A simple experiment was performed to observe in vitro transfer of meca gene from methicillin-resistant Staphylococcus aureus, Malaysian clinical isolates, to methicillin-sensitive one. PCR-based method was used in combination with the selective antibiotic screening method to study the transfer of resistance among clinical isolates carrying different genetic markers. After performing the mixed liquid culture experiments, two types of the transconjugants were obtained. Transconjugants were analyzed by PCR for the presence of meca and ccr recombinase genes. The meca, ccrA3 and ccrB2 genes were detected in donor strains as well as in both types of transconjugants. Transfer of meca, ccrA3 and ccrB2 genes from donor to recipient strains that were present in close proximity during in vitro experiment may suggest possibility of horizontal gene transfer (HGT) among S. aureus clinical strains. Bacterial co-infections in vivo where the bacteria could be in close proximity are likely to be a possible point of horizontal gene transfer (HGT) with regard to certain antibiotic resistant genes.

Key words: Meca, horizontal gene transfer, SCCmec, ccr genes, methicillin-resistant Staphylococcus aureus (MRSA).

INTRODUCTION

Staphylococcus aureus has been recognized as a bacterium that can cause a broad spectrum of diseases and is a major cause of hospital-acquired infections worldwide. Methicillin-resistant S. aureus (MRSA) often referred to as the hospital ‘superbug’ hence clinical isolates, are often resistance to a wide variety of antibiotics. Horizontal gene transfer (HGT) among bacteria and their mobile genetic elements (MGEs) is a primitive mode of transfer and spread of antibiotic resistance in clinically important pathogens. The rapid acquisition of antibiotic resistance in S. aureus in the early 1960s was mediated by plasmid transfer. However, horizontal gene transfer (HGT) events have played a prominent role in the rapid acquisition of antibiotic resistance in S. aureus in recent years (Brody et al., 2008). The cellular mechanism by which S. aureus is capable of withstanding the inhibitory effects of all β-lactam antibiotics, including the semi-synthetic ones such as methicillin, is based on acquisition of the meca gene (Jansen et al., 2006). Transfer of meca is mediated by the staphylococcal cassette chromosome mec (SCCmec) (Wang and Archer, 2010). Although the origins of the meca gene (the principle component of methicillin resistance in staphylococci) are unknown, a meca homologue (88% similarity) is ubiquitous in the antibiotic-susceptible Staphylococcus sciuri, and may be a possible evolutionary precursor of the meca gene of the MRSA strains. Furthermore, S. aureus meca was identical to that identified in a Staphylococcus epidermidis isolate from the same individual, thus suggesting that MRSA strain has arisen in vivo by horizontal transfer of meca between two staphylococcal species (Brody et al., 2008; Wielders et al., 2001; Deurenberg et al., 2007). It has been hypothesized that all MRSA clones have a common ancestor, while another theory suggests that SCCmec was introduced several times into different S. aureus lineages (Juhas et al., 2009).
MecA is carried on a genetic element, SCCmec, which inserts precisely into the *S. aureus* chromosome at orfX (Hiramatsu et al., 2001). Genes encoding cassette chromosome recombinases (ccr) are located within the SCCmec elements. For movement, SCCmec carries specific genes (ccr), which encode recombinases of the invertase/resolvase family. Cassette chromosome recombinase (ccr) genes (ccrC or the pair of ccrA and ccrB) encode recombinases mediating integration and excision of SCCmec into and from the chromosome. Four different homologous pairs of ccr AB genes and one ccr C gene have been reported (Hanssen and Sollid, 2006). These genes are designated as, ccrA1, ccrA2, ccrA3, ccrA4, ccrB1, ccrB2, ccrB3 and ccrB4 (Lto et al., 2004). Integration of a SCCmec element into the chromosome converts drug-sensitive *S. aureus* into hospital pathogen methicillin-resistant *S. aureus* (MRSA), which is resistant to practically all β-lactam antibiotics (Hiramatsu et al., 2001).

The mechanism(s) responsible for mecA transfer is still obscure. The assumption is that the ccr and mec genes were brought together in coagulase-negative staphylococci (CoNS) from an unknown source where deletion in the mec regulatory genes occurred before the genes were transferred into *S. aureus* to generate MRSA (Hanssen et al., 2004). Studies with human staphylococcal strains indicate that *S. epidermidis* is a reservoir of antibiotic resistance genes that can be transferred to *S. aureus* under *in vitro* and *in vivo* conditions (Weidlers et al., 2002). This study was therefore performed to observe *in vitro* resistance transfer of mecA gene from MRSA clinical isolate to methicillin-sensitive one that carries different phenotypic markers. Simple experiment was conducted using PCR-based method in combination with the selective antibiotic screening test for determining direction of resistance transfer.

**MATERIALS AND METHODS**

**Bacterial strains**

Clinical isolates were obtained from clinical samples processed at the diagnostic microbiology laboratory at University Malaya Medical Centre (UMMC, Kuala Lumpur, Malaysia). For strain identification, 386 Af. J. Biotechnol.

**Table 1.** Antibiotic resistance and sensitivity profiles of the donors, recipients and transconjugants that were obtained by disc diffusion method.

<table>
<thead>
<tr>
<th>Strain profile</th>
<th>Antibiotic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ox</td>
</tr>
<tr>
<td>Recipients</td>
<td>S</td>
</tr>
<tr>
<td>Donors</td>
<td>R</td>
</tr>
<tr>
<td>Transconjugant A</td>
<td>R</td>
</tr>
<tr>
<td>Transconjugant B</td>
<td>R</td>
</tr>
</tbody>
</table>

Ox, Oxacillin; Amp, ampicillin; Gen, gentamicin; Ery, erythromycin; Tet, tetracycline; Amik, amikacin; R, resistant; S, sensitive.

**Determining antibiotic resistance profile of bacterial strains**

Resistance to various antibiotics was determined using the method recommended by the Clinical and Laboratory Standards Institute (CLSI, 2006). Disk diffusion was performed on Mueller–Hinton agar using Kirby–Bauer method (Oxoid, Basingstoke, UK). Antibiotics; oxacillin, ampicillin, gentamicin, erythromycin, tetracycline and amikacin, were used for determining the sensitivity of the bacterial strains.

**Criteria for choosing donors and recipients and their antibiotic profiles**

In order to differentiate the donor from recipient, the strain carrying different genetic marker was selected. Donor strains that are resistant to methicillin (oxacillin) were selected based on the presence of mecA gene (mecA+) and recipient strains that are sensitive to methicillin were selected based on absence of mecA gene (mecA−). After studying antibiotic resistant profile of the isolates, the donor strain with the following genetic marker was chosen depending on the availability of the clinical isolates in our laboratory (Table 1). The donor that was chosen was sensitive to gentamicin and resistant to tetracycline and oxacillin, while recipient that was chosen was resistant to gentamicin and sensitive to tetracycline and oxacillin.

**Transfer of antibiotic resistance in mixed liquid cultures**

Bacterial cultures were grown for 12 h in Luria-Bertani (LB) broth. Equal volumes (200 ml) of the donor and recipient strains were mixed in a flask. The cultures were supplemented with 200 ml of LB broth and incubated at 37°C without shaking. Aliquots of 50 ml each were withdrawn after 6 h and placed on two different antibiotics - tetracycline (30 µg/ml) and gentamicin (10 µg/ml) containing agar plates respectively after appropriate dilution. After 24 h, colonies resistant from each plate were picked up as probable transconjugants. The method used was a modification of the experiment performed by Khan et al. (2000). The choice of these antibiotics was purposefully made so that the resistant colonies might represent the transconjugants when the transfer of oxacillin resistance from resistant to sensitive strains or the transfer of gentamicin resistance from sensitive to resistant strains had taken
place. In either case, the transconjugants would be resistant to oxacillin and gentamicin. Colonies from these plates were placed on oxacillin containing agar and blood agar plates.

Detection and amplification of \textit{mecA} and \textit{ccrAB} genes by PCR

DNA extraction was performed following the method described by Unal et al. (1992). The \textit{mecA} primer that could produce 214 bp PCR product was used to screen donors, recipients and transconjugants for the presence of \textit{mecA} gene (Sabet et al., 2006). The donors, recipients and transconjugants were subjected to the PCR for the presence of genes as well. Six primers pairs were used to screen the presence of \textit{ccrAB} genes according to the work by Hanssen et al. (2004). The mastermix gave a final concentration of: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.4 mM MgCl$_2$, 0.09 mM of each dNTP and 0.4 $\mu$M of each of the primers. The 45 $\mu$l mastermix were mixed with 5 $\mu$l template. The following PCR amplification conditions for \textit{ccrA1}, \textit{ccrB1}, \textit{ccrA3} and \textit{ccrB3} primer sets were used: 1 min at 95°C, 30 cycles with 30 s at 95°C, 10 s at 57°C, 60 s at 72°C and the final extension was 4 min at 72°C. For \textit{ccrA2} and \textit{ccrB2} sets of primers, the following conditions were used: 1 min at 94°C, 30 cycles with: 30 s at 94°C 30 s at 62°C, 60 s at 72°C and the final extension was 4 min at 72°C. 10 $\mu$l of PCR-amplified reaction mixture was resolved by electrophoresis on agarose gel. The sizes of amplification product were estimated by comparison with a 100-bp molecular size standard ladder (New England BioLabS, UK).

DNA sequencing of \textit{mecA} and \textit{ccrAB} genes

Polymerase chain reaction products for both \textit{mecA} and \textit{ccrAB} genes were purified using the PCR Purification Kit (Qiagen, Hilden, Germany). The PCR DNA sequencing was carried out using an automated DNA sequencer (an ABI Prism 377 DNA sequencer;
PERKIN-ELMER ABI, WELLESLEY, MA). Nucleotide sequences were analyzed and compared by the use of the BLAST computer program (National Center for Biotechnology Information http://www.ncbi.nlm.nih.gov).

RESULTS

Strain Identification and antibiotic resistance profile of bacterial isolates

The Gram stain characteristics of the all bacterial strains were Gram positive cocci (GPC) in clusters. All the strains were catalase and tube coagulase positive. Using API Staph. system for the identification, all stains were identified as S. aureus. Antibiotic resistance profiles of the donor strains (MRSA) were resistant to oxacillin, ampicillin, erythromycin, tetracycline and amikacin, but sensitive to gentamicin (Table 1). The recipient strains (MSSA) were resistant to ampicillin, gentamicin and erythromycin, but sensitive to oxacillin, tetracycline and amikacin (Table 1).

Antibiotic resistance profile of transconjugants

After performing the mixed liquid cultures experiments and plating on tetracycline and gentamicin-containing agar plates, the growth observed indicated that these colonies are transconjugants. Two types of the transconjugants were obtained (Table 1). The antibiotic resistance profile of the transconjugant A is shown in Figure 1C. The transconjugant A was resistant to oxacillin, ampicillin, gentamicin, erythromycin, tetracycline and amikacin. The transconjugant B was resistant to oxacillin, ampicillin, gentamicin and erythromycin, but sensitive to tetracycline and amikacin (Figure 1D).

Detection of mecA and ccr AB genes at gene level

Prior to mixing, the bacterial lysates from donor and recipient strains were subjected to PCR analysis. The mecA gene was detected in donor strain but not amplified in recipient strains (Figure 2). To determine whether the transfer of the mecA gene had taken place or not, the transconjugants were analyzed by PCR for the presence of mecA gene.

The PCR amplified products from donors, recipients and transconjugant A and transconjugant B are shown in Figure 2. As shown in the Figure, the mecA gene was present in both transconjugant A and B and donor strains. The donors, recipients and transconjugants were subjected to the PCR assay for the presence of ccrAB genes. The PCR results show the amplification of ccrA3 and ccrB2 genes from donors, transconjugant A and transconjugant B. Amplification of ccrA3 gene and ccrB2

Figure 2. An ethidium bromide-stained gel demonstrating PCR amplification of mecA gene in donors, recipients and transconjugants. Lanes 1 and 2, donors; lanes 3 and 4, transconjugants A; lanes 5 and 6, transconjugants B; lanes 7 to 10, recipients; lane 11, negative control; lane 12, 100 bp DNA marker.
Figure 3. An ethidium bromide-stained gel demonstrating PCR amplification of ccrA3 gene in donors, recipients and transconjugants. Lane 1, donor; lane 2, transconjugant A; lane 3, transconjugant B; lanes 4, 5, and 6, recipients; lane 7, negative control; lane 8, positive control (MRSA ATCC 43300); lane 9, 100bp DNA Marker.

Figure 4. An ethidium bromide-stained gel demonstrating PCR amplification of ccrB2 gene in donors, recipients and transconjugants. Lane 1, 100 bp DNA Marker; lane 2, donor; lane 3, transconjugant A; lane 4, transconjugant B; lanes 5 to 7, recipients; lane 8, negative control.

genes produced PCR product of 873 and 1128 bp, respectively (Figures 3 and 4). Among the six primers used for screening ccrAB genes in the recipient strains, no amplification were observed among recipient strains.

DNA sequencing of mecA and ccrAB genes

The mecA gene showed 98% homology with S. aureus, mecA gene for PBP2 (penicillin binding protein 2) (Gene Bank accession number; X52593). The ccrA3 gene showed 100% homology with S. aureus subsp. aureus strain ATCC 33592 CcrA3 (Gene Bank accession number; DQ460218). The ccrB2 gene showed 100% homology with S. epidermidis strain ATCC 12228 CcrB (Gene Bank accession number; AE015929).

DISCUSSION

The resistance mechanism by which the mecA can be transferred has been studied extensively because of the importance of resistant strains in the clinical setting. This study was performed to observe possibility of in vitro transfer of mecA gene from local MRSA to MSSA clinical isolate that do not carry mecA gene, using cost effective and simple approach. Many studies support that horizontal transfer play an important role in the dissemination of the mecA gene in the S. aureus population and specially transfer mecA gene more frequently to MSSA (Weilders et al., 2002). Also, it was suggested that different staphylococcal species acquire SCCmec locally by horizontal gene transfer (Hanssen et al., 2004). The study by Khan et al. (2000) showed the usefulness of PCR and gene-specific probing along with the conven-
tional selective antibiotic screening methods to study the transfer of drug resistance. This resistance transfer was observed between selective isolates that were resistant to the same antibiotic but carry different genetic markers (Khan et al., 2000). The method presented in their study uses a simple preliminary unique approach to analyze the transconjugants.

We have used the same approach in this study to observe and study the transfer of resistant genes with unique focus to study transfer of mecA gene in vitro. The mixing of donor and recipient strains in liquid culture was chosen as it allows transfer of antibiotic resistance between the strains that are in close proximity in vitro. The results obtained showed the use of gentamicin and tetracycline as selective antibiotics was helpful in observing transfer of oxacillin resistance to transconjugants. The transconjugant A is resistant to all the antibiotics, therefore transconjugant A with the phenotype resistant to oxacillin, tetracycline and gentamicin having a new phenotype, resembles neither the recipient nor the donor. The direction of the transfer of oxacillin also could not be determined in the transconjugant A. This could be the donor strain that has received gentamicin resistance from recipient strain or this could be recipient strain that has received the oxacillin and tetracycline resistant donor strain. However, the transconjugant B with the phenotype of resistance to oxacillin, gentamicin and sensitive to tetracycline has the phenotype resembling the recipient, while it is resistant to oxacillin, as well as a new phenotype which is different from the donor but resistant to oxacillin.

Furthermore, studies have found novel SCCmec types or SCC elements without mecA, which could be a reservoir for antibiotic resistance islands in S. aureus. In this study, ccr recombinases genes was not detected in methicillin-sensitive strains (recipient strains) therefore SCCmec were absent in these recipient strains. For integration into and excision of SCC element from the chromosome, genes encoding cassette chromosome recombinases (ccr) are involved. Detection of ccrA3 and ccrB2 genes in donor, transconjugant A and B and their absence originally from the recipient strains showed that the mecA gene transfer have taken place along with ccr recombinases genes. In this study, we focused on using very simple and cost effective experiment to observe in vitro resistance transfer in particular mecA gene between clinical S. aureus isolates.

In conclusion, ccr recombinases genes detection and simple conventional selective antibiotic screening methods was useful in observing mecA gene in vitro transfer among clinical isolates. This observation can further support theory of horizontal gene transfer among same species of clinically important strains of S. aureus.

The observation in this study further highlights the crucial role of horizontal gene transfer in the evolution of bacteria and dissemination of variable genes, including antibiotic resistance and virulence genes.

REFERENCES


