACCTGAACACAATGG$_{47917}$
45	mN22Sn	AB037671	CZ056			$_{48322}$TTCTTGTTGTTTTTTATATGATTCATCA$_{48349}$
	CZ056Sp	AB037671	CZ056		J region in SCC*mercury*	$_{48379}$ATTCTTAGTCCACTCTTCAACCAT$_{48402}$
	mN22b	AB037671	CZ056	SCC*mercury*		$_{48424}$GGTAAACTACAGCCATCTTCAGATAGA$_{48403}$

*S, sense; A, antisense; b, biotin-labelled primers (at the 5′ end); n, non-biotin-labelled primers; p, C^6-amine-labelled-probes (at the 5′ end). All primers labelled with biotin were based on previously published sequences (Kondo *et al.*, 2007). Non-biotin-labelled primers and probes were designed for this study.
†Numbers indicate sequence position in relevant GenBank sequences.

RESULTS AND DISCUSSION

SCC*mec* type characteristics of the reference strains in mPCR/RLB assays

All 42 reference strains were tested by uPCR and mPCR using all 45 primer sets (Table 2). The majority produced amplicons of the expected size in the uPCR, but six (primer sets 19, 20, 25–28) gave negative results. The two groups of primers (the 39 that generated and six that did not generate amplicon signals in the uPCR) were further studied in two separate mPCRs. Fig. 1 shows mPCR/RLB findings using the 39 primers that had given positive results in uPCR. As the other six primer sets did not produce signals with the 42 test isolates, they were removed from the membrane, to save space, and labelled onto a second membrane for use in subsequent studies.

The uPCR results were consistent with those of mPCR/RLB, with the following exceptions: primers βc1Sn and βcb amplified SCC*mec* from strains FH53, SJOG30, RHH58, B827549 and SN39 in the uPCR but produced no signal in mPCR/RLB with probe ccrB1Sp (Fig. 1, lanes 1, 34, 35, 39 and 40, respectively), presumably due to a major sequence variation in the probe region. There were minor differences in intensities of signal dots but, since this is a binary typing system, clear signals similar to or stronger than positive controls on the membrane were regarded as positive. This approach was validated internally by the use of paired (sense/antisense) probes and by comparison of the signals within and between the probes. In our experience, a weak signal reflects minor sequence variation (1–2 bp) and an absent signal represents at least a 2 bp sequence change in the corresponding probe region, as suggested previously (Wang *et al.*, 2008). However, discordant signals between

Fig. 1. RLB patterns of 41 reference strains of MRSA. Lane P, artificial positive control, Lane N, negative control (PCR master mix) Lanes 1–42: 1, FH53; 2; COL; 3, BK2464; 4, E822485; 5, RPAH18; 6, RPAH15; 7, HDG2; 8, 14176-5710; 9, RDH81; 10, AH1; 11, AH13; 12, HU25; 13, K704540; 14, K711532; 15, ANS46; 16, PC8; 17, FH43; 18, RPH85; 19, RHH10; 20, B8-10; 21, PAH58; 22, E804531; 23, CH97; 24, RBH98; 26, IP01M1081; 27, MW2; 28, CH16; 29, CH69; 30, DEN2988; 31, F829549; 32, PAH1, 33, IP01M2046; 34, SJOG30; 35, RHH58; 36, IMVS67; 37, RPH74; 38, J710566; 39, B827549; 40, SN39; 41, RPH2; 42, C801535. [Note: strain 25 (13792-4492) was not included; it gave results identical to those of strain 24 (RBH98).]

probe pairs (one positive – weak or strong – and one negative) can occur but are rare. uPCR and sometimes sequencing, using the same primers as used for that target in the mPCR, can be used to determine whether the discrepancy is due to a modified target (amplicon present) or a non-specific cross-reaction (amplicon absent).

In silico comparison of probe sequences with SCC*mec* gene sequences

The potential sensitivity of the probes designed in the study was assessed in an *in silico* experiment. The GenBank sequences of 14 *S. aureus* whole-genome sequences, 14 partial SCC*mec* sequences and seven non-MRSA genome and SCC*mec* sequences were downloaded and compared with probe sequences from this study using BLASTN pairwise alignments. The results are expressed as virtual RLB profiles in Fig. 2, in which the six probes that gave negative results for the 42 reference strains were also included. This comparative analysis demonstrated that each of the 45 probes found homologous matches with at least one GenBank MRSA *SCCmec* sequence. It suggested that the six additional probes/primer sets could be potentially useful for SCC*mec* typing and should be retained in the mPCR/RLB.

It should be noted that sequences of MRSA SCC*mec* type II 2 (lane 3), type IIB (lane 4), type II.4 (lane 6, accession no. AB261975), type III.1 (lane 7), type IV.4 (lane 13, accession no. AB097677), and type VI (lane 18, accession no. AF411935) were deposited in GenBank as partial-length sequences and may represent only the subtype-definitive regions of the SCC*mec* gene (Kondo *et al.*, 2007). The SCC*mec* type patterns identified by RLB in our 42 reference strains were generally consistent with those extrapolated from MRSA SCC*mec* gene sequences in the public domain. For example, the majority of SCC*mec* type IV strains (Fig. 1, lanes 15–26) had SCC*mec* profiles indistinguishable from that of the genome sequence ID 27 (Fig. 2, lane 27, CP000255). Altogether 15 of 42 strains (35 %) had the same RLB patterns as those of corresponding GenBank sequences. However, there was some heterogeneity in the RLB patterns of other reference strains compared with GenBank sequences of the same SCC*mec* types. For example, RLB patterns of reference strains of SCC*mec* type III differed from the published MRSA SCC*mec* type III subtype 1 sequence (Fig. 2, lane 8, AB037671). This heterogeneity suggests the existence of distinct SCC*mec* type III subtypes (Fig. 2).

Theoretical RLB profiles of seven non-MRSA genome sequences are shown in Fig. 2. Four of five MSSA demonstrated no matches between probes and genomes, but one MSSA, and both meticillin-resistant coagulase-negative *Staphylococcus* strains, contained some SCC*mec* elements, as shown by matching of variable numbers of probes with their genomic sequences. These results are consistent with previous findings of SCC*mec* elements in MSSA and coagulase-negative staphylococci (Mombach Pinheiro Machado *et al.*, 2007; Hanssen & Sollid, 2007). It has been postulated that SCC*mec* elements can be transferred from coagulase-negative staphylococci to *S. aureus* (Shore *et al.*, 2008). Our results suggest that our mPCR/RLB typing system could also be extended to study of the distribution of SCC*mec* elements in other staphylococci.

Discrimination of SCC*mec* genotypes by mPCR/RLB

The mPCR/RLB SCC*mec* typing system developed in this study was based on an mPCR typing scheme described by Kondo *et al.* (2007) using *ccr* and *mec* gene complexes types with further discrimination based on differences in junkyard region 1 and the presence or absence of integrated transposons and plasmids. Another recently developed SCC*mec* typing system used minimal subsets of targets on *ccr* and *mec* gene complexes and three junkyard regions to provide maximal discriminatory capacity (Stephens *et al.*, 2007). The rationales for these two strategies are different: the former is based on known SCC*mec* variants, whereas the latter maximizes discrimination, by recognizing single nucleotide polymorphisms (SNPs) and is not limited to known SCC*mec* types. Our system was designed as an extension of the Kondo scheme, to discriminate major known SCC*mec* types and subtypes rapidly. However, it can be easily modified to accommodate probes for newly identified SCC*mec* elements.

The set of 42 well-characterized MRSA reference strains included isolates representing 5 SCC*mec* types, 9 clonal clusters, 22 *spa* types, 7 *nuc* types, 9 *femA* types, 14 toxin types and 15 MLST types (Cai *et al.*, 2007). The mPCR/RLB assay classified reference strains into 26 SCC*mec* subtypes, including one of SCC*mec* type I (1 strain), two of SCC*mec* II (2 strains), eight of SCC*mec* III (11 strains), eight of SCC*mec* IV (21 strains) and three of SCC*mec* V (3 strains). MRSA SCC*mec* type IV and its variants have increased in importance in recent years because they have been identified in virulent community-acquired strains (Deurenberg & Stobberingh, 2008). Four of the previously unclassified strains demonstrated unique RLB patterns resembling SCC*mec* types IV and V (Fig. 3).

Different sequence-based typing methods targeting different types of gene may not be directly comparable but they are often proposed as alternatives to pulsed-field gel electrophoresis for strain typing. The mPCR/RLB SCC*mec* typing method showed similar discriminatory power to that of MLST and *spa* typing in this small but diverse strain collection, as indicated by Simpson indices of diversity of 0.916 (CI 88.1–95.1 %) for mPCR/RLB typing, 0.882 (CI 85.2–91.2 %) for MLST and 0.926 (CI 90.3–94.9 %) for *spa* typing, respectively. Further investigation is required to determine its practical utility for SCC*mec* typing of a larger collection of clinical isolates. However, combined with GenBank SCC*mec* sequence data, this typing scheme also has the potential to identify novel

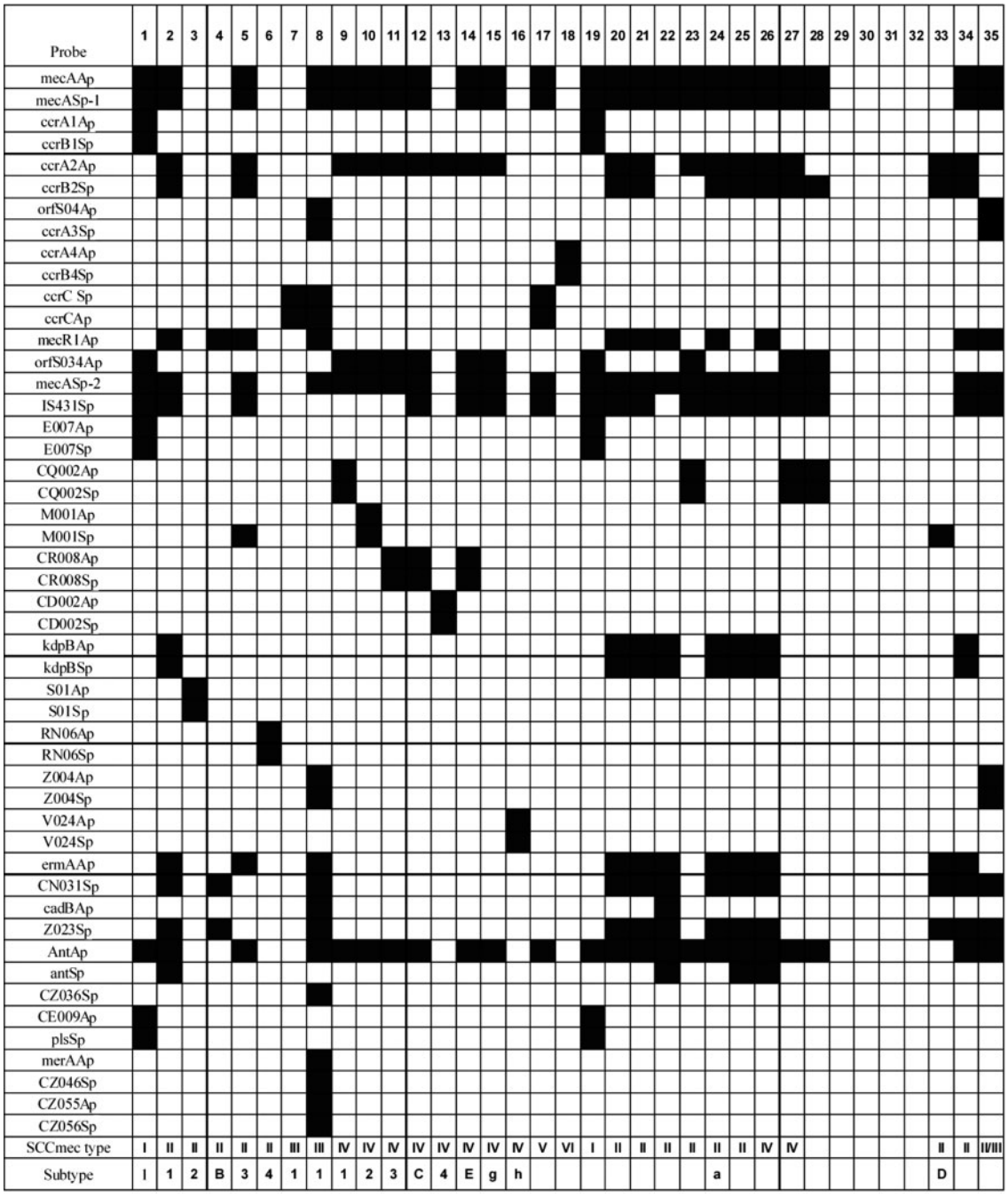
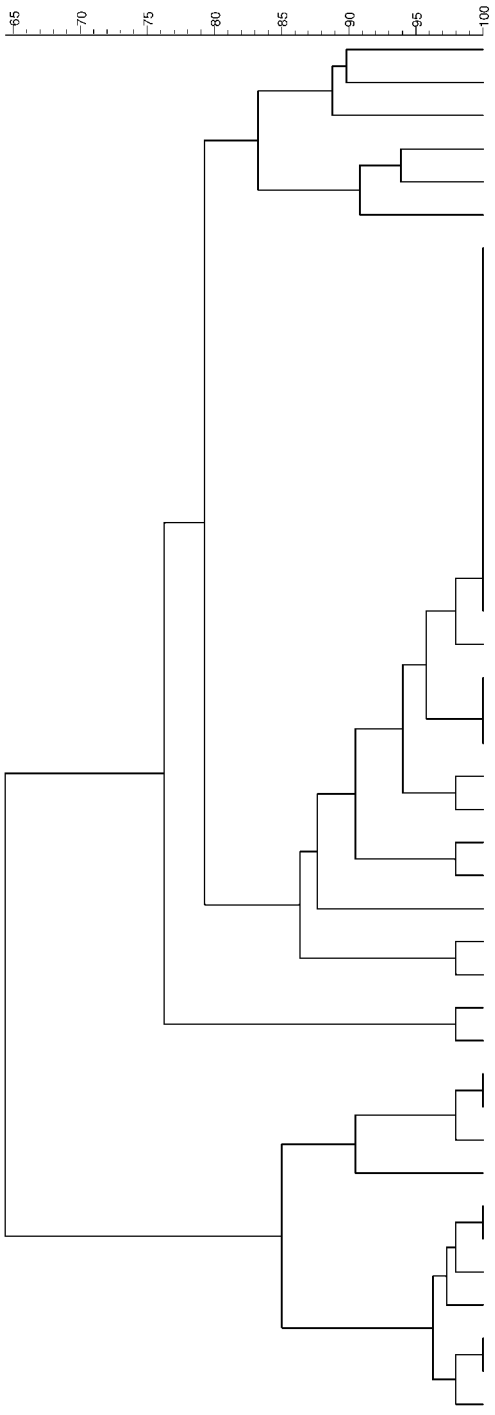


Fig. 2. Simulated *in silico* RLB results based on BLASTN search of RLB probes against SCC*mec* and whole-genome sequences of MRSA and seven non-MRSA staphylococcal genome sequences published in GenBank. Lanes 1–28 (28 MRSA sequences): 1, AB033763 (I.1); 2, D86934 (II.1); 3, AB127982 (II.2); 4, AJ810123 (IIB); 5, AJ810120 (II.3); 6, AB261975 (II.4); 7, AF422696 (III.1); 8, AB037671 (III.1); 9, AB063172 (IV.1); 10, AB063173 (IV.2); 11, AB096217 (IV.3); 12, AY271717 (IVC); 13, AB097677 (IV.4); 14, AJ810121 (IVE); 15, DQ106887 (IVg); 16, AF411936 (IVh); 17, AB121219 (V); 18, AF411935 (VI); 19, CP000046 (I); 20, CP000736 (II); 21, CP000703 (II); 22, BX571856 (II); 23, BA000033 (II); 24, AP009324 (IIa); 25, BA000017 (II); 26, BA000018 (IV); 27, CP000255 (IV); 28, CP000730. Lanes 29–33 (5 MSSA sequences): 29, AJ938182; 30, BX571857; 31, CP000253; 32, AP009351; 33, AM983545 (IID). Lanes 34–35: 34, CP000029 (II) – meticillin-resistant *S. epidermidis* genome; 35, AM904732 (II/III) – meticillin-resistant *S. pseudintermedius*. The black squares represent perfect matches of probe sequences with the GenBank SCC*mec* sequences; the white squares represent probes for which there was no matching GenBank sequence at the time of study (July 2008).



Strain	SCC*mec* type	SCC*mec* subtype	MLST	*spa*	SCC*mec* original type
B827549	NEW		1-1-1-1-1-1-1	t1784	NEW
COL	I	1	3-3-1-1-4-4-16	t008	I
J710566	V	3	10-14-8-6-10-3-2	t065	V
IMVS 67	V	1	3-3-1-1-4-4-3	t008	V
RPH74	V	2	10-14-8-6-10-3-2	t123	V
SN39	NEW		1-1-1-1-1-1-1	t127	NEW
13792-4492	IV	1	6-64-44-2-43-55-51	t202	IV
B8-10	IV	1	3-3-1-1-4-4-3	t711	IV
CH97	IV	1	1-4-27-4-12-1-10	t002	IV
E804531	IV	1	1-4-1-4-12-1-10	t002	IV
FH43	IV	1	1-1-1-1-1-1-1	t127	IV
IP01M1081	IV	1	19-23-15-2-19-20-15	t216	IV
MW2	IV	1	1-1-1-1-1-1-1	t128	IV
PAH 58	IV	1	2-2-2-2-6-3-2	t019	IV
PC8	IV	1	1-1-1-1-1-1-1	t127	IV
RBH98	IV	1	6-64-44-2-43-55-51	t202	IV
RHH10	IV	1	1-1-1-1-1-1-1	t127	IV
RPH85	IV	1	1-1-1-1-1-1-1	t127	IV
IP01M2046	IV	7	22-1-14-23-12-53-31	t1958	IV
FH53	IV	8	1-1-1-1-1-1-1	t127	I
RHH58	IV	8	1-1-1-1-1-1-1	t127	IV
SJOG 30	IV	8	1-1-1-1-1-1-1	t127	IV
CH16	IV	2	7-6-1-5-8-8-6	t032	IV
CH69	IV	3	7-6-1-5-8-8-6	t1963	IV
F829549	IV	5	22-1-14-23-12-4-31	t186	IV
PAH 1	IV	6	2-2-2-2-6-3-2	t019	IV
DEN2988	II	1	3-3-1-1-4-4-3	t008	IVA
C801535	NEW		22-1-14-23-12-4-31	t325	NEW
RPH2	NEW		3-3-1-1-4-4-3	t190	NEW
BK2464	II	2	1-4-1-4-12-1-10	t002	II
E822485	II	2	2-2-2-2-3-3-2	t018	II
RPAH15	III	1	2-3-1-1-4-4-3	t037	III
RPAH18	III	1	2-3-1-1-4-4-3	t037	III
HDG2	III	6	2-3-1-1-4-4-3	t421	IIIB
14176-5710	III	3	2-3-1-1-4-4-3	t1959	III
AH1	III	4	2-3-1-40-4-4-3	t037	IIIA
RDH81	III	4	2-3-1-1-4-4-3	t037	IIIA
AH13	III	5	2-3-1-1-4-4-3	t037	IIIA
HU25	III	8	2-3-1-1-4-4-3	t138	IIIA
K704540	III	7	2-3-1-1-4-4-3	t037	III
K711532	III	7	2-3-1-1-4-4-3	t037	III
ANS46	III	1	2-3-1-1-4-4-3	t037	III

Fig. 3. Relationships between 42 MRSA strains determined by mPCR/RLB and other typing methods. The MLST and *spa* typing results were reported by Cai *et al.* (2007).

SCC*mec* types/subtypes among clinical MRSA isolates with increased accuracy and study relationships between them. Reference strain FH53 (lane 1, Fig. 2) was previously reported to be SCC*mec* type I (Cai *et al.*, 2007). However, it appears to be similar to SCC*mec* IV reference and clinical strains as well as to the published SCC*mec* type IV sequence

(Fig. 3). There was also one genetic marker difference (probe βc2Sp) for strain MW2 between the mPCR/RLB assay and *in silico* results which could be attributed to the polymorphism of the binding site.

Advantages of the mPCR/RLB approach

The SCC*mec* gene typing initially proposed by Kondo *et al.* (2007) involves several mPCRs that produce amplicons of significant size (up to 15 kb) and requires gel-based detection. These features reduce the relevance of the typing scheme for infection-control-based interventions and tracking of introduction or transmission of different SCC*mec* types/subtypes. In contrast, the application of mPCR/RLB to the SCC*mec* typing described here enables the simultaneous screening of up to 43 samples using two mPCRs with a turnaround time (including culturing of isolates, DNA extraction, mPCR setup and running, and RLB hybridization) of about two working days. The preparation of the RLB membrane takes less than 2 h, and it can be reused at least 20 times based on our experience. The consumables cost is ~AU$8 per isolate. The results of mPCR/RLB can be represented as binary data, which are easily transferred and shared between diagnostic and public health laboratories, making the SCC*mec* typing easier to harmonize and suitable for rapid epidemiological studies of large numbers of isolates.

In conclusion, this SSC*mec* typing system improves our capacity to monitor molecular evolution and spread of MRSA and contributes to the development of effective strategies to control its dissemination. The application of the mPCR/RLB hybridization assay to MRSA SCC*mec* typing enhances the specificity, discriminatory power and throughput of the typing procedure. The sequence detection of up to 43 mPCR products in a single hybridization assay can transform MRSA SCC*mec* typing from epidemiological library typing into a tool for near-real-time infection control surveillance and MRSA hospital transmission tracking.

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