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A simplified multiplex PCR assay for fast and easy discrimination of globally distributed staphylococcal cassette chromosome *mec* types in meticillin-resistant *Staphylococcus aureus*

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A multiplex PCR assay was developed for the identification of major types and subtypes of staphylococcal cassette chromosome *mec* (SCC*mec*) in meticillin-resistant *Staphylococcus aureus* (MRSA) strains. The method uses a novel 9 valent multiplex PCR plus two primer pairs for *S. aureus* identification and detection of meticillin resistance. All 389 clinical MRSA isolates from Malaysia and 18 European isolates from the Harmony collection harbouring different SCC*mec* types that we tested were correctly characterized by our PCR assay. SCC*mec* type III and V were by far the most common types among both hospital- and community-acquired Malaysian MRSA isolates, with an apparent emergence of MRSA harbouring the IVh type.

INTRODUCTION

Meticillin-resistant *Staphylococcus aureus* (MRSA), a pathogen responsible for many nosocomial infections, was first reported in 1960 (Jevons, 1961). Over the past few decades it has emerged in the community as well, and it is currently considered a threat to public health (Lindsay & Holden, 2004). MRSA needs to be identified below the species level by rapid and reliable typing methods. Staphylococcal cassette chromosome *mec* (SCC*mec*) typing accompanied with overall genotyping has already provided strong evidence for the independent origins of health-care-associated MRSA and community-acquired MRSA (Naimi *et al.*, 2003). Resistance to meticillin in *S. aureus* is mediated by PBP2a, a penicillin-binding protein with low affinity for β-lactams, that is encoded by the *mecA* gene, which is located on SCC*mec* (Hartman & Tomasz, 1984; Ito *et al.*, 1999). To date, eight (Zhang *et al.*, 2009) different types of SCC*mec* (I–VIII) have been defined on the basis of the combination of *ccr* and *mec* complexes, but only type I–V are globally distributed, whilst others appear to exist as local strains in the country of origin (Oliveira *et al.*, 2006; Zhang *et al.*, 2009). The five allotypes of the *ccr* gene complex have been named *ccrAB1*, *ccrAB2*, *ccrAB3*, *ccrAB4* and *ccrC* (Ito *et al.*, 2001, 2004; IWG-SCC, 2009; Oliveira *et al.*, 2001). Hence, five classes of the *mec* gene complex (A–E) have been described (Ito *et al.*, 2001; Lim *et al.*, 2003). The regions located between these complexes are called J (joining) regions and in every SCC*mec* element there are three J regions (J1–J3) (Ito *et al.*, 2003). Polymorphism in J regions (mainly J1) have been described and used for the definition of SCC*mec* type IV subtypes (Chongtrakool *et al.*, 2006; Pérez-Roth *et al.*, 2004). SCC*mec* type IV has eight individual subtypes, which are characteristic for some of the notorious nosocomial MRSA clones such as EMRSA-15 in European countries, and that are starting to emerge in Asian countries (Cookson *et al.*, 2007; Ghaznavi-Rad *et al.*, 2010; Hsu *et al.*, 2007; Nadig *et al.*, 2010). For SCC*mec* type determination different PCR methods have been described; however, they do not always generate concordant data (Boye *et al.*, 2007; Kondo *et al.*, 2007; Milheiriço *et al.*, 2007b; Shore *et al.*, 2005). Moreover with the available methods more than one multiplex PCR is required for identifying all SCC*mec* types, which again needs lots of optimization and is also time consuming. The availability of an easy and fast assay for the routine monitoring of SCC*mec* types in the hospital or community would be more advantageous. Therefore, the aim of the current study was to establish a simplified multiplex PCR assay with a combination of established PCR primer sets for the rapid and easy detection of globally distributed SCC*mec* types.

Abbreviations: IWG-SCC, International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements; MRSA, meticillin-resistant *Staphylococcus aureus*; SCC*mec*, staphylococcal cassette chromosome *mec*; ST, sequence type.

METHODS

Bacterial isolates. In total, 389 non-duplicate clinical isolates of MRSA from hospitalized patients collected during the years 2007–2008 were used. All isolates were evaluated for the presence of the *mec*A gene by PCR (Milheiriço *et al.*, 2007b). A total of 9 reference strains, including SCC*mec* type I (NCTC 10442), type II (N315), type III (85/2082), type IVa (JCSC 4744), type IVb (JCSC 2172), type IVc (JCSC 4788), type IVd (JCSC 4469), type IVh (HAR 22), type V (strain WIS), and 18 strains from a European reference collection of epidemic MRSA (the Harmony collection) were included as well (Cookson *et al.*, 2007).

PCR primer sets (Table 1) were selected from published papers based on specificity, compatibility and ability to target fragments of SCC*mec* types I–V including type IV subtypes (Cookson *et al.*, 2007). For genotypic confirmation of *S. aureus*, Sa442 *S. aureus* species-specific primers were used (Martineau *et al.*, 1998).

Multiplex PCR. Chromosomal DNA was extracted from MRSA strains using a Qiagen DNeasy kit and a QIAcube robot (Qiagen). DNA concentration was determined using a Biophotometer (Eppendorf).

For multiplex PCR, a Qiagen multiplex PCR kit was used. Initially, the assay was optimized using reference strains. Reaction mixtures contained 1 μg chromosomal DNA, 25 μl master mix with 3 mM $MgCl_2$, 5 μl primer mix (2 μM in TE buffer for each primer) and RNAse-free water to a final volume of 50 μl. The reaction was carried out in a Biometra T PCR system (Biometra) according to the following program: an activation step at 95 °C for 15 min, followed by 30 cycles of initial denaturation at 94 °C for 30 s, 57 °C for 1.5 min and 72 °C for 1.5 min, ending with a final extension step at 72 °C for 10 min, and followed by a holding step at 4 °C.

The PCR amplicons (6 μl) were visualized using UV light (Alpha Imager) after electrophoresis in a 1.8 % (w/v) agarose gel containing 0.01 μl GelRed ml^{-1} (Biotium). In order to distinguish between similarly sized amplicons MetaPhor agarose was used, which can easily discriminate amplicons differing by less than 20 bp. The SCC*mec* type was determined on the basis of the band pattern obtained.

Validation. The multiplex PCR assay was first optimized using control strains; the efficacy of the assay was evaluated with 389 Malaysian MRSA isolates and 18 Harmony isolates (Table 2).

RESULTS

A clear and easily discriminated band pattern was obtained for all five types and subtypes of the main SCC*mec* types using the new multiplex PCR (Fig. 1). Each individual PCR yielded the fragments of expected size: i.e. 613, 287, 243, 776, 1000, 677, 1242, 663 and 325 bp for subtypes I, II, III, IVa, IVb, IVc, IVd, IVh and V, respectively, and 162 and 108 bp for the *mecA* gene and *sa442*, respectively. All the isolates were positive for *mec*A and a certain type of SCC*mec*. Evaluation of our multiplex PCR assay against 389 clinical MRSA isolates, with individual SCC*mec* type sequenced PCR products as the gold standard, showed 100 % sensitivity and specificity (Kondo *et al.*, 2007; Milheiriço *et al.*, 2007b). The vast majority (92.8 %) of the Malaysian isolates carried

Table 1. Primers used for the simultaneous identification and SCC*mec* typing of MRSA by multiplex PCR

Primer	Orientation	Oligonucleotide sequence (5′→3′)	Target gene	Strain used as standard	Size (bp)	Reference
Type I	Forward	GCTTTAAAGAGTGTCGTTACAGG	ORF E008 of strain NCTC10442	NCTC 10442	613	Zhang *et al.* (2005)
	Reverse	GTTCTCTCATAGTATGACGTCC				
Type II	Forward	GATTACTTCAGAACCAGGTCAT	*kdpE* of strain N315	N315	287	Kondo *et al.* (2007)
	Reverse	TAAACTGTGTCACACGATCCAT				
Type III	Forward	CATTTGTGAAACACAGTACG	J1 region of SCC*mec* type III	85/2082	243	Milheiriço *et al.* (2007b)
	Reverse	GTTATTGAGACTCCTAAAGC				
Type IVa	Forward	GCCTTATTCGAAGAAACCG	ORF CQ002 of strain CA05	JCSC 4744	776	Zhang *et al.* (2005)
	Reverse	CTACTCTTCTGAAAAGCGTCG				
Type IVb	Forward	AGTACATTTTATCTTTGCGTA	J1 region of SCC*mec* type IVb	JCSC 2172	1000	Okuma *et al.* (2002)
	Reverse	AGTCATCTTCAATATGGAGAAAGTA				
Type IVc	Forward	TCTATTCAATCGTTCTCGTATT	IVc element of strain 81/108	JCSC 4788	677	Ma *et al.* (2005)
	Reverse	TCGTTGTCATTTAATTCTGAACT				
Type IVd	Forward	AATTCACCCGTACCTGAGAA	CD002 in type IVd	JCSC 4469	1242	Kondo *et al.* (2007)
	Reverse	AGAATGTGGTTATAAGATAGCTA				
Type IVh	Forward	TTCCTCGTTTTTTCTGAACG	J1 region strain HAR22	HAR22	663	Milheiriço *et al.* (2007b)
	Reverse	CAAACACTGATATTGTGTCG				
Type V	Forward	GAACATTGTTACTTAAATGAGCG	ORF V011 of strain JCSC3624	WIS	325	Zhang *et al.* (2005)
	Reverse	TGAAAGTTGTACCCTTGACACC				
mecA	Forward	TCCAGATTACAACTTCACCAGG3	*mec*A gene	ATCC29247	162	Milheiriço *et al.* (2007b)
	Reverse	CCACTTCATATCTTGTAACG				
Sa442	Forward	AATCTTTGTCGGTACACGA-TATTCTTCACG	Species-specific target	–	108	Martineau *et al.* (1998)
	Reverse	CGTAATGAGATTTCAGTAGA-TAATACAACA				

Table 2. Comparison of the present study with the traditional PCR SCC*mec* typing method for Harmony isolates

Isolates shown in bold (97151, 1000/93, 98/26821, 76167) were newly subtyped using our protocol.

Isolate	Country	Ridom *spa* type	MLST ST	MLST CC	ST by traditional SCC*mec**	ST by SCC*mec* typing in current study
1155-1/98	Germany	t001	228	5	I	I
13	Slovenia	t001	228	5	I	I
18	Slovenia	t001	228	5	I	I
M307-I	UK	t001	5	5	I	I
97S98 (95/5101/1)	Belgium	t303	247	8	I	I
61974-II	Finland	t002	5	5	II	II
96/32010	UK	t018	36	30	II	II
99/1139	UK	t018	36	30	II	II
99/159	UK	t018	36	30	II	II
97S101 (95/1119/3)	Belgium	t045	5	5	III	III
98541	Finland	t037	241	8	III	III
AK 541	Sweden	t037	239	8	III	III
37481	Finland	t234	239	8	III	III
3680	Greece	t037	239	8	III	III
97151	France	t024	8	8	IV	**IVh**
1000/93	Germany (Hannover)	t009	254	8	IV	**IVh**
98/26821	UK	t1275	22	22	IV	**IVh**
76167	Finland	t015	45	45	IV	**IVc**

CC, Clonal complex; MLST, multilocus sequence typing; *spa*, staphylococcal protein A encoding gene.
*Kondo *et al.* (2007).

SCC*mec* type III, while 22 (5.7 %) isolates harboured SCC*mec* type V and 6 (1.5 %) were demonstrated to contain SCC*mec* type IVh. The results obtained for the European Harmony collection are summarized in Table 2. All results are in accordance with those obtained by established methods (Cookson *et al.*, 2007). All the type V isolates were confirmed by the presence of the *ccrC* gene, and six sequence type (ST)-22 IVh strains were assigned by the *ccrA2B2* and type B *mec* element.

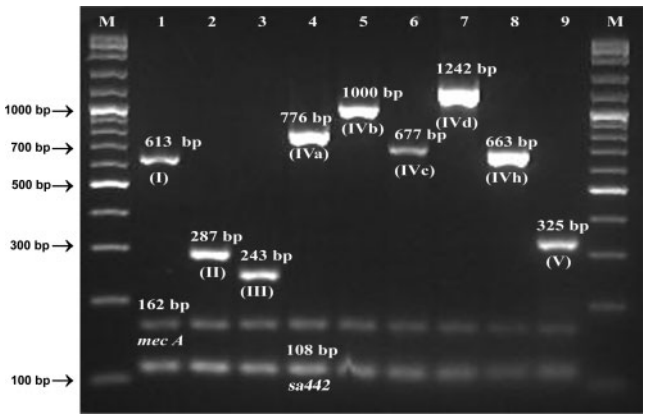

Fig. 1. Validation and application of the SCC*mec* multiplex PCR. Lane 1, SCC*mec* type I (NCTC 10442); lane 2, type II (N315); lane 3, type III (85/2082); lane 4, type IVa (JCSC 4744); lane 5, type IVb (JCSC 2172); lane 6, type IVc (JCSC 4788); lane 7, type IVd (JCSC 4469); lane 8, type IVh HAR22; lane 9, type V (WIS); M, DNA molecular mass size marker (VC 100 bp Plus DNA ladder; Vivantis).

DISCUSSION

The multiplex PCRs optimized by Oliveira & de Lencastre (2002) have been used for assigning SCC*mec* elements based on the identification of eight loci (A to H), mostly located in the J regions. The PCRs were updated for the identification of the SCC*mec* type V but the system is still limited due to its inability to discriminate SCC*mec* subtypes IVa, b, c, d and h; however, an individual multiplex PCR dedicated for the subtyping of the class IV was described simultaneously (Milheiriço *et al.*, 2007a). In addition, due to the presence of common elements, such as *ccrA2B2* in type II and IV or the class A *mec* gene in types II and III, complex comparison procedures and sometimes difficult interpretation may compromise the final results (Milheiriço *et al.*, 2007b). The most informative and robust method to determine SCC*mec* types was introduced by Kondo *et al.* (2007), but five different multiplex PCRs with 34 pairs of primers are required. Hence, routine application is considered cumbersome. Recently, a new and apparently simple method was introduced by Boye *et al.* (2007) using four sets of primers only. However, this method will only identify major SCC*mec* types. The advantages of the method we present here, as compared to the method of Boye *et al.* (2007), is that subtyping of type IV

SCC*mec* is feasible. We found that six of our clinical isolates and three Harmony MRSA strains, which were earlier reported as type IV (ST-254-MRSA-IV, ST-22-MRSA-IV and ST-8-MRSA-16), were categorized now as type IVh. MRSA with such cassettes are currently penetrating Asian countries, but have thus far been hard to detect in Singapore and Taiwan (Hsu *et al.*, 2007; Huang *et al.*, 2006). The possible emergence of a new SCC*mec* type in the future cannot be neglected as seen with SCC*mec* type VI to VIII; hence, reconfiguration of the assay will be necessary when significant numbers of new SCC*mec* type strains have evolved.

In Malaysian hospitals, the MRSA prevalence ranges from 5.6 to 33.7 %, with an overall rate of 26 % (Ministry of Health, 2008). Most hospital-acquired MRSA belong to ST239 III/IIIA (Ghaznavi-Rad *et al.*, 2010) and EMRSA 15 IVh strains are emerging. In addition, increasing numbers of community-acquired MRSA of ST188, SCC*mec* type V are being detected (Nor Shamsudin *et al.*, 2008). The new assay developed herein was successful in typing MRSA regardless of their clinical source, clonality, demography, period of isolation and ethnicity of the people the MRSA isolates came from. Although the majority of the isolates tested were of SCC*mec* type III, the correct typing of harmony isolates confirms the reliability of the assay.

Comparison of our new assay with the traditional PCR SCC*mec* typing method (including *mec* and *ccr* gene complex typing) and the assay of Milheiriço *et al.* (2007b) demonstrated 100 % sensitivity and specificity when testing a number of control strains. Our multiplex PCR assay does not conflict with any of the PCRs reported before (Kondo *et al.*, 2007; Milheiriço *et al.*, 2007b). The new method is a combination of earlier PCRs, but with an optimized PCR protocol that can be applied effectively for the routine diagnosis and epidemiological typing of SCC*mec* in MRSA.

Conclusions

We have successfully simplified the protocol of SCC*mec* typing to allow rapid and reliable assignment of SCC*mec* types to MRSA strains. This strategy is more robust in its ability to characterize most known SCC*mec* types and subtypes, and genotypically confirms *S. aureus* as well as meticillin resistance in a single tube.

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