Malaysian Journal of Microbiology, Vol 13(4) December 2017, pp. 308-317

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Genotypic and phenotypic characterization of methicillin resistance determinants and β-lactamase in *Staphylococcus* species

Abdul Rahim Abdul Rachman[1], Norhidayah Mat Azis[1], Pung Hui Ping[1], Zarizal Suhaili[1,2], Syafinaz Amin Nordin[3], Zulkefley Othman[1], Mohd Nasir Mohd Desa[1,4*]

[1]Department of Biomedical Science, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Malaysia.
[2]School of Animal Sciences, Faculty of Bioresources and Food Industry, Universiti Sultan Zainal Abidin, Besut Campus, 22200 Besut, Terengganu, Malaysia.
[3]Department of Medical Microbiology and Parasitology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Malaysia.
[4]Halal Products Research Institute, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia.
Email: mnasir@upm.edu.my

Received 21 March 2017; Received in revised form 20 April 2017; Accepted 24 April 2017

ABSTRACT

Aims: To characterize the genotypic distribution of mec complex, bla complex, methicillin-resistance level (cefoxitin-MIC) and β-lactamase activity in carriage methicillin-resistant *Staphylococcus* species for a potential correlation.
Methodology and results: Biochemical test, 30 µg cefoxitin diffusion disc test, cefoxitin E-test, mec and bla complexes distributions, Pbp2a and β-lactamase assays were conducted to characterize phenotypic and genotypic of MRSA and MRCoNS in our collection. Phylogenetic tree was constructed using MEGA6 software to trace the diversity of *blaZ* gene of MRSA and MRCoNS. Sixteen MRSA and nineteen MRCoNS were identified by biochemical tests followed by 30 µg cefoxitin antibiotic disc susceptibility test and *mecA* gene screening. Twenty nine isolates carry complete *mecA* genes (2.1 kb), incomplete mec regulator (negative or truncated) and positive Pbp2a assay for both MRSA and MRCoNS. Only MRCoNS SC177 isolate with cefoxitin MIC of 32 µg/mL carries complete mec complex. Thirty-one of thirty-five isolates carry complete bla complex (*blaZ*, *blaRI*, *blaI*) with 10 MRSA produce strong β-lactamase and cefoxitin MIC of ≥12 µg/mL. Only 4 MRCoNS with cefoxitin MIC of ≤8 µg/mL produce strong β-lactamase. The diversity of *blaZ* gene was demonstrated by phylogenetic analysis and unusual amino acid mutation at position 145 for MRSA SA60 isolate may compromise its β-lactamase activity with low cefoxitin MIC level (2 µg/mL).
Conclusions, significance and impact of the study: Isolates that carry complete complete *mecA* gene were largely consistent with the expression of Pbp2a. Nevertheless, there is no clear correlation of mec regulator genes in relation to cefoxitin-MIC in both methicillin resistant (MR) Isolates that carry *Staphylococcus* species. On the other hand, various expression level of β-lactamase may correlate with cefoxitin-MIC level in MRSA as compared to MRCoNS.

Keywords: cefoxitin-MIC, mec complex, bla complex, β-lactamase, MR *Staphylococcus*

INTRODUCTION

Methicillin-resistant *Staphylococcus* species are resistant toward β-lactam antibiotics and cause a variety of problems such as skin and bloodstream infections, and pneumonia worldwide (Ahlstrand *et al*., 2011). The complexity of the Staphylococcal Chromosomal Cassette (SCC) mec gene structure that carried with it methicillin-resistance determinant gene named as *mecA* and its regulatory gene regiment (*mecRI* and *mecI*) in *Staphylococcus* species remains to be elucidated. *MecA* gene encodes for Pbp2a and serves as a benchmark to diagnose the presence of methicillin-resistant *Staphylococcus* since it is highly conserved among *Staphylococcus* species (Al-Abbas, 2012). Regulation of *mecA* is controlled by mec regulator genes consisting *mecRI* (transmembrane inducer) and *mecI* (mec repressor). Both are located immediately upstream of the *mecA* promoter. *mecI* encodes a repressor for *mecA* gene. In the absence of β-lactam antibiotics, it binds *mecA* promoter and blocks the transcription of *mecA* gene. *MecRI* will induce the detachment of *MecI* by proteolytic cleavage in the presence of β-lactam antibiotics (Arêde *et al*., 2012). However, the regulatory mec structure seems unstable with the varying length of *mecI* and *mecRI*

***Corresponding author**

nucleotide sequences due to truncation and mutation events (Shore and Coleman, 2013).

Previous reports suggested that the expression of methicillin resistance may correlate with over production of β-lactamase, which would partially hydrolyze methicillin and related penicillins (McDougal and Thornsberry 1986; Massiddaa *et al*., 2006; Olayinka *et al*., 2009). The enzyme is encoded by *blaZ*, which is part of bla complex consisting of *blaI* and *blaRI*. Bla complex (*blaI-blaRI-blaZ*) has a similar gene orientation as of mec complex (*mecI-mecRI-mecA*) (Arêde *et al*., 2013). Interestingly, *mecI* and *blaI* have a similar structure and function although they only share 60% nucleotide sequence homology (García-Castellanos, 2003; Safo *et al*., 2005). Both repressors act interchangeably by binding to the promoter region of either *blaZ* or *mecA* operon. On the other hand, the trans-membrane inducers (*blaRI* and *mecRI*) of the respective operons have a different action; *blaRI* only reacts with *blaI* and *mecRI* only cleaves *mecI* (McKinney *et al*., 2001). Although, the respective regulator systems are responsible for their own *blaZ* or *mecA* transcription process, a previous study demonstrated that presence of bla locus is associated with a dramatical increase of *mecA* transcription and the mechanism remains unknown (Katayama *et al*., 2003).

Some studies also claimed that overexpression of Pbp2a in methicillin-resistant strains doesn't guarantee that the strains will have a high resistance against methicillin (De Lencastre *et al*., 1994; Rohrer *et al*., 2003; Plata *et al*., 2009). This event usually occurs in methicillin-resistant *Staphylococcus* species with cefoxitin-MIC level < 8 µg/mL, which is recognized as heterogeneous methicillin-resistant *Staphylococcus* species (CLSI, 2014). This group of methicillin-resistant (MR) *Staphylococcus* strains was frequently reported to produce Pbp2a constitutively, and also had an overproduction of β-lactamase. (Boyce and Medeiros, 1987; Malachowa and DeLeo, 2010; Hiramatsu *et al*., 2013). The role of beta-lactamase in relation to methicillin resistance is not well understood. Nevertheless, it was claimed that *mecRI* regulation system is much slower compared with the *blaRI* regulation system, taking hours instead of minutes. Therefore, it is possible that β-lactamase system may immediately interfere with the mec system instead of the mec system itself (Hiramatsu *et al*., 2013). With such ambiguities, this study was preliminarily undertaken to characterize mec and bla complexes genes distributions, Pbp2a and β-lactamase activity towards methicillin-resistance level (cefoxitin-MIC), and mutation in *blaZ* gene in relation to β-lactamase activity in a collection of MR *Staphylococcus* species for any potential explanation on the genotypic and phenotypic associations.

MATERIALS AND METHODS

Bacterial isolate

Sixteen MRSA and nineteen MRCoNS were available in our collection to be further analyzed in this study. The isolates were previously identified from nasal swab of healthy students at the Faculty of Medicine and Health Sciences, UPM in years 2013 and 2014 (Azis *et al*., 2014). Biochemical test such as Gram stain, coagulase and catalase test (H_2O_2) and mannitol salt agar (MSA) (Merck, Germany) were performed to differentiate *S.aureus* and CoNS. Antibiotic susceptibility pattern was determined by disc diffusion method using 30 µg cefoxitin diffusion disc based on a guideline from Clinical and Laboratory Standards Institute (CLSI) (2014) (CLSI, 2014). The presence of *mecA* gene with size 530 bp was screened in all isolates according to Petinaki *et al*. (2001).

Cefoxitin E-test

All *mecA* positive isolates were subjected for minimum inhibitory concentration (MIC) by standard E-test method (0.016-256 ug/mL) (Oxoid, UK). The concentration of 8 ug/mL was made as a breakpoint for resistance category as recommended by Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2014).

Mec operon screening

For detection of full and partial presence of *mecA* gene sequence, all *mecA* positive isolates were analyzed by PCR (Biometra Thermocycler, Germany) using different primer sets targeting *mecA* gene with expected sizes of 1.0 kb and 2.1 kb according to Petinaki *et al*., 2001 and Shore *et al*. (2011). Each PCR reaction was carried out in a 25 µL mixture composed of 12.5 µL of Green GoTaq® PCR mastermix (2X Green GoTaq® Reaction Buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP and 3 mM $MgCl_2$) (Promega, USA), 7.5 µL nuclease free water (Promega, USA), 2.5 µL of DNA template (25-100 ng) and 1.25 µL of 0.025 µM of each specific primer pairs (Petinaki *et al*., 2001; Shore *et al*., 2011). *MecI* and *mecRI* genes were also screened using PCR in all *mecA* positive isolates using different primer sets targeting different sites described by Petinaki *et al*. (2001) to trace alteration or deletion part of mec regulator genes. Primer for *mecI* amplifies 1196 bp targeting the whole *mecI* including penicillin-binding domain (PB), while primers for the *mecRI* gene were of two separate sets targeting two part of *mecRI*, the PB and membrane spanning part (MS) for 747 bp and 310 bp, respectively. PB is located at upstream of *mecRI* whereas MS is located at the mid-internal region of the *mecRI* sequence. Each PCR reaction was carried out in a 25 µL mixture composed of 12.5 µL of Green GoTaq® PCR mastermix (2X Green GoTaq® Reaction Buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP and 3 mM $MgCl_2$) (Promega, USA), 7.5 µL nuclease free water (Promega, USA), 2.5 µL of DNA template (25-100 ng) and 1.25 µL of 0.025 µM of each specific primer pairs (Petinaki *et al*., 2001).

Bla operon screening and protein analysis

All *mecA* positive isolates were subjected to *blaZ* amplification by targeting a gene sequence area of the

functional part according to Kaase *et al*. (2008). Expected PCR products with size ~0.5 kb were purified, sequenced and submitted to GenBank (http://www.ncbi.nlm.nih.gov/genbank/). *BlaZ* nucleotide sequences were translated into an amino acid sequence using Expasy Translate tool (http://web.expasy.org/translate). Multiple sequence alignment using Biology workbench server (http://workbench.sdsc.edu) was conducted for tracing amino acid mutation on *blaZ* sequences of all *blaZ*-positive isolates. The phylogenetic tree was constructed using the neighbor-joining method with a bootstrap value of 1000 replication using MEGA 6 based on the amino acid sequence of all *blaZ*-positive isolates. *BlaZ*-positive isolates were classified based on β-lactamase signature types and classes according to *blaZ* protein sequence (Olsen *et al*., 2006; Bush and Jacoby, 2010). All *blaZ* sequence were submitted to GenBank and assigned with accession number KX985816 until KX985846.Bla regulator genes (*blaRI* and *blaI)* were also screened using primers sets targeting 0.9 kb *blaI* gene and 1.1 kb *blaRI* gene sequence as described by Chen *et al*. (2015). Each PCR reaction was carried out in a 25 µL mixture composed of 12.5 µL of Green GoTaq® PCR mastermix (2X Green GoTaq® Reaction Buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP and 3 mM $MgCl_2$) (Promega, USA), 7.5 µL nuclease free water (Promega, USA), 2.5 µL of DNA template (25-100 ng) and 1.25 µL of 0.025 µM of each specific primer pairs (Chen *et al*., 2015).

PBP2a and β-lactamase assays

All *mecA* positive isolates were tested for the presence of Pbp2a using penicillin-binding protein (PBP2') latex agglutination test (Oxoid,UK) and β-lactamase enzyme using nitrocefin (Oxoid,UK) according to manufacturers instruction. Pbp2a assay consisted of 2 major steps which are culture extraction and latex agglutination assay. For protein extraction, several loops of overnight colonies of each isolate (~1.5 $\times 10^9$ cells) were suspended into 1.5 mL microcentrifuge containing Extraction Reagent 1 solution. The suspension culture was placed in a water bath (Memmert) at 100 °C for 3 min followed by cooling at room temperature (~27 °C). One drop of Extraction Reagent 2 solution was added and the solution was centrifuged at 1500 x *g* for 5 min and the supernatant was used for protein assay. For latex agglutination assay, one circle was labeled on the test card for testing with Test Latex (monoclonal antibody against PBP2') and another for testing with Control Latex (monoclonal antibody with negative binding towards *S. aureus* proteins). One drop of each Test Latex and Control Latex were added to each labeled circle. After that, 50 µL of extracted supernatant from each isolate were placed on test circle and control circle and mix thoroughly. Finally, the mixture for both circles was incubated for 3 min at room temperature (~27 °C). Any agglutination on latex circle was observed and recorded for each isolate as the presence of Pbp2a protein. For β-lactamase enzyme assay, one drop of 0.5 mM nitrocefin solution (1.0 mg of lyophilized nitrocefin powder + 1.9 mL of rehydrating fluid) was added to the surface of an overnight culture of each isolate. The β-lactamase production of each isolate was determined based on colour changes from yellow to red.

RESULTS

Pre-screening

All 35 isolates were amplified for 0.5 kb *mecA* gene to be considered as MRSA or MRCoNS in this study (Table 1) with 98-99% sequence homology upon BLAST analysis databases (data not shown).

Mec complex and PBP2a assay

Different part of mec complex (*mecA*, *mecRI*, *mecI*) was amplified to ensure the presence of complete mec complex in methicillin-resistant *Staphylococcus* species. For MRSA (n=16), 88% (n=14) isolates were positive for 1.0 kb while, only 75% (n=12) carried full length of *mecA* gene (2.1 kb). For MRCoNS (n=19), 95% (n=18) isolates were positive for both *mecA* gene fragments (1.0 kb and 2.1 kb) except isolate MRCoNS SC164 (positive in 1.0 kb; negative in 2.1 kb) (Table 1). For *mecRI*, two different locations were amplified named as PB part and MS part. 94% (n=15) MRSA were positive for PB part (but present in truncated form) and 31% (n=5) were positive for MS part. While MRCoNS (n=19) were 21% (n=4) positive for PB part (complete+truncated) and 73% (n=14) positive for MS part (Table 1). For *mecI*, 18% of MRSA (n=3) and 21% of MRCoNS (n=4) had a positive PCR amplification. Only one MRCoNS SC177 with MIC 32 µg/mL had a complete mec complex (2.1 kb *mecA*, *mecRI*, *mecI*). Isolates that carried full-length *mecA* gene (2.1 kb) gave a positive reaction in Pbp2a assay (MRSA=11; MRCoNS=18) except MRSA SA13 isolate with MIC 24 µg/mL; positive for 2.1 kbp *mecA* gene but negative in Pbp2a assay (Table 1).

Bla complex and nitrocefin assay

Among the 35 methicillin-resistant *Staphylococcus*, thirty-one isolates consisting of sixteen MRSA and fifteen MRCoNS had a positive *blaZ* and *blaRI* genes amplification with only four of MRCoNS (SC67, SC126, SC144 and SC70) had a negative detection for both genes. For *blaI* gene, 93% of MRSA (n=15) and 63% of MRCoNS (n=12) were positive for *blaI* gene. Isolates that were detected for the *blaZ* gene were also detected for the *blaRI* gene but not *blaI* gene in both MRSA and MRCoNS species. Moreover, more MRSA (n=15) carry complete bla complex genes compared to MRCoNS (n=12), respectively. In nitrocefin assay, red, orange and yellow colours indicate a strong, weak and no β-lactamases production, respectively. 69% of MRSA (n=11) and 27% of MRCoNS (n=4) had a red colour, whereas 25% of MRSA (n=4) and 60% of MRCoNS (n=9) showed orange colour in nitrocefin assay. Only 6% of MRSA (n=1) and 33% of MRCoNS (n=5) showed yellow in

Table 1: Genotypic distribution of mec and bla complexes and Pbp2a and nitrocefin assays in relation to methicillin-resistance level (cefoxitin-MIC) of MR *Staphylococcus* species.

Isolate	FOX-MIC test (0.016-256 ug/mL)	*mecA*			*mecRI*		*mecI*	pbp2a assay	*blaZ* (AN)	*blaRI*	*blaI*	Nitrocefin assay
		0.5 kb*	1.0 kb	2.1 kb	MS part	PB part						
SA23	12	+	+	+	-	Δ	-	+	+(KX985838)	+	+	Red
SA13	24	+	+	+	+	Δ	-	-	+(KX985819)	+	+	Red
SA6	48	+	+	+	-	Δ	+	+	+(KX985829)	+	+	Red
SA3	12	+	+	+	-	Δ	-	+	+(KX985836)	+	+	Red
SA4	16	+	+	-	+	Δ	-	-	+(KX985835)	+	+	Red
SA55	12	+	+	+	-	Δ	-	+	+(KX985820)	+	+	Red
SA5	24	+	+	+	-	-	-	+	+(KX985837)	+	+	Red
SA2	16	+	+	+	+	Δ	-	+	+(KX985834)	+	+	Red
SA34	24	+	+	+	+	Δ	-	+	+(KX985818)	+	+	Red
SA65	32	+	+	-	-	Δ	-	-	+(KX985844)	+	+	Orange
SA60	2	+	-	-	-	Δ	+	-	+(KX985824)	+	-	Yellow
SA18	24	+	+	+	-	Δ	-	+	+(KX985831)	+	+	Orange
SA45	3	+	-	-	+	Δ	+	-	+(KX985823)	+	+	Orange
SA42	6	+	+	+	-	Δ	-	+	+(KX985817)	+	+	Red
SA7	6	+	+	+	-	Δ	-	+	+(KX985821)	+	+	Orange
SA32	12	+	+	+	-	Δ	-	+	+(KX985822)	+	+	Red
SC67	4	+	+	+	+	-	-	+	-	-	-	Yellow
SC6	12	+	+	+	+	-	-	+	+(KX985839)	+	+	Orange
SC140	2	+	+	+	+	Δ	-	+	+(KX985846)	-	-	Orange
SC8	8	+	+	+	+	-	-	+	+(KX985826)	+	+	Orange
SC129	12	+	+	+	-	-	-	+	+(KX985842)	+	+	Orange
SC165	16	+	+	+	+	-	-	+	+(KX985828)	+	+	Orange
SC111	32	+	+	+	+	-	-	+	+(KX985841)	+	+	Orange
SC37	12	+	+	+	+	-	-	+	+(KX985840)	+	+	Orange
SC57	12	+	+	+	+	-	-	+	+(KX985827)	+	+	Orange
SC156	8	+	+	+	-	-	-	+	+(KX985843)	+	+	Red
SC126	24	+	+	+	+	-	-	+	-	-	-	Yellow
SC144	16	+	+	+	+	-	-	+	-	-	-	Yellow
SC175	4	+	+	+	-	-	-	+	+(KX985825)	+	+	Red
SC119	6	+	+	+	-	-	-	+	+(KX985832)	+	+	Red
SC91	1.5	+	+	+	+	+	-	+	+(KX985830)	+	-	Red
SC164	8	+	+	-	+	+	-	-	+(KX985845)	+	+	Orange
SC89	24	+	+	+	+	-	-	+	+(KX985833)	+	+	Yellow
SC177	32	+	+	+	+	+	+	+	+(KX985816)	+	-	Yellow
SC70	4	+	+	+	-	-	+	+	-	-	-	Yellow

FOX: cefoxitin; SA: MRSA; SC: MRCoNS;*: pre-screening result; +: amplified; -: not amplified; Δ: truncated: AN; Genbank accession number of the partial nucleotide sequence of a *blaZ* gene: Red; strong β-lactamase production: Orange; weak β-lactamase production: Yellow; no β-lactamase production.

nitrocefin assay (negative β-lactamase) (Table 1). Overall, all MRSA that produced strong β-lactamase (red colour) have MIC ≥12 µg/mL, while for MRCoNS, only isolates with MIC ≤ 8 µg/mL produce strong β-lactamase (red colour).

Protein signature type of *blaZ*

A total of 31 from 35 isolates had a positive *blaZ* gene amplification. Only four MRCoNS (SC67, SC126, SC144, SC70) showed a negative bla complex (*blaZ*, *blaRI,blaI*) and nitrocefin assay, respectively. The phylogenetic tree produced four major groups (I,II,III and IV) consisting of five protein signature types (type 1,2,3,5 and 6) with three β-lactamase family classes based on deduced amino acid sequences of *blaZ* from the thirty-one isolates (Figure 1).

In the phylogenetic analysis, group I composed of protein signature type 2 and 5 from β-lactamase class D family. Fifteen isolates (MRCoNS=11; MRSA=4) contained protein signature type 5 and became the most prevalent signature type in this study while two isolates (MRSA=2) contained protein signature 2. A majority of isolates (n=13) in group I have cefoxitin resistance level of ≥8 µg/mL except two MRCoNS [SC175 (4 µg/mL), SC119 (6 µg/mL)] for protein signature type 5 and two MRSA [SA7 (6 µg/mL), SA45 (3 µg/mL)] for protein signature 2. Two MRSA (SA7, SA45) for protein signature type 2 had an identical amino acid sequence with *S.aureus* M25254. For protein signature type 5, majority isolates (n=12) had two point mutations at amino acid residue 119 from a threonine to alanine and residue 145 from glycine to glutamic acid (Table 2); most isolates (n=11) produced β-lactamase except MRCoNS SC89 although it carried *blaZ* gene. Other three MRCoNS isolates (SC175=MIC 4, SC119= MIC 6 and SC129= MIC 12) carrying protein signature type 5 had an identical amino acid sequence with *E. faecalis* U43087 and showed a positive β-lactamase assay (Figure 1).

Group II contained 10 isolates (MRCoNS=2; MRSA=8) of protein signature type I from β-lactamase class A family. Eight isolates had cefoxitin resistance level of ≥8 µg/mL except for MRSA SA42 (6 µg/mL) and MRCoNS SC140 (2 µg/mL) with all of the isolates showed a positive reaction in the β-lactamase assay. Group III composed of one MRSA (SA4=MIC 16 µg/mL) of protein signature type 3 from β-lactamase class C family with a positive β-lactamase assay. Group IV contained three isolates (MRCoNS=2; MRSA=1) with protein signature type 6 from β-lactamase B family. Only MRCoNS SC91 (MIC 1.5 µg/mL) showed a positive reaction in the β-lactamase assay (Figure 1). Besides, MRSA SA60 isolate had a single amino acid mutation at residue 145 as compared to that in *S.aureus* AF086644 (Table 2).

Table 2: Protein signature types of *blaZ*.

Signature type[a]	Isolates[b]	*BlaZ* variable protein[c]											
		119	131	139	141	142	145	193	196	203	207	217	220
1	X52734 (*S. aureus*)[c], SC156, SA23, SA5, SA3, SA2, SA32, SA34, C13, SA42,SC140	T	K	V	Q	R	E	E	K	L	S	P	Y
2	M25254 (*S. aureus*), SA45, SA7	T	K	V	Q	R	E	E	K	L	S	S	C
3	AP004832 (*S. aureus*), SA4	T	K	V	Q	R	E	K	N	F	N	P	Y
5	U43087 (*E. faecalis*), SC129, SC119, SC175, SC8, SA55, SC57, SC165, SA6, SA18, SC89, SC6, SC37, SC111, SA65, SC164,	T A	K	V	Q	R	G E	E	K	L	S	S	C
6	AF086644 (*S. aureus*), SC91, SC177, SA60	K	N	I	K	R	K N	K	N	L	N	P	Y

[a] Signature type based on Olsen *et al*., 2006, Malik *et al*., 2007 and Chen *et al*., 2015.
[b] Strain in bold represents strain from GenBank.
[c] Variable position according to deduced protein sequence of X52734.

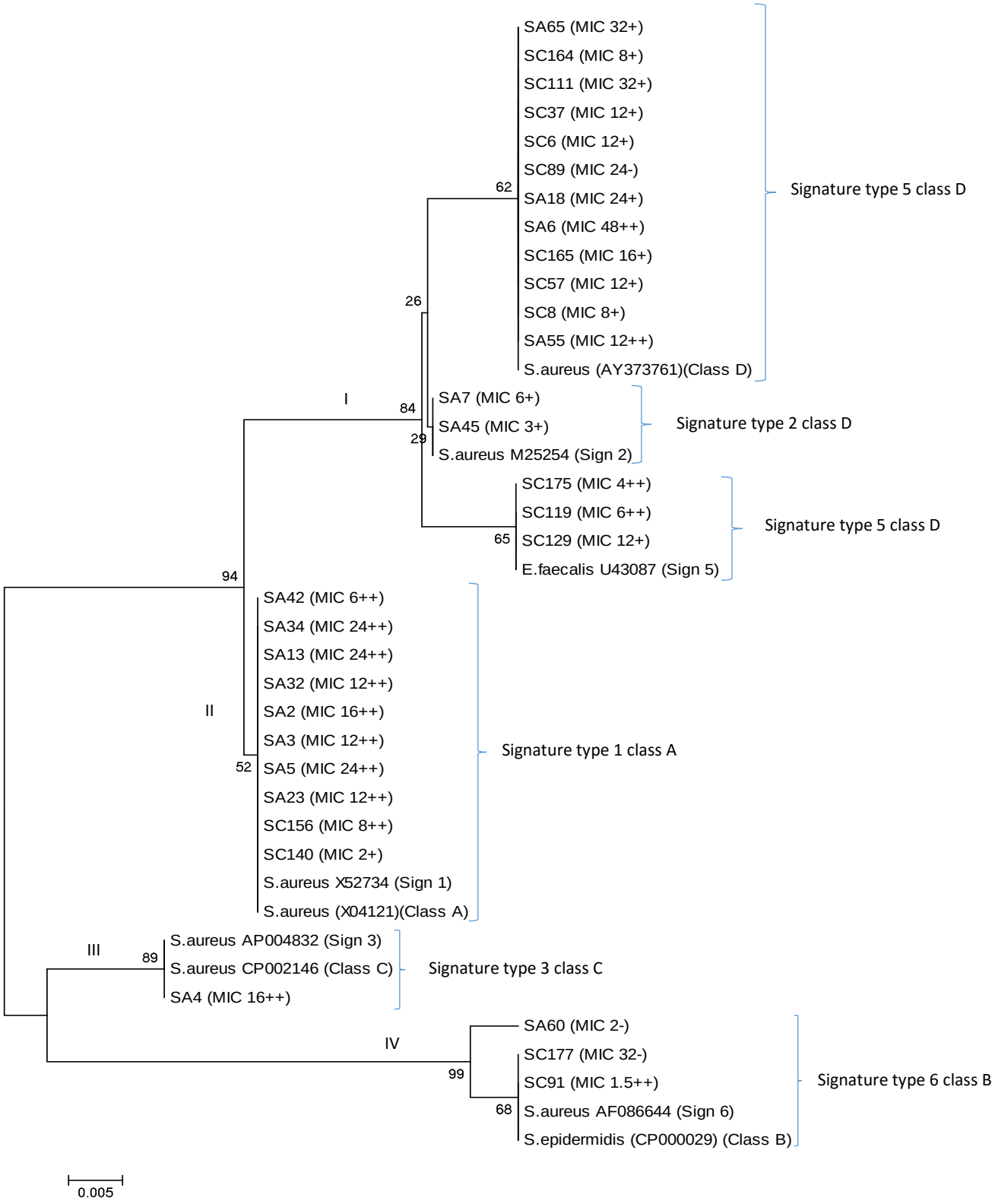

Figure 1: Phylogenetic analysis of *blaZ* protein sequences using neighbor-joining method. Bootstrap value constructed using 1000 replication. SA, MRSA; SC, MRCoNS; MIC, Cefoxitin; ++: strong β-lactamase; +: weak β-lactamase; -: no β-lactamase; AY373761, M25254, U43087, X52734, X04121, AP004832, CP002146, AF086644, CP000029: Reference strains retrieved from NCBI.

DISCUSSION

All MR *Staphylococcus* species that carried full length *mecA* gene (2.1 kb) in this study showed a positive reaction in the Pbp2a assay except isolate MRSA SA13. Isolate MRSA SA13 carried a full-length *mecA* gene but had a negative Pbp2a assay. This could be due to potential mutation or alteration in *mecA* nucleotide sequence in the isolate that may lead to failure in Pbp2a production (Lowy, 2003). Regardless of the species, most MR *Staphylococcus* in this study carried partial *mecRI* with the absence of *mecI* gene. This situation may be caused by disruption of the mec regulator sequences particularly by the activities of insertion sequences such as *IS431* and *IS1272* (Malachowa and DeLeo, 2010). The regulator sequences in *Staphylococcus* species have long been recognized as part of mobile genetic elements whereby genetic exchange, deletion and insertion are common to affect the functionality of the genes (Ito *et al*., 1999; Jansen *et al*., 2007; Malachowa and DeLeo, 2010). Overall, no obvious correlation was observed between mec regulator gene distributions toward methicillin-resistant level in *Staphylococcus* species. Nevertheless, Oliveira *et al*. (2011) proposed that full resistance toward β-lactam antibiotics in MR *Staphylococcus* strain usually does not require functional mec regulator (*mecRI-mecI*) system (Oliveira *et al*., 2011).

MRSA SA6 isolate had MIC level of 48 µg/mL which is the highest MIC level in this study, although the isolate carried mec repressor gene (*mecI*). Presumptively, *mecI* may not affect methicillin resistance level in *Staphylococcus* species as a previous observation showed that overexpression of *mecI* had no effect on the expression of methicillin resistance in many *S. aureus* strains (Oliveira and de Lencastre, 2011). This suggests the potential involvement of other regulatory factors such as *mecR2* (same function as *mecRI*), a previously unrecognized gene located downstream of *mecI* by interfering with the binding of *mecI* into *mecA* promoter and facilitating proteolysis of *mecI* (Arêde *et al*., 2012). This study did not attempt to detect the presence of *mecR2* in SA6 isolate. Nevertheless, since MRSA SA6 isolate carried non-functional inducer gene (partial *mecRI*), the involvement of *mecR2* could be postulated. Furthermore, *blaI* was also detected in MRSA SA6 isolate and *blaI* can interfere with the role of *mecI* as repressor toward *mecA* by binding with *mecI* and forming mecI:blaI heterodimers. This may block *mecI* from binding with *mecA* gene promoter (Arêde *et al*., 2013).

Only MRCoNS SC177 isolate with cefoxitin MIC 32 µg/mL carried complete mec complex and was positive in Pbp2a and β-lactamase assay. None of MRSA isolates carried complete mec structure in this study. Although this study has no evidence to claim MRCoNS as mec complex reservoir but the presence of the one isolate SC177 among MRCoNS could be consistent with the earlier claim that MRCoNS may serve as a carrier for a complete sequence of mec complex (Shore and Coleman, 2013). Many findings suggested that MRCoNS isolates are the reservoirs for mec complex and have been transferring the genetic determinants into *S. aureus* (Ito *et al*., 2009; Shore *et al*., 2011; Tulinski *et al*., 2012).

Overall, a majority of MRSAs carried a complete bla operon (*blaZ*, *blaRI*, *blaI*) with a strong β-lactamase production and cefoxitin-MIC level of ≥ 12 µg/mL although two MRSA isolates (SA13, SA4) were negative in the Pbp2a production (Table 1). Meanwhile, a few MRCoNS which have high cefoxitin-MIC level in this study such as SC177 (32 µg/mL), SC126 (24 µg/mL) and SC144 (16 µg/mL) did not carry bla operon or only contained partial bla operon and showed no β-lactamase activity (Table 1). Overproduction of β-lactamase was claimed as one of the factors of methicillin-resistance determinant especially in heterogeneous MRSA since MRSA with low level cefoxitin-MIC (2 µg/mL-4 µg/mL) but with overproduction of β-lactamase had been observed. On the other hand, this situation was not common in MRCoNS (Petinaki *et al*., 2002). This observation agrees with previous reports that claimed β-lactamase production in association with MR to be more frequent in *S. aureus* than in CoNS (Saini *et al*., 2012; Robles *et al*., 2014).

As for beta-lactamase, its production had been compromised in seven MR isolates and three of them (SA60, SC89, SC177) carried the *blaZ* gene. There is a correlation with the mutation observed on the amino acid sequence of the two (SA60, SC89) from the three isolates. First, *blaZ* amino acid of SA60 isolate has an unusual mutation at position 145 involving lysine (K) to asparagine (N) and separated from others isolates in protein signature type 6 (Figure 1). This mutation may give a major impact on β-lactamase production considering mutation on that position did not occur in protein signature type 6 in *Staphylococcus* species isolated from human that was previously documented by Olsen (Olsen *et al*., 2006). MRSA SA60 isolate also had the lowest cefoxitin-MIC level (2 µg/mL) among MR isolates in this study and maybe related to negative β-lactamase production or the incomplete *mecA* in MRSA SA60 isolate. For the latter, although MRSA SA60 isolate showed a negative Pbp2a expression (Table 1) which could be the main factor for its lower MIC level, a few studies claimed that a diverse MIC level was not related to Pbp2a expression alone. Interestingly, a same level of Pbp2a expression was found in isolates with low cefoxitin-MIC level (1.5 µg/mL) as well as that with high cefoxitin-MIC level (1.5 mg/mL) (Berger-Bachi *et al*., 1992; De Lencastre and Tomasz, 1994; Peacock and Paterson, 2015).

On the other hand, MRCoNS SC89 have an amino acid modification at position 119 involving threonine (T) to alanine (A) (Table 2). Mutation at position 119 may not play an important role in the β-lactamase production and may not affect cefoxitin MIC level (24 µg/mL) since other isolates with the same mutation in signature type 5 positive had a positive β-lactamase production with cefoxitin-MIC level ≥ 8 µg/mL (Figure 1). Besides, mutation was not observed in *blaZ* amino acid of MRCoNS SC177 isolate suggesting that the *blaZ* gene detection cannot be used as an indicator for β-lactamase production since other studies also observed the same situation; no β-lactamase production but presence of *blaZ*

gene (Pereira *et al*., 2014; Papanicolas *et al*., 2014). Another study also highlighted the importance of mutation of bla regulator (*blaRI*, *blaI*) rather than *blaZ* as the primary reason for the different level of β-lactamase production (Milheirico *et al*., 2011).

Five protein signature types (type 1,2,3,5 and 6) and four β-lactamase classes (A,B,C,D) were demonstrated in phylogenetic tree indicating the diverse protein sequences of *blaZ* in this study. Surprisingly, class A and class D β-lactamase genes were detected in 25 isolates (15 isolates= class D β-lactamase, 10 isolates= class A β-lactamase) which was previously documented as the largest group of β-lactamases (Bush and Jacoby, 2010). Certain MRSA and MRCoNS shared same protein signature (type 2 and 5) (Figure 1) suggesting possible genetic transfer of *blaZ* gene that could have taken place within *Staphylococcus* population although previous finding reported *blaZ* gene exchange and horizontal spread of *blaZ* within *Staphylococcus* species is a rare event (Olsen *et al*., 2006).

CONCLUSION

As a whole, the comparative analysis in this study indicates a potential correlation between β-lactamase production toward methicillin-resistance particularly in MRSA since MRSA with cefoxitin-MIC ≥ 12 µg/mL have a strong β-lactamase production. Furthermore, a majority of MRSA with cefoxitin-MIC ≥12 µg/mL with a strong β-lactamase production carry a complete bla operon. This demonstrates the importance of bla operon and β-lactamase production in rendering a higher methicillin-resistance level among MRSA as compared to MRCoNS. However, these correlations are only based on comparative observation while the exact mechanism on how the production of β-lactamase affecting the methicillin-resistance level is still ambiguous. Perhaps many other factors are involved and remain to be investigated. In the phylogenetic analysis, four groups represent five protein signature types and four classes of β-lactamase indicating a level of diversity of *blaZ* protein sequence although within a small collection of isolates (n=35) in this study. However, no obvious pattern was observed between β-lactamase classes and cefoxitin-MIC distribution among both MR *Staphylococcus* species since a majority of MR *Staphylococcus* was clustered into a group that homogenously carried β-lactamase class D but varied from low to high cefoxitin-MIC level. This study only looks at the distribution in a small sample size of both species which is still subjected to ambiguity and required further validation involving more MR isolates with a better experimental model.

ACKNOWLEDGEMENT

Funding: This work was supported by the Research University Grant Scheme, University Putra Malaysia [grant numbers: 9455000]; the Fundamental Research Grant Scheme, Ministry of Higher Education, Malaysia [grant number: 5524924].

REFERENCES

Ahlstrand, K., Svensson, K., Persson, L., Tidefelt, U. and Söderquist, B. (2011). Glycopeptide resistance in coagulase-negative staphylococci isolated in blood cultures from patients with hematological malignancies during three decades. *European Journal of Clinical Microbiology Infection Disease* **30, 1349-1354.**

Al-Abbas, M. A. (2012). Antimicrobial susceptibility of *Enterococcus faecalis* and a novel Plano-microbium isolate of bacteremia. *International Journal of Medicine and Medical Sciences* **4, 19–27.**

Arêde, P., Milheiriço, C., de Lencastre, H. and Oliveira, D. C. (2012). The anti-repressor *MecR2* promotes the proteolysis of the *mecA* repressor and enables optimal expression of β-lactam resistance in MRSA. *Plos Pathogen* **8(7), e1002816.**

Arêde, P., Ministro, J. and Oliveira, D. C. (2013). Redefining the role of the β-lactamase locus in methicillin-resistant *Staphylococcus aureus*: Lactamase regulators disrupt the *mecI* mediated strong repression on *mecA* and optimize the phenotypic expression of resistance in strains with constitutive *mecA* expression. *Antimicrobial Agents and Chemotherapy* **57, 3037-3045.**

Azis, N. M., Ab Hamid, A., Pung, H. P, Abdul Ra-Fee, P. A., Yahya, F. A., Amin Nordin, S., Neela, V., Suhaili, Z. and Mohd Desa M. N. (2014). *Staphylococcus aureus* infection risk in a population of health sciences students at a public university. *Iranian Journal of Public Health* **43, 112-116.**

Berger-Bachi, B., Strässle, A., Gustafson, J. E. and Kayser, F. H. (1992). Mapping and characterization of multiple chromosomal factors involved in methicillin resistance in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* **36, 1367-1373.**

Boyce, J. M. and Medeiros, A. A. (1987). Role of β-lactamase in expression of resistance by methicillin-resistant *Staphylococcus aureus*. *Antimicrobial Agents of Chemotherapy* **31, 1426-1428.**

Bush, K. and Jacoby, G. A. (2010). Updated functional classification of β-Lactamases. *Antimicrobial Agents and Chemotherapy* **54, 969-976.**

Chen, M. M. S., Boardman, W. S., Smith, I., Goodman, A. E. and Brown, M. H. (2015). Characterisation of β-lactam resistance mediated by *blaZ* in staphylococci recovered from captive and free-ranging wallabies. *Journal of Global Antimicrobial Resistance* **3, 184-189.**

CLSI. (2014). Performance Standards for Antimicrobial Susceptibility Testing. Available from **http://www.gxccl.com/download/upload/CLSIM100-S24 sample.pdf.** [Retrieved on January 2014].

De Lencastre, H. and Tomasz, A. (1994). Reassessment of the number of auxiliary genes essential for expression of high-level methicillin resistance in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* **38, 2590-2598.**

De Lencastre, H., de Jonge, B. L., Matthews P. R. and Tomasz A. (1994). Molecular aspects of methicillin resistance in *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy* **33, 7-24.**

García-Castellanos, R. (2003). Three-dimensional structure of *mecI*. Molecular basis for transcriptional regulation of staphylococcal methicillin resistance. *The Journal of Biological Chemistry* **278, 39897-39905.**

Hiramatsu, K., Ito, T., Tsubakishita, S., Sasaki, T., Takeuchi, F., Morimoto, Y., Katayama, Y., Matsuo, M., Kuwahara-Arai, K., Hishinuma, T. and Baba, T. (2013). Genomic basis for methicillin resistance in *Staphylococcus aureus*. *Infectious of Chemotherapy* **45, 117-136.**

Ito, T., Katayama, Y. and Hiramatsu, K. (1999). Cloning and nucleotide sequence determination of the entire mec DNA of pre-methicillin-resistant *Staphylococcus aureus* N315. *Antimicrobial Agents and Chemotherapy* **43, 1449-1458.**

Ito, T., Hiramatsu, K., Oliveira, D. C., de Lancastre, H., Zhang, K., Giffard, P. M., O'Brien, F., Coleman, B., Tenover, F. C., Skov, R. C., Enright, M. C., Ko, K. S., Grundmann, H., Laurent, F., Kearns, A. M., Goering, R., John, J. F., Daum, K. and Soderquist, B. (2009). International working group on the classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC): Classification of Staphylococcal cassette chromosome mec (SCCmec): Guidelines for reporting novel SCCmec elements. *Antimicrobial Agents of Chemotherapy* **53, 4961-4967.**

Jansen, A., Türck, M., Szekat, C., Nagel, M., Clever, I. and Bierbaum, G. (2007). Role of insertion elements and yycfg in the development of decreased susceptibility to vancomycin in *Staphylococcus aureus*. *International Journal of Medical Microbiology* **297, 205-215.**

Kaase, M., Lenga, S., Friedrich, S., Szabados, F., Sakinc, T., Kleine, B. and Gatermann, S. G. (2008). Comparison of phenotypic methods for penicillinase detection in *Staphylococcus aureus*. *Clinical Microbiology and Infection* **14, 614-616.**

Katayama, Y., Takeuchi, F., Ito, T., Ma, X. X., Ui-Mizutani, Y., Kobayashi I. and Hiramatsu, K. (2003). Identification in methicillin-susceptible *Staphylococcus hominis* of an active Primordial Mobile Genetic Element for the Staphylococcal Cassette Chromosome mec of methicillin-resistant *Staphylococcus aureus*. *Journal of Bacteriology* **185, 2711-2722.**

Lowy, F. D. (2003). Antimicrobial resistance: The example of *Staphylococcus aureus*. *The Journal of Clinical Investment* **111, 1265-1273.**

Malachowa, N. and DeLeo, F. R. (2010). Mobile genetic element. *Cellular of Molecular Life Sciences* **67, 3057-3071.**

Malik, S., Christensen, H., Peng, H. and Barton, M. D. (2007). Presence and diversity of the beta-lactamase gene in cat and dog staphylococci. *Veterinary Microbiology* **123, 162-168.**

Massiddaa, O., Mingoia, M., Fadda, D., Whalen, M. B., Montanari, M. P. and Varaldo P. E. (2006). Analysis of the β-lactamase plasmid of borderline methicillin-susceptible *Staphylococcus aureus*: Focus on bla complex genes and cadmium resistance determinants cadD and cadX. *Plasmid* **55, 114-127.**

McDougal, L. K. and Thornsberry, C. (1986). The role of β-lactamase in staphylococcal resistance to penicillinase-resistant penicillins and cephalosporins. *Journal of Clinical Microbiology* **23, 832-839.**

McKinney, T. K., Sharma, V. K., Craig, W. A. and Archer, G. L. (2001). Transcription of the gene mediating methicillin resistance in *Staphylococcus aureus* (*mecA*) is co-repressed but not co-induced by cognate *mecA* and β-lactamase regulators. *Journal of Bacteriology* **183, 6862-6868.**

Milheirico, C., Portelinha, A., Krippahl, L., de Lencastre, H. and Oliveira, D. C. (2011). Evidence for a purifying selection acting on the β-lactamase locus in epidemic clones of methicillin-resistant *Staphylococcus aureus*. *BMC Microbiology* **11, 76.**

Olayinka, B. O., Olayinka, A. T., Obajuluwa, A. F., Onaolapo, J. A. and Olurinola, P. F. (2009). Absence of *mecA* gene in methicillin-resistant *Staphylococcus aureus* isolates. *African Journal of Infectious Disease* **3, 49-56.**

Oliveira, D. C., Barros, L. S. S., Silva, V. C. and Cirqueira, M. G. (2011). Study of *Staphylococcus aureus* in raw and pasteurized milk consumed in the Reconcavo area of the State of Bahia, Brazil. *Journal of Food Process Technology* **2, 1-5.**

Oliveira, D. C. and de Lencastre, H. (2011). Methicillin resistance in *Staphylococcus aureus* is not affected by the overexpression in trans of the *mecA* gene repressor: A surprising observation. *PLoS ONE*, **6(8), e23287.**

Olsen, J. E., Christensen, H. and Aarestrup, F. M. (2006). Diversity and evolution of *blaZ* from *Staphylococcus aureus* and coagulase negative staphylococci. *Journal of Antimicrobial Chemotherapy* **57, 450-460.**

Papanicolas, L. E., Bell, J. M. and Bastian, I. (2014). Performance of phenotypic tests for detection of penicillinase in *Staphylococcus aureus* isolates from Australia. *Journal of Clinical Microbiology* **52, 1136-1138.**

Peacock, S. J. and Paterson, G. K. (2015). Mechanisms of methicillin resistance in *Staphylococcus aureus*. *Annual Review of Biochemistry* **84, 577-601.**

Pereira, L. A., Harnett, G. B., Hodge, M. M., Cattell, J. A. and Speers, D. J. (2014). Real-time PCR assay for detection of *blaZ* genes in *Staphylococcus aureus* clinical isolates. *Journal of Clinical Microbiology* **52, 1259-1261.**

Petinaki, E., Arvaniti, A., Dimitracopoulos, G. and Spiliopoulou, I. (2001). Detection of *mecA*, *mecRI* and *mecI* genes among clinical isolates of methicillin-resistant staphylococci by combined polymerase chain reactions. *Journal of Antimicrobial Chemotherapy* **47, 297-304.**

Petinaki, E., Arvaniti, A., Bartzavali, C., Dimitracopoulos, G. and Spiliopoulou, I. (2002). Presence of mec genes and overproduction of beta-lactamase in the expression of low-level methicillin resistance among staphylococci. *Chemotherapy* **48, 174-181.**

Plata, K., Rosato, A. E. and Wegrzyn G. (2009). *Staphylococcus aureus* as an infectious agent: Overview of biochemistry and molecular genetics of its pathogenicity. *Acta Biochimica Polonica* **56, 597-612.**

Robles, B. F., Nóbrega, D. B., Guimarães, F. F., Wanderley, G. G. and Langoni, H. (2014). Beta-lactamase detection in *Staphylococcus aureus* and coagulase-negative *Staphylococcus* isolated from bovine mastitis. *Pesquisa Veterinária Brasileira* **34, 325-328.**

Rohrer, S., Maki, H. and Berger-Bächi, B. (2003). What makes resistance to methicillin heterogeneous?. *Journal of Medical Microbiology* **52, 605-607.**

Safo, M. K., Zhao, Q., Ko, T. P., Musayev F. N., Robinson, H., Scarsdale, N., Wang, A. H. and Archer, G. L. (2005). Crystal structures of the *blaI* repressor from *Staphylococcus aureus* and its complex with DNA: Insights into transcriptional regulation of the bla and mec operons. *Journal of Bacteriology* **187, 1833-1844.**

Saini, V., McClure, J. T., Scholl, D. T., DeVries, T. J. and Barkema, H. W. (2012). Herd-level association between antimicrobial use and antimicrobial resistance in bovine mastitis *Staphylococcus aureus* isolates on Canadian dairy farms. *Journal of Dairy Science* **95, 1921-1929.**

Shore, A. C., Deasy, E. C., Slickers, P., Brennan, G., O'Connell, B., Monecke, S., Ehricht, R. and Coleman D. (2011). Detection of staphylococcal cassette chromosome mec type XI carrying highly divergent *mecA*, *mecI*, *mecR1*, *blaZ*, and *ccr* genes in human clinical isolates of clonal complex 130 methicillin-resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* **55, 3765-3773.**

Shore, A. C. and Coleman, D. C. (2013). Staphylococcal cassette chromosome mec: Recent advances and new insights. *International Journal of Medical Microbiology* **303, 350-359.**

Tulinski, P., Fluit, A. C., Wagenaar, J. A., Mevius, D., van de Vijver, L. and Duim, B. (2012). Methicillin-resistant coagulase-negative staphylococci on pig farms as a reservoir of heterogeneous staphylococcal cassette chromosome mec elements. *Applied and Environmental Microbiology* **78, 299-304.**