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Revelation of staphylococcal cassette chromosome mec types in methicillin-resistant *Staphylococcus aureus* isolates from Thailand and Vietnam

R. Lawung [a,b], L.V. Chuong [a,c], R. Cherdtrakulkiat [a], A. Srisarin [d], V. Prachayasittikul [a,*]

[a] Department of Clinical Microbiology and Applied Technology, Faculty of Medical Technology, Mahidol University, Bangkok, Thailand
[b] Center of Medical Laboratory Services, Faculty of Medical Technology, Mahidol University, Bangkok, Thailand
[c] Department of Microbiology, Faculty of Medicine, University of Medicine and Pharmacy, Ho Chi Minh City, Vietnam
[d] Department of Clinical Microscopy, Faculty of Medical Technology, Mahidol University, Bangkok, Thailand

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ABSTRACT

Methicillin resistant *Staphylococcus aureus* (MRSA) is highly prevalent, and its typing plays a crucial role in epidemiology and evolution in both health and community settings. Multiplex PCR and staphylococcal cassette chromosome *mec* (SCC*mec*) typing based on *mec* complexes and cassette chromosome recombinase (*ccr*) allotypes have been developed for MRSA identification. The first of these procedures can identify 4 *mec* classes (A, B, C1, and E) and 2 *ccr* allotypes (*B2* and *B4*) in one tube, and the second can identify *mecA*, *mec* class C2, and 3 allotypes (*A1*, *A3*, and *C*). Our method offers a novel means to further differentiate between the main SCC*mec* types I through XI and is both highly sensitive (detectable up to 0.3ηg DNA) and specific (100%). Several SCC*mec* types (I, III, IV, V and a non-typeable group) were found in 66 MRSA isolates obtained from Ho Chi Minh City, Vietnam and Nakhon Pathom, Thailand. SCC*mec* type III was highly predominant in both regions. The designed assay is rapid, convenient, flexible, and reliable. Therefore, this assay is suitable for the high-throughput screening of the main SCC*mec* types of MRSA isolates.

1. Introduction

Staphylococcus aureus infection represents a potentially life-threatening event in health care facilities and community settings (Lowy, 1998). It is highly prevalent and can develop multi-drug resistance, as in the case of methicillin-resistance *Staphylococcus aureus* (MRSA). MRSA is mostly derived from a *mecA* gene insertion into the chromosome of methicillin-susceptible *S. aureus* (MSSA). The *mecA* gene encodes a 78-kDa penicillin binding protein (PBP), PBP2a or PBP2′, that binds with low affinity to all β-lactams (Berger-Bachi and Rohrer, 2002). The *mecA* gene is situated on a mobile genomic island on the staphylococcal cassette chromosome *mec* (SCC*mec*) (Ito et al., 2003). The SCC*mec* elements share several characteristics: (i) they carry the *mecA* gene in the *mec* gene complex, (ii) they carry the *ccr* gene(s) in the *ccr* gene complex (*ccrAB* and/or *ccrC*), (iii) they integrate at a specific site in the *S. aureus* chromosome, referred to as the integration site sequence (ISS) for SCC, and (iv) they are flanked by direct repeat (DR) sequences containing the ISS (IWG-SCC, 2009). SCC*mec* elements are classified into types and subtypes. Types are classified by the combination of the type of *ccr* gene complex (*ccr* gene allotype) and the class of the *mec* gene complex. These are the key elements of the cassette responsible for the integration and excision of SCC*mec* and the beta-lactam resistance phenotype (IWG-SCC, 2009). Another region that differs between the *ccr* and *mecA* gene complexes is the joining region (J1–J3), which can be used to identify SCC*mec* subtypes within the same SCC*mec* type. Five classes of the *mec* gene complex (class A, B, C1, C2, and D) have been described, depending on the structural diversity of the *mecA* regulatory genes (*mecR1* and *mecI*) caused by the integration site of the insertion sequences (*IS1272* and *IS431*) (IWG-SCC, 2009). Recently, a new *mec* class (class E) was reported (Shore et al., 2011; Turlej et al., 2011). Three phylogenetically distinct *ccr* genes (*ccrA*, *ccrB* and *ccrC*) have been found in *S. aureus*. The *ccr*A and the *ccr*B genes have been differentiated into four and five allotypes, respectively, while the *ccr*C variants have a high nucleotide similarity and only one allotype, defined as *ccrC1* (Turlej et al., 2011). Based on the types of the *mec* gene complex and the *ccr* gene complex, eleven major SCC*mec* elements have been identified (Shore et al., 2011; Turlej et al., 2011).

To control the dissemination of MRSA, epidemiological studies are required. SCC*mec* typing is regarded as a useful tool for the high-throughput classification of MRSA. During the last decade, several methods based on multiplex PCR have been developed to detect SCC*mec*

Abbreviations: MRSA, methicillin resistant *Staphylococcus aureus*; *SCCmec*, staphylococcal cassette chromosome mec; *ccr*, cassette chromosome recombinase; HCMC-Vietnam, Ho Chi Minh City, Vietnam; NP-Thailand, Nakhon Pathom, Thailand.

* Corresponding author at: Department of Clinical Microbiology and Applied Technology, Faculty of Medical Technology, Mahidol University, Bangkok 10700, Thailand. Tel.: +66 2 4414376; fax: +66 2 4414380.
E-mail address: virapong.pra@mahidol.ac.th (V. Prachayasittikul).

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types I to V based on the analysis of variants in the J regions and the classification of *ccr* and *mec* complexes (Boye et al., 2007; Ghaznavi-Rad et al., 2010; Kondo et al., 2007; Milheirico et al., 2007; Zhang et al., 2005). The methods that targeted the J regions were systemically flawed because they could not differentiate between SCC*mec* types I and VI and types IV and VI (Turlej et al., 2011). Furthermore, new SCC*mec* typing systems must be developed to address the increasing diversity of the known SCC*mec* subtypes and the discovery of new SCC*mec* types, including SCC*mec* types VII, X, and XI (Turlej et al., 2011). Therefore, we developed a multiplex PCR (M-PCR) assay for the identification of MRSA and the SCC*mec* types. The *mecA* gene and ten target genes of *ccr* and *mec* complexes were used for the MRSA identification and the SCC*mec* classification, respectively. This method represents a rapid, robust, and convenient way to effectively distinguish between major reported SCC*mec* types of MRSA.

2. Materials and methods

2.1. Bacterial strains

Sixty-six MRSA isolates collected from Ho Chi Minh City, Vietnam (HCMC-Vietnam) and Nakhon Pathom, Thailand (NP-Thailand) were used for this study. Forty-five isolates were collected between 2008 and 2010 from four tertiary hospitals in Vietnam (Cho Ray Hospital, Gia Dinh People's Hospital, 175 Military Hospital, and the Thong Nhat Hospital) and twenty-one isolates were collected in 2011 from the Nakhon Pathom Hospital of Thailand. Eight known SCC*mec* strains, which were kindly supplied by Teruyo Ito (Jutendo University, Tokyo, Japan), were used for method validation. These included NCTC10442 (SCC*mec* type I), JCSC3069 (SCC*mec* type II), 85/2082 (SCC*mec* type III), JCSC4474 (SCC*mec* type IVa), JCSC2172 (SCC*mec* type IVb), JCSC4488 (SCC*mec* type IVc), JCSC4469 (SCC*mec* type IVd), and WIS (SCC*mec* type V). Furthermore, twelve MSSA and twenty-eight non-staphylococcal isolates were also used to evaluate the specificity of our developed method.

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by disk diffusion and agar dilution methods, as recommended by the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2011). MRSA was identified using a disk diffusion method with cefoxitin (30 μg) and oxacillin (1 μg). All MRSA isolates was also tested with non-β lactam antibiotics, including erythromycin (15 μg), clindamycin (2 μg), trimethoprim–sulfamethoxazole (1.25/23.75 μg), tetracycline (30 μg), rifampin (5 μg), linezolid (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), quinupristin–dalfopristin (15 μg), and gentamicin (10 μg) (Oxoid, England). The detection of inducible clindamycin resistance was accomplished with the D-zone test. Vancomycin susceptibility testing was performed using the agar dilution method. *S. aureus* ATCC 25923 and *S. aureus* ATCC 29213 were used as the control strains for the disk diffusion and agar dilution method, respectively.

2.3. The SCCmec typing system

2.3.1. Preparation of a DNA template

DNA template isolation was accomplished according to a method described previously (Chen et al., 2009). Briefly, a loopful of overnight bacterial growth on nutrient agar was suspended in 100 μL of freshly prepared 20 μg/mL lysostaphin solution (Sigma-Aldrich, USA), then incubated at 37 °C for 10 min and heated at 100 °C for 10 min. The solution was centrifuged at 10,000 *g* for 5 min, and 2 μL of the supernatant was used as a target for the PCR reaction.

2.3.2. Primer design

Primers were designed from thirty-seven SCC*mec* sequences published in the National Center for Biotechnology Information (NCBI) database. ClustalW2 was applied to check the variance and homology of specific genes, and Primer3 online software was used to find the primers. The BLAST and The OligoAnalyzer 3.0 programs were also used. The designed primers are presented in Table 1.

For the classification of both the *ccr* and *mec* complexes, two M-PCR reactions (20 μL reaction mixture) containing PCR buffer, 1.5 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate (dNTP), 1.5 U i-Taq (Intron Biotechnology, Korea) and 4.5 μL of primer mix were prepared (Table 2). The primers in the first reaction tube were composed of 2.0 μM mcABC1-F primer, 2.0 μM mcA-R primer, 0.5 μM mcB-R primer, 0.5 μM mcC1-R primer, 0.625 μM mcE primers (forward and reverse), 0.625 μM ccrB2 primers (forward and reverse), and 0.625 μM ccrB4 primers (forward and reverse), while the other tube contained 0.125 μM mecA primers (forward and reverse), 0.125 μM mcC2 primers (forward and reverse), 0.188 μM ccrC primers (forward and reverse), 0.125 μM ccrA1/3-F primer, 0.125 μM ccrA1-R primer, and 0.125 μM ccA3-R primer. The reaction was performed in a Thermal Cycler (Bio-Rad, USA) under the following conditions: 1 cycle of initial denaturation at 95 °C in 5 min, 35 cycles at 95 °C for 30 s, 57 °C for 45 s, and 72 °C for 1 min, and a final extension step at 72 °C for 10 min. The PCR products (5 μL) were subjected to electrophoresis on a 1.5% agarose gel. The SCC*mec* type was identified by the band patterns obtained (Table 2).

2.3.3. Detection limits

The detection limit was evaluated using serial two-fold dilutions of the DNA template (0.016–0.5 ng DNA) in a multiplex PCR system. The limit of detection is defined as the positive results of the band patterns at the lowest DNA concentration.

2.3.4. Specificity of M-PCR

We assessed the specificity of the developed method using 12 MSSA and a variety of 28 clinically non-staphylococcal isolates including *Enterococcus* spp. (6 isolates), *Streptococcus* spp. (3 isolates), *Micrococcus luteus*, *Shigella boydii*, *Salmonella typhi*, *Edwardsiella tarda*, *Escherichia coli*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Acinetobacter baumanii*, *Stenotrophomonas maltophilia*, *Aeromonas hydrophila*, *Plesiomonas shigelloides*, *Pasteurella multocida*, *Bacillus cereus*, *Corynebacterium diphtheriae*, *Listeria monocytogenes*, *Neisseria mucosa*, and *Moraxella catarrhalis*.

2.4. Statistical analysis

The chi-square test was used to analyze the prevalence rates of the MRSA isolates from HCMC-Vietnam and NP-Thailand in each antibiogram (Eveillard et al., 2005). The results were considered statistically significant when p-value was < 0.05.

3. Results

Sixty-six MRSA isolates from HCMC-Vietnam and NP-Thailand were found to have similar antibiograms. All isolates were susceptible to vancomycin, linezolid, and quinupristin–dalfopristin. They showed high rates of resistance to ciprofloxacin, erythromycin, clindamycin, gentamicin, tetracycline, and trimethoprim–sulfamethoxazole but were susceptible to rifampin and chloramphenicol. Furthermore, all MRSA isolates had multiple drug resistance (MDR) to at least four groups of non-β-lactam antibiotics (Table 3).

All target genes in our method were clearly illustrated and easily identified. In addition, all primer sets exhibited a high specificity to their corresponding SCC*mec* targets. No cross-reaction was found among the *mec* classes or the *ccr* allotypes. The results exhibited 100% in accordance with all tested reference strains (Fig. 1), with the detection limits of the template at 0.312 ng for all SCC*mec* types except *mec*