Research Journal of Biological Sciences 4 (8): 937-942, 2009
ISSN: 1815-8846

Novel Molecular Analysis for Characterization of Staphylococcal Cassette Chromosome in a Methicillin-Resistant *Staphylococcus aureus* Isolated from Malaysian Hospital

[1]Nagi A. AL-Haj, [2]E. Amghalia, [3]Mariana N. Shamsudin, [4]Rasedee Abdullah,
[5]Rahmah Mohamed and [1]Zamberi Sekawi
[1]Department of Medical Microbiology, Faculty of Medicine and Health Sciences,
Sana'a University, Sana'a, Yemen
[2]Department of Microbiology and Parasitology, Faculty of Medicine and Health Sciences,
University of Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[3]Department of Marine Science and Aquaculture,
[4]Laboratory of Immunotherapeutic and Vaccine, Institute of Bioscience,
University of Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[5]Malaysia Genome Institute, University of Kebangsaan Malaysia,
Malaysian Technology Development Corporation,
Smart Technology Centre, 43600 Bangi, Malaysia

Abstract: Methicillin-resistant *Staphylococcus aureus* strains have appeared in countries worldwide and continue to be one of the most common hospital pathogens and it has become increasingly prevalent in community-acquired infections and provided strong evidence for the independent origins of health care-associated Methicillin-resistant *Staphylococcus aureus* and community-acquired. It has been shown that methicillin-susceptible *S. aureus* strains become MRSA strains by the acquisition of a staphylococcal cassette chromosome mec element carrying the mecA gene, which is responsible for methicillin resistance and has become essential for the characterization of *Staphylococcus aureus* clones in epidemiological studies. The objective of this study to identify the staphylococcal cassette chromosome mec types of methicillin-resistant *Staphylococcus aureus* isolated from different Malaysian Hospitals. PCR amplification and sequencing analysis were performed to determine the SCCmec type of MRSA. The present research successfully established molecular characteristics of local MRSA contribute as initial database of these isolates in order to fully understand the epidemiology, microbiology and pathophysiology of these infections.

Key words: Methicillin-Resistant *Staphylococcus aureus* (MRSA), Staphylococcal Cassette Chromosome (SCC), Community-Acquired MRSA (C-MRSA), mutation, Polymerase Chain Reaction (PCR), Malaysia

INTRODUCTION

Staphylococci are often found in the human nasal cavity (and on other mucous membranes) as well as on the skin. There are five species of staphylococci commonly associated with clinical infections: *Staphylococcus aureus*, *S. epidermidis*, *S. haemolyticus*, *S. hominis* and *S. saprophyticus*. Molecular typing techniques have been used with increasing frequency in studies of the epidemiology of Methicillin-Resistant *Staphylococcus aureus* (MRSA) and also for a better understanding of the evolutionary relationships among MRSA chromosomal cassette mec (SCCmec) type IV or V (Crisostomo *et al*., 2001; Enright *et al*., 2000, 2002; Goering, 1993; Oliveira *et al*., 2001). One of the conclusions emerging from these studies was that a complete characterization of MRSA lineages requires not only identification of the genetic background of the bacteria but also identification of the structural types of the large and heterologous mec genes, which

Corresponding Author: Mariana N. Shamsudin, Department of Microbiology and Parasitology, Faculty of Medicine and Health Sciences, University of Putra Malaysia, 43400, Serdang, Selangor, Malaysia

carries methicillin resistance (Hiramatsu *et al*., 2001; Oliveira *et al*., 2001, 2002). Previous studies by Ito *et al*. (1999, 2001) have elucidated the complete structure of three major mec elements, also referred to as (SCCmec). Type 1 (34 kb) was identified in the first MRSA strain isolated in 1961 in the United Kingdom (strain NCTC10442), type 2 (52 kb) was identified in an MRSA strain isolated in 1982 in Japan (strain N315) and type 3 (66 kb) was identified in an MRSA strain isolated in 1985 in New Zealand (strain 82/2082) (Ito *et al*., 1999, 2001).

More recently, a smaller fourth mec element, SCCmec type 4 (20-24 kb), was independently identified among representatives of the Pediatric clone Oliveira *et al*. (2001) and in two community-acquired MRSA strains Ma *et al*. (2002). Present study we described, the characterization of methicillin resistant *S. aureus* genes bases for epidemiological identification, which were all characterized by PCR amplification and DNA sequencing, based on the information described in references Ito *et al*. (1999, 2001). However, these methods are laborious and time-consuming.

This study focusing on the strategy, which was designed to detect the structural variations observed in the mec genes as in order to provide a useful tool for the rapid tentative identification of the structural type of the mec elements in MRSA isolates.

MATERIALS AND METHODS

Bacterial isolates: A total of thirty isolates of *S. aureus* were used in this study. Two reference strains of multiple drug resistant *S. aureus* obtained from University Hospital Petaling Jaya. All of them were obtained from different patients visiting Hospital Seremban, Hospital Miri, Sarawak and from the Laboratory Gribbel's Petaling Jaya.

Boiling DNA extraction: After overnight culture on Blood agar plates, one colony of each sample was re-suspended in 25 μL of sterile ultra pure water and the suspension was then placed in a 100°C heat block for 12 min. From this suspension, a 1 μL volume was directly used as a template for PCR amplification.

Detection of mecA, mecR1 and mecI genes: Based on the design primer (Table 1) PCR amplification reaction was performed in 25 μL volume mixtures containing 80 mM $MgCl_2$, PCR buffer, 3.75 mM DNTP mix (Fermentas), 10 picomole of single 10-base primer (OPERON Technologies), 100-300 ng of template and 1 unit of Taq polymerase (BioSyntech Technologies).

Amplifications were carried out using a thermal cycler (Biometra-Trio Thermoblock) programmed for 3 min at 96°C followed by 25 cycles, each consisting of 1 sec at 95°C, 30 sec at 59°C and 30 sec at 55°C and a final extension period of 7 min at 72°C for the detection of mecA and mecR1 genes and for the mecI gene 1 min at 95°C followed by 30 cycles, each consisting of 1 min at 95°C, 30 sec at 59°C and 30 sec at 55°C and a final extension period of 2 min at 72°C.

After amplification, 10 μL of the reaction mixture was loaded onto a 1.4% agarose gel and electrophoresed. The gel was then stained with ethidium bromide and photographed using an imager (Alpha Imager™ 2200, Alpha Innotech Corporation).

DNA sequencing of mecA, mecR1 and mecI genes: The methicillin resistant genes were sequenced in this study 5 MRSA isolates (isolate number A5, A10, A15, St1 and St4) and 5 MSSA isolates (isolate number 11, S2, N12, G4 and G8) for 3 different methicillin resistant genes mecA, mecR1 and mecI found in *S. aureus* was sequenced.

The purified amplified products were sequenced commercially and compared with the respective gene sequences in the Gene Bank (National Center for Biotechnology Information).

Table 1: Primers used for MRSA genes amplifications

Target	Primer sequences (5'-3')	Amplicon size (bp)
mecA forward	AAA ATC GAT GGT AAA GGT TGG C	
mecA reverse	AGT TCT GCA GTA CCG GAT TTG C	533
mecI forward	AATGGCGAAAAAGCACAACA	
mecI reverse	GACTTGATTGTTTCCTCTGTT	481
mecR1 penicillin binding domaine, forward	GTCTCCACGTTAATTCCATT	310
mecR1 penicillin binding domaine, reverse	GTCGTTCATTAAGATATGACG	
mecR1 transmembrane domaine, forward	CAGGGAATGAAAATTATTGGA	
mecR1 transmembrane domaine, reverse	CGCTCAGAAATTTGTTGTGC	318

RESULTS AND DISCUSSION

Detection of mecA, mecR1 and mecI gene by PCR: The PCR technique was applied to five strains of MRSA and five isolates of MSSA. The mecA, mecR1and mecI genes were found only in MRSA isolates of *S. aureus* tested. MecA, mecR1 and mecI gene successfully amplified and isolated from MRSA isolated however, these genes are not amplified in any of non MRSA isolates. The amplification of genes produced a single band of 533 bp of mecA gene, 310 bp of the mecR1 penicillin binding domaine gene, 318 bp of the mecR1 transmembrane domaine gene and 481 bp of mecI gene (Fig. 1-3).

MecA, mecR1 and mecI sequencing genes: The BLASTN query results of the genes amplified for the local MRSA isolates (Table 2 and 3) were compared with gene sequences of mecA, mecR1 and mecI obtained from GenBank under the accession number X52593.1 for mecA gene, accession number X63598.1 for mecR1 gene and accession number X63598.1 for mecI gene. Gene sequences analysed showed variable percent similarity, when compared to the GeneBank database. The amplification products for all the genes, respectively were consistent with the predicted sizes. Sequencing data confirmed that the actual sizes of the mecA amplification products matched the predicted sizes of 533 bp, respectively and 310 bp of the mecR1 penicillin binding domain gene, 318 bp of the mecR1 transmembrane domain gene and 481 bp of the mecI gene. The sequences of the amplified fragments for the mecA gene from 4 isolates showed 99% homology to the GeneBank, while another isolate was 100% homology. The mecR1 gene sequences gave 100% for all isolates. The sequences of the amplified fragments for the mecI gene gave 99% homology for 4 isolates and 98% for 1 isolate.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most significant human pathogens that causes both nosocomial and community-acquired infections worldwide (McDonald, 2006; Drews *et al*., 2006). This dangerous bacterium could also cause a wide range of infectious diseases from mild conditions, such as skin and soft tissue infections, to severe, life-threatening debilitation, such as Toxic Shock Syndrome (TSS) and bacterial endocarditis (Lowy, 1998). MRSA was first detected in the early 1960s, shortly after methicillin (a β-lactam antibiotic) came into clinical usage (Livermore, 2001; Lowy, 1998). However, treatment of these infections has become more difficult since *S. aureus* has become resistant not only to the regularly used penicillin-related (β-lactams) antibiotics, but also to other various structurally-unrelated antibiotics, such as tetracycline, rifampicin and chloramphenicol (Enright *et al*., 2002). Methicillin-resistance in *S. aureus* is

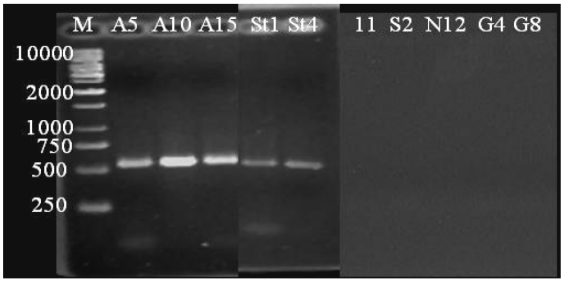

Fig. 1: The detection of mecA gene fragments by PCR. Lane M represents 1 kp DNA marker ladder. Lanes A5, A10, A15, St1, St4 represents MRSA isolates, lanes 11, S2, N12, G4 and G8 represents non-MRSA isolates. All the MRSA isolates have a single band at position 533 bp of the mecA gene

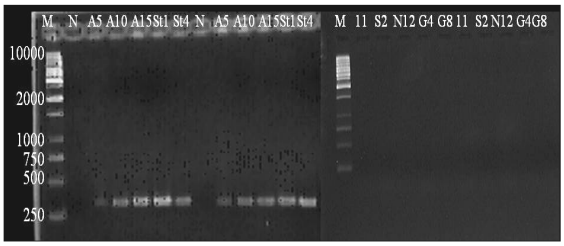

Fig. 2: The detection of mecR1 gene fragments by PCR. Lane M represents 1kp DNA marker ladder. Lanes A5, A10, A15, St1, St4 represents MRSA isolates, lanes 11, S2, N12, G4 and G8 represents non-MRSA isolates. All the MRSA isolates have a single band at position 310 bp of the mecR1 penicillin binding domaine gene, 318 bp of the mecR1 transmembrane domaine gene

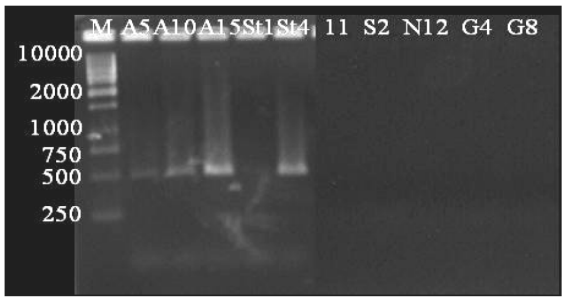

Fig. 3: The detection of mecI gene fragments by PCR. Lane M represents 1kp DNA marker ladder. Lanes A5, A10, A15, St1, St4 represents MRSA isolates, lanes 11, S2, N12, G4 and G8 represents non-MRSA isolates. All the MRSA isolates have a single band at position and 481 bp of mecI gene

Table 2: Mutations detected in mecA, mecR1 P, mecR1 T and mecI of MRSA

	mecA				mecI	
Isolate	Nucleotide position change	Codon change	mecR1 P	mecR1 T	Nucleotide position change	Codon change
A5	440 (C→A)	Thr→Asn	-	-	260 (T→A)	Tyr→Phe
A10	440 (C→A)	Thr→Asn	-	-	260 (T→A)	Tyr→Phe
-	-	-	-	-	445 (T→A)	Lys→Ile
-	-	-	-	-	447 (C→A)	Thr→pro
A15	-	-	-	-	260 (T→A)	Tyr→Phe
St1	440 (C→A)	Thr→Asn	-	-	260 (T→A)	Tyr→Phe
St4	440 (C→A)	Thr→Asn	-	-	260 (T→A)	Tyr→Phe

Table 3: Mutations detected in mecA, mecR1 P, mecR1 T and mecI of MRSA strains after treat with honey

Isolate	mecA	mecR1 P	mecR1 T	mecI
A5	Deletion of 5 bases, insertion of 2 bases, substitution 7 bases	Deletion of 1 base, substitution 3 bases	Deletion of 2 bases, insertion of 2 bases, substitution 4 bases	Deletion of 1 base
A10	Deletion of 7 bases, insertion of 1 base, substitution 5 bases	Deletion of 1 base, substitution 4 bases	Deletion of 1 base, insertion of 3 bases, substitution 3 bases	Deletion of 1 base, substitution 5 bases
A15	Deletion of 6 bases, substitution 8 bases	Deletion of 1 base, insertion of 1 bases, substitution 5 bases	Deletion of 1 base, insertion of 2 bases, substitution 3 bases	Deletion of 1 base, substitution 2 bases
St1	Deletion of 6 bases, insertion of 3 bases, substitution 8 bases	Deletion of 1 base, insertion of 2 bases, substitution 13 bases	Deletion of 1 base, insertion of 2 bases, substitution 4 bases	Deletion of 1 base, substitution 2 bases
St4	Deletion of 6 bases, insertion of 3 bases, substitution 8 bases	Deletion of 1 base, insertion of 2 bases, substitution 5 bases	Deletion of 1 base, insertion of 2 bases, substitution 2 bases	Deletion of 1 base, insertion of 2 bases, substitution 7 bases

mediated by the presence of Penicillin-Binding Protein 2a (PBP-2a), which is expressed by an exogenous gene, mecA. This gene is carried by a genetic element, designated as Staphylococcal Cassette Chromosome mec (SCCmec), which is inserted near the chromosomal origin of replication (Hiramatsu *et al*., 2001). Usually, the identification of MRSA using conventional gel-based PCR assay has become a handy molecular detection method as compared to the traditional phenotypic cell culture-microscopy biochemical-based method (Sakoulas *et al*., 2001). A valuable consequence of advances in molecular biology is the applicability of molecular approach for determining molecular relatedness of isolates for epidemiologic investigation through new technologies based on DNA, or molecular analysis. These DNA-based molecular methodologies include PCR-based typing methods of genomic DNA or relevant target genes such as virulent factors or antibiotic determinants. A rapid and reliable diagnosis of infection by MRSA is of major importance. Although, *S. aureus* is relatively easy to cultivate, conventional identification methods may yield false-positive or false-negative results (Frebourg *et al*., 1998; Wallet *et al*., 1996). Standard susceptibility tests are time-consuming. The correct identification of *S. aureus* and the detection of the methicillin resistant genes based on molecular methods have evolved as the method of choice for definitive identification. The target genes mecA, mecR1 and mecI in local MRSA isolates successfully amplified at 533, 310, 318 and 481 bp. The acquisition of mecA is considered to be the first genetic requisite for methicillin resistance of staphylococci (Kobayashi *et al*., 1998) and his presence of the mecI and mecR1 genes in MRSA strains has been confirmed previously. The mecA, mecR1 and mecI containing the 533, 310, 318 and 481 bp marker was found to be inadequate for the confirmation of the identification of MRSA from bacterial cultures. PCR assay that was performed from genomic DNA was optimized to be simple and performed in <1 1/2 h. The PCR assay developed is found to be specific for MRSA as it amplified 5 *S. aureus* isolates. This primer was studied by blast analysis of the genetic marker identified. This genetic marker was found to be conserved in *S. aureus* genome. The PCR assay, which was performed with DNA samples from 5 *S. aureus* isolates showed excellent result. PCR-DNA sequencing based analysis of genes mecA showed 99-100% homology to *S. aureus* genome and 533 bp fragment was found to be present in all of the MRSA isolates tested 100% homology to (X52593.1) but sequencing analysis result indicate a point mutation in isolates A5, A10, St1 but the strains still MRSA even if the new codon code another amino acid the function of the mecA gene not change may be because the amino acid is in nonvital portion of the gene. MecR1 P 318 bp showed 100% homology to (X63598.1) of *S. aureus* genome, which found to be present in all of the MRSA isolates tested. MecRI 481 bp is a signal transducing protein to mecA. MecI showed 98-99% homology to *S. aureus* genome also found to be present in all of the MRSA isolates tested. The MRSA strains examined in this study have the mecI gene. These isolates were, therefore, able to express methicillin-resistance because these strains found to have a substitution in mecI at nucleotide 260, it has previously been assumed that a mecI gene with the same sequence

as the functioning gene in pre-MRSA N315 and with the same upstream regulatory sequence is capable of producing the mecI peptide (Suzuki *et al*., 1993; Hiramatsu, 1995; Kobayashi *et al*., 1998). Expression of PBP-2a is controlled by mecI and mecR1, located upstream of mecA, which encode mecA repressor protein and signal transducer protein, respectively (Hiramatsu *et al*., 1997; Kuwahara-Arai *et al*., 1996). An MRSA carrying intact mecI and mecR1 together with mecA has been called pre-MRSA, which is represented by prototype *S. aureus* strain N315 (Hiramatsu, 1995). Since, intact mecI product strongly represses the expression of PBP-2a, the pre-MRSA is apparently methicillin susceptible (Hiramatsu, 1995; Kuwahara-Arai *et al*., 1996).

Hence, it is hypothesized that removal of the repressor function for mecA is a prerequisite for constitutive expression of methicillin resistance in *S. aureus* with mec DNA. Indeed, the deletion of mecI or point mutations in the mecI gene has been found in a number of methicillin-resistant staphylococcal isolates (Hiramatsu, 1995; Hurlimann-Dalel *et al*., 1992; Kobayashi *et al*., 1998; Suzuki *et al*., 1993). We previously studied the presence of mec regulator genes in a number of clinical isolates of MRSA. Most strains were found to possess mecI and mecR1 genes and the possibility of mutation in the mecI was suggested (Kobayashi *et al*., 1998). In the present study, of 5 MRSA strains one point mutation have been most frequently detected in the mecI gene (base substitutions T to A at position 260) (Hiramatsu, 1995; Suzuki *et al*., 1993). In all the MRSA strains, a mutation was detected in mecI gene sequences, except in one strain (A10), which had mutations in three points. A nucleotide substitution at position 260 generated an amino acid change and a new termination codon, respectively. Since, a methicillin resistance phenotype can be due to mutations in mecI the emergence of a methicillin-resistant mutant will be a function of an overall mutability value that results from the independent mutability values for this gene. Classical genetic analysis indicates that when mutations in either one or another gene can produce antibiotic resistance, the overall mutability will be the sum of the independent mutability values. Establishment of databases on molecular properties of local strains is a sound approach in an epidemiological-linked health infection management. Among molecular properties of pathogens that are useful in an epidemiology based management include sequence analysis of species specific target genes, universal 16sRNA, antibiotic resistant determinant genes, relevant infective site genes or virulent factor genes as well as genetic diversity and clonal types. Other important molecular trait useful in infection management is colonization versus infection gene markers as well as source tracing gene markers. These properties can contribute immensely to surveillance program to reduce and control infection from multiple drug resistant pathogens.

CONCLUSION

An important finding of the research is the optimization of molecular methods for simple amplification of various genes useful in epidemiological-linked infection management. These genes include those involved in methicillin-resistant *S. aureus*. By using *S. aureus* strains to be tested as templates, various oligonucleotides primers amplified the 533 bp region of mecA, 310 bp region of the mecR1 penicillin binding domaine gene, 318 bp region of the mecR1 transmembrane domaine gene and 481 bp region of the mecI gene.

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