THE IMPACT OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) EVOLUTION ON CLINICAL, PHENOTYPIC AND GENOTYPIC CHARACTERISTICS OF MRSA STRAINS ISOLATED IN A TERTIARY TEACHING HOSPITAL

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DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF MEDICAL SCIENCE

FACULTY OF MEDICINE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2018

UNIVERSITY OF MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: Sit Pik San

Matric No: MGN15

Name of Degree: Master of Medical Science

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"): The impact of Methicillin-Resistant *Staphylococcus aureus* (MRSA) evolution on clinical, phenotypic and genotypic characteristics of MRSA strains isolated in a tertiary teaching hospital.

Field of Study: Medical Microbiology

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a notorious bacterial pathogen causing well-publicized healthcare-associated (HA) and community-associated (CA) infections. Vancomycin has been used as the primary treatment for MRSA infections. However, reduced vancomycin susceptibility and vancomycin MIC creep have been described in many studies. Furthermore, the emergence of CA-MRSA isolates which cause nosocomial infections and HA-MRSA isolates circulating in the community has also been reported. In spite of that, only limited studies were done in monitoring the trends in MRSA infections in Malaysian hospitals. Therefore, this study attempted to characterized HA– and CA-MRSA infections using phenotypic and genotypic approaches. In addition, the changing epidemiology and the evolution of HA- and CA-MRSA strains isolated from 2011 to 2013 were also assessed and correlated with patient's clinical data. A total of 278 MRSA strains from year 2011 to 2013 collected from a tertiary teaching hospital in Malaysia were studied. Overall, MRSA acquisitions were significantly high in patients more than 50 years of age and male gender. Sixty-five percent of HA-MRSA were found carrying SCC*mec* types I to V while 26.9% were CA-MRSA strains that harboured SCC*mec* types III, IV and V. The presence of SCC*mec* types IV and V strains that caused nosocomial infections and SCC*mec* type III strains that associated with CA-infections were also observed. Majority of the strains were HA-MRSA and belonged to SCC*mec* type III which were found to be persistent and remained as endemic strains in this hospital. The overall PVL-positive rate among the MRSA strains in this hospital was 1.8%. All 2013 MRSA strains were multidrug-resistant which showed high resistance towards erythromycin, gentamicin, ciprofloxacin and clindamycin. During the study period, no vancomycin MIC creep was detected based on

the significant fluctuation in the rate of MRSA strains with vancomycin MIC ≥ 1.5 µg/mL (38.9% in 2011, 57.9% in 2012 and 56.5% in 2013). High vancomycin MIC was observed to be significantly associated with SCC*mec* type III. There was no significant association between the mortality rate and high vancomycin MIC. Age, gender, diagnosis, comorbidities and the infection severity scores were the independent factors associated with patient's mortality. Among 158 MRSA bacteraemia episodes, 80.4% occurred in patients more than 50 years old, male gender and were caused by the HA-MRSA strains. The incidence of bacteraemia appeared to be mostly primary bacteraemia. In addition, diabetes mellitus, chronic kidney disease and hypertension were major comorbidities associated with bacteraemia. MRSA bacteraemia patients with SCC*mec* type IV strains were significantly associated with cardiovascular disease. Compared with other SCC*mec* types, MRSA bacteraemia with SCC*mec* type V strains were implant-related. These strains associated with bacteraemia were further subtyped by MLST which grouped strains into 13 sequence types and were assigned into 12 pulsotypes by PFGE. The most common pulsotype was pulsotype E which was exhibited by SCC*mec* type III-ST239 strains. The presence of MRSA clones such as ST152-I, untypeable- ST508, ST1-IV, ST1137-IV, ST5-V, ST45-V and ST951-V in this study were the first reported in Malaysia.

(488 words)

ABSTRAK

Methicillin-resistant *Staphylococcus aureus* (MRSA) adalah bakteria patogen yang terkenal dalam menyebabkan jangkitan "healthcare-associated" (HA) dan "community-associated" (CA). Vancomycin merupakan rawatan utama untuk jangkitan MRSA. Tetapi penurunan sensitifitas terhadap vancomycin dan "vancomycin MIC creep" telah dilaporkan. Di samping itu, kemunculan isolat CA-MRSA dalam menyebabkan jangkitan nosokomial dan penyebaran isolat HA-MRSA dalam kommuniti juga telah dilaporkan. Walau bagaimanapun, kajian yang dilakukan untuk memantau trend jangkitan MRSA di hospital di Malaysia adalah terhad. Oleh itu, kajian ini telah mencirikan jangkitan HA- dan CA-MRSA melalui kaedah phenotip dan genotip. Tambahan pula, perubahan epidemiologi dan evolusi strain-strain HA- dan CA-MRSA dari tahun 2011 hingga 2013 juga telah dinilaikan and dikaitkan dengan data klinikal pesakit. Sebanyak 278 strain MRSA dari tahun 2011 hingga 2013 dikumpulkan daripada sebuah hospital pengajaran pengajian tinggi di Malaysia telah dipelajari. Peningkatan yang signifikan telah diperhatikan terhadap pesakit 50 tahun ke atas dan jantina lelaki dalam memperolehi MRSA. Enam puluh lima peratus jangkitan HA-MRSA didapati membawa SCC*mec* taip I hingga V manakala 26.9% adalah strain CA-MRSA membawa SCC*mec* taip III, IV dan V. Kewujudan strain SCC*mec* taip IV dan V dalam menyebabkan jangkitan nosokomial dan strain SCC*mec* taip III dalam menyebabkan jangkitan kommuniti telah diperhatikan. Majoriti strain-strain adalah HA-MRSA dan SCC*mec* taip III didapati kekal sebagai strain endemik di hospital ini. Prevalens gen PVL antara strain-strain MRSA dalam hospital ini adalah 1.8%. Semua strain-strain MRSA dari tahun 2013 adalah rintang terhad pelbagai antibiotik dimana kadar rintangan yang tinggi terhadap erythromycin, gentamicin, ciprofloxacin and clindamycin telah diperhatikan. Semua strain-strain MRSA adalah sensitif terhadap vancomycin. Sepanjang tempoh kajian ini, tiada kehadiran "vancomycin

MIC creep" diperhatikan berdasarkan penaikan dan penurunan yang signifikan terhadap kadar strain MRSA dengan vancomycin MIC ≥ 1.5 μg/mL (38.9% dalam 2011, 57.9% dalam 2012 and 56.5% dalam 2013). Vancomycin MIC yang tinggi adalah berkaitan dengan SCC*mec* taip III. Tiada hubungan yang signifikan diperhatikan antara kadar kematian dan vancomycin MIC ≥ 1.5μg/mL. Umur, jantina, diagnosis, komorbiditas dan markah keparahan jangkitan adalah antara faktor yang berkaitan dengan kematian pesakit. Antara 158 episod bakteremia MRSA, 80.4% berlaku pada pesakit 50 tahun ke atas, jantina lelaki dan disebabkan oleh strain HA-MRSA. Kebanyakan kes bakteremia adalah "primary bacteraemia". Tambahan pula, kencing manis, penyakit buah pinggang kronik dan tekanan darah tinggi adalah komorbiditas yang utama berkaitan dengan bakteremia. Pesakit bakteremia MRSA dengan SCC*mec* taip IV adalah berkaitan dengan penyakit kardiovaskular. Berbanding dengan SCC*mec* taip yang lain, bakteremia MRSA dengan SCC*mec* taip V adalah berkaitan dengan implan. Strain-strain MRSA yang berkaitan dengan bakteremia telah dicirikan oleh MLST dan PFGE dimana strain-strain tersebut dibahagikan kepada 13 jenis jujukan dan 12 pulsotaip. Pulsotaip yang paling biasa adalah pulsotaip E yang dipamerkan oleh strain SCC*mec* III-ST239 Kewujudan klon MRSA seperti ST152-I, "untypeable"- ST508, ST1-IV, ST1137-IV, ST5-V, ST45-V and ST951-V dalam kajian ini telah dilaporkan untuk kali pertama di Malaysia.

(458 patah perkataan)

ACKNOWLEDGEMENTS

First of all, I would like to express my appreciation to my supervisors; Assoc Prof Dr Sasheela A/p Sri La Sri Ponnampalavanar, Dr Cindy Teh Shuan Ju and Dr Nuryana binti Idris for their guidance and support throughout the course of my project.

I would also like to thank Prof Dr Thong Kwai Lin, who has provided me with the materials and equipment to do PFGE as well as the control strains for SCC*mec* typing. I am grateful to Ngoi Soo Tein and Koh Xiu Pei for providing guidance to do PFGE and also to my lab mates for their guidance.

My sincere gratitude to University of Malaya for providing PPP grant (PG174-2015B), Graduate Research Assistantship Scheme (GRAS) and also Ministry of Higher Education for providing FRGS grant (FP016-2014B) which played a huge role in providing financial assistance for my study.

Finally, I would like to thank my mother, sister and brothers for their unceasing encouragements and supports.

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LIST OF SYMBOLS AND ABBREVIATIONS

MRSA	:	Methicillin-resistant *Staphylococcus aureus*
HA	:	healthcare-associated
CA	:	community-associated
SSTIs	:	skin and soft tissue infections
CoNS	:	coagulase-negative staphylococci
PFGE	:	pulsed-field gel electrophoresis
MLST	:	multilocus sequence typing
UMMC	:	University Malaya Medical Centre
HKL	:	Hospital Kuala Lumpur
SCC*mec*	:	Staphylococcal cassette chromosome *mec*
VISA	:	vancomycin-intermediate *Staphylococcus aureus*
US	:	United States
VRSA	:	vancomycin-resistant *Staphylococcus aureus*
MIC	:	minimum inhibitory concentration
PCR	:	polymerase chain reaction
PVL	:	Panton Valentine Leukocidin
S. aureus	:	*Staphylococcus aureus*
β	:	beta
MSCRAMMs	:	microbial surface components recognizing adhesive matrix molecules
TSST-1	:	Toxic shock syndrome toxin-1
MHC	:	major histocompatibility class
RBCs	:	red blood cells
WBCs	:	white blood cells

α	:	alpha
γ	:	gamma
σ	:	sigma
agr	:	accessory gene regulator
sae	:	staphylococcal accessory element
sarA	:	staphylococcal accessory regulator A
PBPs	:	penicillin-binding proteins
UK	:	United Kingdom
CDC	:	Centers for Disease Control and Prevention
PSMs	:	phenol-soluble modulins
ACME	:	arginine catabolic mobile element
SOP		Standard operating procedure
CLSI	:	Clinical and Laboratory Standards Institute
µg	:	microgram
mm	:	millimetre
M-PCR	:	multiplex PCR
>	:	more than
≤	:	less than or equal to
<	:	less than
≥	:	more than or equal to
%	:	percent
Mb	:	Megabyte
UPGMA	:	unweighted pair group matching analysis
bp	:	base pair
ST	:	sequence type
CC	:	clonal complex

TMP/SMX	:	trimethoprim-sulfamethoxazole
µg/mL	:	microgram per millilitre
CSF	:	cerebrospinal fluid
°C	:	degree Celsius
TSA	:	Tryptic Soy Agar
TSB	:	Tryptic Soy Broth
EDTA	:	ethylenediaminetetraacetic acid
dNTP	:	deoxynucleotide triphosphate
BSA	:	Bovine Serum Albumin
DNA	:	deoxyribonucleic acid
$MgCI_2$	:	magnesium chloride
µL	:	microlitre
ddH_2O	:	double distilled water
mL	:	millilitre
rpm	:	revolutions per minute
mM	:	millimolar
µM	:	micromolar
ng	:	nanogram
U	:	unit
CAZ	:	Ceftazidime
ERY	:	Erythromycin
GEN	:	Gentamicin
FOX	:	Cefoxitin
RIF	:	Rifampicin
FUS	:	Fusidic Acid
CMX	:	Cotrimoxazole

CIP	:	Ciprofloxacin
CLI	:	Clindamycin
CRO	:	Ceftriaxone
AMC	:	Amoxicillin-clavulanate
CST	:	Colistin
PEN	:	Penicillin
TZP	:	Piperacillin-Tazobactam
SAM	:	ampicillin-sulbactam
SLV		Single-locus variant
V	:	volt
TBE	:	Tris-borate-EDTA
CSB	:	cell suspension buffer
OD	:	optical density
mg/mL	:	milligram per millilitre
TE	:	Tris-EDTA
V/cm	:	volt per centimetre
VSSA	:	Vancomycin-susceptible *Staphylococcus aureus*
MSSA	:	Methicillin-sensitive *Staphylococcus aureus*
CKD	:	chronic kidney disease
OR	:	Odds Ratio
CI	:	Confidence Interval
APACHE	:	Acute Physiology and Chronic Health Evaluation
CCI	:	Charlson Comorbidity Index
MDROs	:	multidrug-resistant organisms
UKM	:	Universiti Kebangsaan Malaysia
mg/L	:	milligram per litre

g/mol	:	gram per mole
NA	:	Not available

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 General Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a significant cause of healthcare-associated (HA) and community-associated (CA) infections which include pneumonia, bacteraemia, skin and soft tissue infections (SSTIs), endocarditis and osteomyelitis (Boucher *et al.*, 2010; Gorwitz *et al.*, 2006). The incidence of MRSA infections have extensively increased worldwide where high rates of more than 50% were reported in North and South America, Asia and Malta (Hand in Scan, 2014). In Malaysia, the prevalence of MRSA is reported to have increased from 17% in 1986 to 44.1% in 2007 (Lim *et al.*, 2013).

Nosocomial bacteraemia is common in hospitals worldwide and is associated with high mortality rates (Gopal Katherason *et al.*, 2010). *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Acinetobacter* spp, MRSA and coagulase-negative staphylococci (CONS) were reported as causative pathogens of nosocomial bacteraemia (Gopal Katherason *et al.*, 2010; Karchmer, 2000; Tak *et al.*, 2013). MRSA bacteraemia is one of the major concerns worldwide because of its high incidence rates (Cuervo *et al.*, 2015) and causes significant morbidity and mortality (Chong *et al.*, 2015). In Malta, the rate of MRSA bacteraemia in 2007 was 52.4% (Naber, 2009). Whereas in Taiwan, the incidence of MRSA bacteraemia from 2000 to 2010 remained above 55% (Chen & Huang, 2014). In Malaysia, 21% of MRSA bacteraemia cases were reported in 2008 (Ahmad *et al.*, 2010).

Phenotypic methods such as antibiotic susceptibility testing and various discriminative molecular methods including SCC*mec* typing, pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) are used for MRSA typing in order to describe the epidemiological trends (Ostojić & Hukić, 2015). SCC*mec* type III-MLST type ST239 were reported as the predominant clone in the previous studies conducted in 2003 and 2008 strains in University Malaya Medical Centre (UMMC) as well as 2007 to 2008 strains in Hospital Kuala Lumpur (HKL) (Ghaznavi-Rad *et al.*, 2010a; Lim *et al.*, 2013). In addition, CA-MRSA clones including SCC*mec* type IV-ST6, ST22, ST30, ST1178 and SCC*mec* type V-ST772 are also present in hospitals in Malaysia (Ahmad *et al.*, 2009; Ghaznavi-Rad *et al.*, 2010a; Lim *et al.*, 2013; Sam *et al.*, 2008).

There are relatively limited antimicrobial treatments available for MRSA infections (Kurosu *et al.*, 2013). To date, vancomycin has been a key antibiotic agent to treat MRSA infections. However, there were reports on the emergence of vancomycin-intermediate *Staphylococcus aureus* (VISA) in causing treatment failures in the United States (US) and Japan as well as presence of vancomycin-resistant *Staphylococcus aureus* (VRSA) in the US (Carroll, 2013b) which has led to great concern in global public health.

No study has shown the presence of VRSA strains in Malaysia. However, heterogenous VISA (hVISA) strains were detected among MRSA isolates in 2009 from Malaysian hospitals including Hospital Selayang and HKL (Norazah *et al.*, 2012; Ramli *et al.*, 2012). In 2009, Ahmad *et al.* (2010) has reported a greater proportion of MRSA isolated from blood from six major hospitals in Malaysia with high vancomycin MIC of 2µg/mL (Ahmad *et al.*, 2010). The increase in vancomycin MIC within the susceptible range (vancomycin MIC creep) is of major concern as it could be one of the factors causing vancomycin treatment failure and patient's mortality as well as the emergence of VISA and VRSA strains in the future.

Few studies have reported the emergence of CA-MRSA isolates causing nosocomial MRSA outbreaks and presence of HA-MRSA isolates in the community (Ahmad *et al.*, 2009; Rodriguez-Noriega & Seas, 2010; Song *et al.*, 2011). In spite of that, only limited studies were done in Malaysia to monitor the trends in MRSA infections as well as vancomycin susceptibility. In addition, there is no local study done to correlate between molecular and phenotypic characteristics of MRSA strains with the patient's clinical outcome as well as to study the characteristics of MRSA bacteraemia. Therefore, the aim of this study is to determine the prevalence of MRSA infection and the relationships between clinical, phenotypic and molecular evolution of MRSA with the patient's outcome.

1.2 Objectives of the study

a) To characterize HA– and CA-MRSA infections by SCC*mec* PCR typing, pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST).

b) To detect the presence of Panton-Valentine leukocidin (PVL) gene among the MRSA strains.

c) To evaluate the vancomycin susceptibility in MRSA strains by E-test.

d) To determine the changing epidemiology of HA- and CA-MRSA.

e) To investigate the MRSA evolution from year 2011 to 2013 and correlate with patient's clinical data.

1.3 Research hypotheses

Hypothesis 1: The epidemiology of HA- and CA-MRSA would have changed during the 3-year study period in this hospital.

Hypothesis 2: The clinical and genotypic differences between MRSA with high and low vancomycin MIC.

Hypothesis 3: The clinical, phenotypic and genotypic characteristics are associated with the patient's outcomes.

CHAPTER 2: LITERATURE REVIEW

2.1 *Staphylococcus aureus*

2.1.1 Characteristics

Staphylococcus aureus (*S. aureus*) is a gram positive cocci arranged in a grape-like clusters, non-motile, non-spore forming, and facultative anaerobe (Carroll, 2013b; Costa *et al.*, 2013) which belongs to the *Staphylococcaceae* family (Todar, 2012). It forms round, smooth, raised and glistening deep golden yellow colonies when growing on nutrient agar and produces β-haemolysis on blood agar (Carroll, 2013b). *S. aureus* is tolerant to high salt concentration and ferment mannitol where it produces yellow colonies on mannitol salt agar (Todar, 2012). *S. aureus* also produces catalase which differentiates them from streptococci and enzyme coagulase which differentiates them from coagulase-negative staphylococci (CoNS) (Levinson, 2012).

S. aureus is a transient colonizer of the anterior nares, upper respiratory tract (Wilson *et al.*, 2011) and other anatomical locales including the skin, hair and gastrointestinal tract (Todar, 2012). It has been estimated that approximately 20 to 50% of the general populations are *S. aureus* carriers (Carroll, 2013b). This colonization significantly increases the risk of infections by providing a reservoir for the pathogen from which it is introduced when the host defence is compromised (Costa *et al.*, 2013).

2.1.2 Pathogenesis

S. aureus is one of the major nosocomial pathogen which causes multitude of infections from minor skin infections (pimples and boils) or food poisoning to severe life-threatening infections (scalded skin syndrome, osteomyelitis, toxic shock syndrome, pneumonia, bacteraemia and endocarditis) (Carroll, 2013b; Levinson, 2012; Plata *et al.*, 2009). An array of virulence factors are involved in the pathogenesis of infection, allowing the adherence of bacterium to tissue, invading the host's immune system and causing harmful effects to the host (Costa *et al.*, 2013). These factors are categorized into (1) cell surface-associated (adherence) which include microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), staphyloxanthin and polysaccharides capsules; and (2) secreted factors (exotoxins) which include superantigens, cytolytic toxins and enzymes. A brief summary of each virulence factor and their respective roles are shown in Table 2.1.

Table 2.1: List of virulence factors and their respective roles (Carroll, 2013b; Costa *et al.*, 2013; Gordon & Lowy, 2008; Levinson, 2012; Reygaert, 2013).

Virulence factors	Putative roles
Cell-surface (adherence) factors	
Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs)	
• Protein A	Binds to the Fc portion of IgG molecules.
• Fibronectin-binding proteins	Fibronectin and plasma clot attachment.
• Collagen-binding protein	Collagenous tissues and cartilage adherence.
• Clumping factor proteins	Adhere organism to fibrinogen and fibrin. Forms clumps once it combines with plasma.
Polysaccharides capsule	Inhibit phagocytosis. 11 serotypes have been identified.
Staphyloxanthin	Neutrophil reactive oxidant-based phagocytosis resistance.
Secreted factors (exotoxins)	
Superantigens	
• Enterotoxins (SEA, B, C, D, E, G and Q)	Causes food poisoning. It is secreted when *S. aureus* grows in carbohydrate and protein food and it acts on neural receptors in the gut.

Table 2.1, continued

Virulence factors	Putative roles
• Toxic shock syndrome toxin-1 (TSST-1)	Binds to major histocompatibility class (MHC)-II, activates T-cells which promotes protean manifestations of the toxic shock syndrome.
• Exfoliative toxins ETA and ETB	Cause staphylococcal scalded skin syndrome. The accumulation in the skin can cause desmosomes disruption through proteolytic cleavage of desmoglein I
Cytolytic toxins	
• α-haemolysin	Acts on a wide spectrum of eukaryotic cell membrane.
• β-haemolysin	Cause sphingomyelin degradation and is toxic to human red blood cells (RBCs).
• γ-haemolysin	Lyse leucocytes and erythrocytes.
• σ-haemolysin	Disrupts biologic membrane.
• Panton Valentine Leukocidin (PVL)	Lyse white blood cell (WBC) by causing pore formation.
Enzymes	
• Hyaluronidase	Degrades hyaluronic acid.
• Staphylokinase	Plasminogen activation and inactivate antimicrobial peptides.
• Lipases	Inactivates fatty acid.
• Nucleases	Cleave nucleic acid.
• Catalase	Converts hydrogen peroxide to water and oxygen.
• Coagulase	Binds to prothrombin and initiates fibrin polymerization.

The regulation of the virulence genes in *S. aureus* begins with the attachment of bacterium to the host by up-regulating the gene expression coding for surface proteins involved in the attachment and defence against the host's immune system. During an infection, *S. aureus* up-regulates the toxins production that facilitate the spread in tissues (Costa *et al.*, 2013). The expression of the above virulence genes is regulated by several systems in response to bacterial cell density and environmental signals which include two-component systems (consist of two proteins; accessory gene regulator, *agr* and staphylococcal accessory element, *sae*) and global regulatory systems (consist of staphylococcal accessory regulator A, *sarA*) (Carroll, 2013b).

agr controls the expression of surface adhesins (protein A, coagulase and fibronectin binding protein) and production of exoproteins (α-haemolysin, TSST-1 and enterotoxins). It also down-regulates the synthesis of cell-wall associated proteins. *sae* regulates the expression of genes at the transcriptional level and is important for α- toxin, β- haemolysins and coagulase productions. *sarA* positively control *agr* and down-regulates several proteases (Carroll, 2013b).

2.1.3 Antimicrobial Resistance

Penicillin, a β-lactam antibiotic was introduced in 1942 and used to treat wounded soldiers during World War II (American Chemical Society International Historic Chemical Landmarks, n.d). β-lactam drugs inhibit the bacterial cell wall synthesis in which the initial process involves the binding of the drug to the cell receptors penicillin-binding proteins (PBPs). Once attached, the transpeptidation reaction is inhibited and the synthesis of peptidoglycan is blocked. This is followed by the inactivation of an inhibitor of autolytic enzymes in the cell wall. As a result, lytic enzyme is activated leading to cell lysis. There are at least six PBPs which are under chromosomal control and any mutation can change their affinities for β-lactam drugs (Carroll, 2013a).

Due to the widespread use of penicillin, β-lactamase (penicillinase)-producing *S. aureus* was reported in mid 1940s (DeLeo & Chambers, 2009). This enzyme is plasmid-mediated which exposes the β-lactam ring of penicillin and obliterates its antimicrobial activity. β-lactamase inhibitors such as clavulanic acid, tazobactam and sulbactam have high affinities for and bind to some β-lactamase but cannot be hydrolyzed by β-lactamase. They simultaneously protect hydrolyzable penicillins such as ampicillin, ticarcillin and amoxicillin from destruction (Carroll, 2013a).

The advent of methicillin (semi-synthetic β-lactamase-resistant penicillin) in 1960 served as a countermeasure against the resistant strains as it is stable against hydrolysis by β-lactamase. However, soon after the introduction of methicillin, the first case of MRSA was reported in the United Kingdom (UK) in 1961 and has since become widespread (Yamamoto *et al.*, 2013).

2.2 Methicillin-resistant *Staphylococcus aureus* (MRSA)

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a bacterium that is resistant to methicillin and other β-lactam antibiotics (penicillin, carbapenems, cephalosporins and cloxacillin) (Lindsay, 2013). It is usually formed by the acquisition of a mobile genetic element called staphylococcal cassette chromosome *mec* (SCC*mec*) that carries the *mec*A gene (Deurenberg *et al.*, 2007). MRSA is also referred to as "superbug"; a term used to define any microorganism that is resistant to the common antibiotics that are supposed to treat infections (Moore, 2008).

SCC*mec* consists of direct and terminal repeats, *mec* and *ccr* gene complexes and the junkyard (J) regions. The *mec* gene complex consists of *mec*A as well as *mec*I and *mec*R1, the regulatory genes. Based on the structural diversity of *mec*I and *mec*R1 region, six *mec* gene complexes (Class A, B, C1, C2, D and E) have been defined by Ito *et al.* (2009). The *ccr* gene complex consists of *ccr* genes surrounded by *orfs* which are responsible for cassette mobilization and encodes DNA recombinases of the invertase-resolvase family. Two *ccr* gene complexes have been reported based on the *ccr* genes composition; one carrying *ccrA* and *ccrB* genes while the other carries *ccrC* genes. To date, Type 1 to Type 8 *ccr* gene complexes as shown in Table 2.2 have been identified. The J regions (J1 to J3) are situated around and between *mec* and *ccr* complexes and consist of various non-essential genes that carry additional antibiotic resistance

determinants in some cases (Ito *et al.*, 2009; Turlej *et al.*, 2011; Zhang *et al.*, 2005). Various SCC*mec* types can be generated by the combinations of these complex classes and allotypes. Thus far, 11 SCC*mec* types (I to XI) have been defined as shown in Table 2.2 (Ito *et al.*, 2009). In spite of that, SCC*mec* types I to V are the only SCC*mec* types that are distributed globally whereas the other SCC*mec* types are not frequently found and exist only as local strains in their country of origin (Ghaznavi-Rad *et al.*, 2010b). SCC*mec* types are further differentiated into subtypes based on the differences in the J region DNA (Milheirico *et al.*, 2007a).

Table 2.2: Characteristics of SCC*mec* types (Ito *et al.*, 2009).

SCC*mec* types	***mec* gene complexes**	***ccr* genes complexes**
I	B	*ccr*A1B1 (Type 1)
II	A	*ccr*A2B2 (Type 2)
III	A	*ccr*A3B3 (Type 3)
IV	B	*ccr*A2B2(Type 2)
V	C	*ccr*C (Type 5)
VI	B	*ccr*A4B4 (Type 4)
VII	C1	*ccr*C1 (Type 5)
VIII	A	*ccr*A4B4 (Type 4)
IX	C2	*ccr*A1B1 (Type 1)
X	C1	*ccr*A1B6 (Type 7)
XI	E	*ccr*A1B3 (Type 8)

The 78-kDa penicillin-binding protein 2a (PBP 2a or PBP2') is encoded by 2.1kb *mec*A gene that has low affinity to β-lactams in which despite the presence of β-lactams, it retains its activity by allowing cell wall biosynthesis to continue. The expression of PBP 2a is regulated by *mec*I and *mec*R1 system (Kawada-Matsuo & Komatsuzawa, 2012).

MRSA is known as one of the leading cause of healthcare- and community-associated infections. HA-MRSA strains are multidrug-resistant and often contain large SCC*mec* types I to III and seldom carry Panton-Valentine leukocidin (PVL) genes (File, 2008). SCC*mec* types II and III carry additional genetic elements such as Tn554 which encodes clindamycin, macrolides and streptogramin B resistances as well as pT181 which encodes tetracyclines resistance (Appelbaum, 2007). HA-MRSA predominantly causes nosocomial infections such as pneumonia, bacteraemia, endocarditis, catheter-related infections and post-operative wound infections (Guggenbichler *et al.*, 2011). Centers for Disease Control and Prevention (CDC) defined HA-MRSA as cultures that were found positive more than 48 hours after admission and met all of the following healthcare-associated risk factors; old age, recent hospitalization or surgery, prolonged antibiotic treatment, presence of underlying medical illnesses (such as chronic liver disease or diabetes), presence of indwelling catheters or medical devices, contact with healthcare facilities (nursing home or haemodialysis) or residence in a long-term care facility. (Boucher & Corey, 2008; David & Daum, 2010; David *et al.*, 2008; Haddadin *et al.*, 2002; Kumari *et al.*, 2016; Millar *et al.*, 2007; Sydnor & Perl, 2011).

In the 1990s, changes in the epidemiology of MRSA infections were described as a result of the emergence of CA-MRSA strains (Millar *et al.*, 2007). CA-MRSA strains are sensitive to antibiotics with the exception of β-lactams and macrolides (erythromycin, azithromycin and clarithromycin) and contain smaller SCC*mec* types IV or V (File, 2008; Gorwitz *et al.*, 2006). They predominantly cause SSTIs, necrotizing pneumonia and fasciitis, pyomyositis, sepsis and osteomyelitis (Watkins *et al.*, 2012). The risk factors associated with CA-MRSA include intravenous drug user, chronic skin disease, children, athletes, military personnel, prisoner, close body contact with CA-MRSA patient and poor personal hygiene (Matouskova & Janout, 2008; Millar *et al.*, 2007; Watkins *et al.*,

2012). CDC defined CA-MRSA as positive culture obtained from an outpatient or within 48 hours of admission without any exposure to healthcare-associated risk factors (Watkins *et al.*, 2012). CA-MRSA strains also express virulence determinants such as phenol-soluble modulins (PSMs) and type I arginine catabolic mobile element (ACME) which contribute to the severity of the disease as well as fitness and transmissibility of the isolates, respectively (Szabó, 2014; Watkins *et al.*, 2012).

Panton-Valentine leukocidin (PVL) toxin is among the leukocidins that requires much attention due to its connection with CA-MRSA. PVL is a bi-component exotoxin encoded by *lukF-PV* and *lukS-PV* genes and transmitted by bacteriophages (David & Daum, 2010). It forms pores in the leucocyte membrane causing leucocyte destructions (Carroll, 2013b), and is reported to be associated with severe necrotizing infections (Rostamzad & Rostamneia, 2016).

According to CDC, MRSA is responsible for more than 90,000 serious infections and 18,000 hospital-related deaths per year in the US. These MRSA strains were involved in many serious SSTIs as well as pneumonia (Wilson *et al.*, 2011). While widely known as a growing cause of SSTIs, MRSA also represents a significant proportion of invasive infections, particularly bacteraemia (Burkey *et al.*, 2008). The risk factors for MRSA bacteraemia were identified as increased age, male gender, severe underlying disease (such as diabetes, liver disease and renal failure), recent hospitalization, intravenous drug use, nursing home resident, previous antibiotic treatment, history of MRSA infection and presence of catheter or invasive procedure (Weigelt, 2016) .

MRSA bacteremia and its life-threatening complications such as infective endocarditis may result in significant morbidity and mortality (Yilmaz *et al.*, 2016). The mortality rate among patients with MRSA bacteremia were ranged from 20 to 50% (MRSA Survivors Network, n.d). In 2008, a study done in Canada has reported the fatality rate in MRSA bacteraemia patients (39%) was higher compared to those of MSSA (24%) (Laupland *et al.*, 2008). Similarly, in a meta-analysis of 31 cohort studies (1980-2000), MRSA bacteraemia was found to have higher mortality rate compared to MSSA (Cosgrove *et al.*, 2003). Ok *et al.* (2013) also documented that patients with persistent MRSA bacteraemia had significantly higher mortality rate (58.1%) than those with non-persistent MRSA bacteraemia (16.7%) (Ok *et al.*, 2013).

A number of risk factors for mortality in MRSA and *S. aureus* bacteraemia patients have been enumerated in some studies. Those factors are old age, female gender, presence of comorbidities (alcoholism, cirrhosis, congestive cardiac failure, malignancy and chronic renal failure), immune status (immunosuppression), severity scores, onset of bacteremia and microbiological factors (such as methicillin resistance, high vancomycin MIC and bacterial characteristics). In addition, factors like staying in a nursing home and organ impairment are also associated with mortality in MRSA bacteraemia (Pastagia *et al.*, 2012; van Hal *et al.*, 2012).

2.2.1 Phenotypic and genotypic methods for MRSA

MRSA strains can be typed by phenotypic and genotypic methods. The most common phenotypic method is antibiotic susceptibility testing (Ostojić & Hukić, 2015). The Clinical and Laboratory Standards Institute (CLSI) recommends cefoxitin disk (30µg) screen test for the detection of MRSA (Appelbaum, 2007). According to CLSI guidelines, strains with the cefoxitin zone diameter of ≤ 21mm, are identified as MRSA

(Broekema *et al.*, 2009). The main disadvantages of phenotypic method are variability in resistance expression, lack of discriminatory and reproducibility power. In addition, the environment may easily affect the antibiotic resistance patterns where unrelated strains may develop same resistance pattern as a result of the similar selective pressure exerted on them. In addition, it is also possible to have different antibiograms between two strains from the same clone due to the loss or acquisition of plasmids carrying resistance genes. Hence, phenotypic method alone should not be used as the typing method for MRSA (Montesinos *et al.*, 2002; Weller, 2000).

Various molecular typing methods were utilized for epidemiological surveillance in order to develop effective strategies to control and prevent the spread of MRSA. Such commonly used methods include SCC*mec* typing, PFGE and MLST. Although PFGE and MLST methods are expensive and time-consuming, they remain as gold standards for comparative typing of MRSA strains because they are highly discriminatory, stable and reproducible (Deurenberg *et al.*, 2007; Laplana *et al.*, 2007; Sabat *et al.*, 2013; Stefani *et al.*, 2012).

MRSA characterization can be done by SCC*mec* typing which assigns SCC*mec* types to MRSA strains to differentiate between HA- and CA-MRSA strains. This is done by identifying and determining the presence of different *ccr* genes and *mec* complex using PCR amplification (Szabó, 2014; Turlej *et al.*, 2011). Various multiplex SCC*mec* typing methods have been developed. Oliveira and de Lencastre (2002) developed the first multiplex PCR which identified SCC*mec* types I to IV by detecting the eight loci on SCC*mec* types using eight pairs of primers and one internal primer for *mec*A gene detection (Oliveira & de Lencastre, 2002). The primers to detect SCC*mec* types IVa and IVb were designed by Okuma *et al.* (2002) (Okuma *et al.*, 2002). Hisata *et al.* (2005)

developed multiplex PCR to identify SCC*mec* types IIa, IIb, IVc and IVd (Hisata *et al.*, 2005). Two different multiplex PCR were developed by Zhang *et al.* (2005) and Milheirico *et al.* (2007b) to characterize SCC*mec* types I to VI. Zhang *et al.* (2005) uses nine pairs of primers to target SCC*mec* types I-III, IVa, IVb, IVd and V as well as *mec*A gene. Milheirico *et al.* (2007b) uses 10 pairs of primers to identify SCC*mec* types I to VI (Milheirico *et al.*, 2007b; Zhang *et al.*, 2005). Kondo *et al.* (2007) developed a PCR scheme which allows for the detection of SCC*mec* types I to IX except for SCC*mec* types VII and X by combining six multiplex PCR (M-PCR) reactions namely; M-PCR 1 for *ccr* type and *mec*A gene amplification, M-PCR 2 for *mec* A, B and C2 class amplification, M-PCR 3 and M-PCR 4 for open reading frames (ORFs) from J1 region of SCC*mec* types I and V amplification, M-PCR 5 and M-PCR 6 for gene alleles in J2 and J3 regions, respectively. In addition, both M-PCR 5 and M-PCR 6 are also used for Tn554 and pT181 identification, respectively (Kondo *et al.*, 2007). New SCC*mec* type V variants (5C2 & 5) were detected using primers for *ccrC1* allele 2 and c*crCI* allele 8, *orf*33 and *orf*35 and *mec* class C2 variant provided by Higuchi *et al.* (2008) (Higuchi *et al.*, 2008). However, the disadvantages of SCC*mec* typing have also been reported. For example, the complexity of this system since SCC*mec* region is variable and the newly SCC*mec* types are permanently being defined (Szabó, 2014).

PFGE is used for short term local epidemiological studies as well as outbreak investigations. It provides global chromosomal overview scanning for more than 90% of chromosomes and is used to separate DNA fragments up to approximately 10Mb. However, any single detectable change in the chromosome would results in at least two band position differences. In brief, cell suspension of MRSA strains are embedded in agar, lyzed and large DNA fragments are generated from the digestion of agarose plugs by restriction enzyme such as *Sma*I. Separation of DNA fragments are done by

electrophoresis using an electrical field of alternating polarity as well as other parameters such as temperature and agarose concentration. The resultant banding patterns are analyzed using BioNumerics software which generated dendrograms based on the Dice coefficient and unweighted pair group matching analysis (UPGMA) (Bio-Rad, n.d; Enright *et al.*, 2000; Singh *et al.*, 2006; Szabó, 2014).

MLST is used for long-term global epidemiological studies and to assess the evolutionary relationships among the MRSA strains. It assesses the sequences of 450bp internal fragments of seven housekeeping genes (*arc*, *aro*, *glp*, *gmk*, *pta*, *tpi* and *ygi*). The sequences of the genes are compared to the reference databases (http://www.mlst.net) and an allelic profile or sequence type (ST) is obtained. The eBURST software (http://eburst.mlst.net/v3/mlst_datasets/) assigns MRSA strains in a clonal complex (CC) when they shared six of the seven alleles. The disadvantages of MLST include it cannot be used for infection control or outbreak investigations due to high cost. In addition, phylogenetic relationship and clones resolution can be masked by using slow evolving housekeeping genes (Enright *et al.*, 2000; Obert *et al.*, 2007; Robinson & Enright, 2004; Szabó, 2014).

2.2.2 Epidemiology of MRSA in Malaysia and worldwide

In 1961, MRSA isolates were first identified in the UK and also recovered from Europe, Australia and the US from the 1960s to the early 1970s (David & Daum, 2010; Enright *et al.*, 2002). North and South America, Asia and Malta are among the regions reported with the highest incidence of MRSA. Intermediate rates of 25-50% were reported in China, Africa, Australia, Italy, Greece, Portugal and Romania (Hand in Scan, 2014). In the US and UK, the MRSA infections reported a 62% increase from 1999 to 2005 (Klein *et al.*, 2007) and 40% from 1999 to 2002 (Giannoudis *et al.*, 2005), respectively.

The prevalence of MRSA in Malaysia ranges from 17% in 1986 to 44.1% in 2007 (Lim *et al.*, 2013). These variations can be explained by the different populations, environmental control and interpretation guidelines (Chen & Huang, 2014).

MRSA is globally distributed with HA-MRSA strains arising from the 1960s and CA-MRSA strains from the 1990s. In Korea, the average MRSA rates remained above 70% from 1998 to 2011 whereas in Japan, the rates of HA-MRSA infections decreased from 71.6% in 2001 to 41% in 2011. Proper hand hygiene practices, isolation measures, antibiotic stewardship and surveillance programs were considered as the factors for the decrease in the MRSA infections (Chen & Huang, 2014). Molecular epidemiology studies have reported that SCC*mec* type III-ST239 as the predominant HA-MRSA clone in Asia and SCC*mec* type II-ST5 as the dominant HA-MRSA clone in Japan, Korea, China, Hong Kong, Taiwan, Europe and North America (Chuang & Huang, 2013). In a Malaysian study on MRSA isolates from 2003 and 2008, SCC*mec* type III-MLST type ST239 was reported to be the predominant clone (Lim *et al.*, 2013) which was similar to the report by Ghaznavi-Rad *et al.* (2010a) where majority of the MRSA strains in a tertiary hospital in Malaysia (HKL) belonged to SCC*mec* type III-MLST ST239 (Ghaznavi-Rad *et al.*, 2010a).

The emergence and outbreaks of CA-MRSA were reported worldwide. In the US, Canada and Europe, ST1 (USA400), ST8 (USA300) and ST80 were mostly reported. ST59 was reported in the Asia-Pacific region (Taiwan and Australia), SCC*mec* type IV-ST45 in Hong Kong, SCC*mec* type IV-ST30 was reported worldwide including in the US, Europe and Japan; and SCC*mec* type IV-ST22 in Southeast Asia. In Korean hospitals, CA-MRSA clone SCC*mec* type IV-ST72 accounts for significant fraction of healthcare-associated infections (Chuang & Huang, 2013). In Malaysia, a study conducted by Ahmad

et al. (2009) in 2006 to 2008 has shown that majority of the CA-MRSA belonged to *SCCmec* type IV-ST30. Lim *et al.* (2013) reported that one CA-MRSA strain in UMMC belonged to SCC*mec* V-ST772 and Sam *et al.* (2008) also stated the multi-sensitive CA-MRSA strains in the Malaysian hospital belonged to SCC*mec* IV-ST22, ST6, ST30, ST1178 and ST1179 (Ahmad *et al.*, 2009; Lim *et al.*, 2013; Sam *et al.*, 2008).

HA- and CA-MRSA strains were reported to carry PVL genes in several epidemiological studies. For instance, a study in Minnesota in 2000, reported 77% of CA-MRSA isolates and 4% of HA-MRSA isolates were PVL-positive. From 2004 to 2005, the University of Chicago reported 35.9% of HA-MRSA isolates were PVL-positive (David & Daum, 2010). PVL-positive strains were found carrying SCC*mec* type IV as indicated by Vandenesch *et al.* (2003), Diep *et al.* (2004) and David *et al.* (2008) (David *et al.*, 2008; Diep *et al.*, 2004; Vandenesch *et al.*, 2003). PVL-positive SCC*mec* type IV-ST1 isolates were first reported in the US. In addition, PVL-carrying CA-MRSA strains were noted to cause SSTIs, necrotizing pneumonia and bacteraemia (Vandenesch *et al.*, 2003).

In Japan, the first CA-MRSA isolate that carried PVL gene was reported in 2003 (David & Daum, 2010). In Taiwan, PVL-positive CA-MRSA strains were found in children whom had SSTIs (Huang *et al.*, 2007). In Singapore, PVL-positive MRSA isolates belonging to SCC*mec* type IV-ST30 and SCC*mec* type V-ST8 were reported (Hsu *et al.*, 2006). In Malaysia, PVL gene is common among SCC*mec* type V-ST1 and SCC*mec* type V-ST188 CA-MRSA strains (Ghaznavi-Rad *et al.*, 2010a). Based on previous studies conducted by Lim *et al.* (2013) and Ghaznavi-Rad *et al.* (2010a), the prevalence of PVL gene in UMMC and HKL were 2% (Lim *et al.*, 2013) and 4.9% (Ghaznavi-Rad *et al.*, 2010a), respectively.

2.2.3 Antimicrobial treatments for MRSA

Antimicrobials agents such as vancomycin, linezolid, daptomycin, quinupristin-dalfopristin, tigecycline, ceftobiprole, ceftaroline fosamil, telavancin, gentamicin, trimethoprim-sulfamethoxazole (TMP/SMX) and clindamycin are used for the treatment of MRSA infections (David & Daum, 2010; Kurosu *et al.*, 2013). Carroll (2013b) reported that daptomycin, linezolid and quinupristin-dalfopristin are used as treatments for MRSA bacteraemia, pneumonia, SSTIs and endocarditis. In addition, ceftaroline has been approved to treat SSTIs and community-acquired pneumoniae (Carroll, 2013b). Ceftobiprole, on the other hand, is reported to have a promising role in treating hospital-acquired pneumonia (Edwards *et al.*, 2014) whereas TMP/SMX and clindamycin are used for community-acquired SSTIs (Moon, 2009). Gentamicin is reported to be used in combination of cloxacillin and benzylpenicillin, rifampicin or vancomycin to treat endocarditis (University Malaya Medical Centre, 2014). Tigecycline is used for complicated skin and intra-abdominal infections and the recently approved telavancin is used to treat skin infections (Rasmussen *et al.*, 2011).

Based on the National Antibiotic Guidelines 2014, erythromycin, gentamicin, co-trimoxazole, rifampicin, tetracycline, fusidic acid, ciprofloxacin, clindamycin, vancomycin and linezolid are the commonly used antibiotics from year 2008 to 2013 (Ministry of Health Malaysia, 2014). However, Thong *et al.* (2009) has reported high resistance of MRSA to TMP/SMX (73%), tetracycline (47%), erythromycin (92%), gentamicin (76%), netilmicin (24%), ciprofloxacin (94%), rifampicin (12%), clindamycin (18%) and fusidic acid (11%) (Thong *et al.*, 2009).

Vancomycin, a glycopeptide antibiotic remains as the main drug used for treating severe MRSA infections. Vancomycin inhibits the early stages of cell wall peptidoglycan biosynthesis by binding to the -peptidyl–D-Ala–D-Ala sequence on the bacterial cell wall in which disrupts the transglcosylase and transpeptidase enzyme responsible for the cell wall construction (Dengler *et al.*, 2011).

Since 1980s, there has been an increasing use of vancomycin in the US as well as in several countries. Subsequently, in 1997, the first MRSA strain with reduced vancomycin susceptibility was reported in Japan (Gardete & Tomasz, 2014). In the US and several other countries, vancomycin intermediate-resistant *S. aureus* (VISA) strains were observed among patients that have complex infections with prolonged vancomycin treatments (Carroll, 2013b). The mechanisms of VISA strains are delineated by the bacterial cell wall thickening and reduced cross-linking that prevents vancomycin from entering the cell by trapping vancomycin within the layers of cell wall peptidoglycan (Reygaert, 2013). VISA strains are susceptible to quinupristin-dalfopristin and oxazolidinones (Carroll, 2013b). In 2002, vancomycin-resistant *S. aureus* (VRSA) was first reported (Gardete & Tomasz, 2014). VRSA resistance is mediated by the acquisition of enterococcal *van*A gene from enterococci which decreases the binding affinity of vancomycin by modifying the peptidoglycan precursor structure, changing a D-alanine-D-alanine structure to D-alanine-D-lactate (Reygaert, 2013). According to the National Antibiotic Guideline 2014 and the National antibiotic resistance surveillance report 2016, no VRSA was reported in Malaysia (Institute for Medical Research, 2016; Ministry of Health Malaysia, 2014).

Several studies have demonstrated the existence of vancomycin MIC creep. However, reports on its associations with clinical outcomes are limited and contradictory. Vancomycin MIC creep refers to the increase in vancomycin MIC within a susceptible range. Based on the study by Wang *et al.* (2006) in the US, the presence of vancomycin MIC creep was noted by a shift in the vancomycin MICs from ≤ 0.5 to 1µg/mL during the 5-year period (from 2000 to 2004). The percentages of isolates with MIC 1µg/mL were significantly higher in 2004 (70.4%) compared to 2000 (19.9%) (Wang *et al.*, 2006). Yeh *et al.* (2012) reported the existence of vancomycin MIC creep among bacteraemic MRSA isolates in Taiwan. However, there was no significant association between in-hospital mortality and high vancomycin MICs (Yeh *et al.*, 2012). In Hong Kong, Ho *et al.* (2010) found vancomycin MIC creep among blood MRSA isolates from 1997 to 2008 (Ho *et al.*, 2010). Ahmad *et al.* (2010) has stated that MRSA strains causing bacteraemia in Malaysia were observed to have high vancomycin MIC (2µg/mL). However, there is no data available to support the presence of vancomycin MIC creep as well as vancomycin treatment failure and clinical outcomes with high vancomycin MICs (Ahmad *et al.*, 2010).

CHAPTER 3: MATERIALS AND METHODS

3.1 Ethics

The Medical Ethic Committee of University Malaya Medical Centre had approved this study on the 7th of June 2014 (MEC ID: 20145-168).

3.2 Materials

3.2.1 Bacterial strains

This study was a single centre retrospective cohort study where adult patients (16 years of age and older) who fulfilled the criteria for MRSA infection clinically were included. A total of 278 MRSA clinical strains were collected from the microbiology diagnostic laboratory in UMMC. The MRSA strains were isolated from sterile sites including blood, tissue, cerebrospinal fluid (CSF), bone, synovial fluid and pus in January 2011 to December 2013. The details of the strains are listed in Appendix A. The strains were identified as *S. aureus* and MRSA using standard microbiological methods according to standard operating procedures (SOPs) by laboratory staff of the microbiology diagnostic laboratory. All the clinical specimens were cultured on blood and MacConkey agar and the selected *S. aureus* colonies growing on blood agar (presence of β-haemolysis with golden yellow colonies) after overnight incubation at 35°C, were further tested with DNase, tube coagulase and cefoxitin disk diffusion test. The strains with positives result on the tube coagulase and DNase tests were identified as *S. aureus*. Furthermore, the strains with zone diameter of ≤ 21mm for cefoxitin were identified as MRSA and confirmed with PCR-targeting *mec*A gene.

Upon receiving the cultures, the strains were confirmed as *S. aureus* by PCR-targeting *fem*A gene and were stored in Tryptic Soy Agar (TSA) stab culture at room temperature as well as in Tryptic Soy Broth (TSB) containing 50% glycerol at both -20^{o}C and -80^{o}C. Antimicrobial susceptibility testing and molecular characterization by SCC*mec* typing and the detection of PVL genes were done on 278 non-replicate MRSA strains. A total of 158 MRSA strains isolated from blood were further typed by PFGE and MLST.

Reference strains including NCTC10442 (SCC*mec* type I), N315 (SCC*mec* type II), 85/2082 (SCC*mec* type III), JCSC4744 (SCC*mec* type IVa), JCSC2172 (SCC*mec* type IVb), MR108 (SCC*mec* type IVc), JCSC4469 (SCC*mec* type IVd) and WIS (SCC*mec* type V) were used as positive control strains for SCC*mec* typing, PVL genes detection and PCR assay targeting *fem*A and *mec*A genes. *Salmonella* serotype Braenderup strain (H9812) was used as reference strain for PFGE. All the control strains were kindly provided by Prof Thong Kwai Lin, University of Malaya.

3.2.2 Consumables, chemicals and reagents

Table 3.1: List of media, agarose, buffers and solutions that were used in this study. All media, buffers and solutions preparation is described in Appendix B.

Name	Source
Media, agarose and buffers	
Tryptic Soy Agar	BD Difco™, New Jersey, USA
Tryptic Soy Broth	BD Bacto™, New Jersey, USA
Seakem gold agarose	Lonza Walkersville, MD, USA
Agarose, LE, Analytical Grade	Promega, Madison, WI, USA
EDTA Disodium salt	
Boric acid	
Tris Base	
N-Lauroylsarcosine sodium salt	Sigma-Aldrich, St Louis, MO, USA
Sodium chloride	
Type 1 agarose, low EEO	
Lysostaphin	
Solutions	
Glycerol	Promega, Madison, WI, USA
Proteinase K (10 mg/mL)	
SYBR Safe DNA gel stain	Life Technologies, Carlsbad, CA, USA
Lysozyme solution (10 mg/mL)	Sigma-Aldrich, St Louis, MO, USA
Hydrochloric acid	
Sodium hydroxide	
Absolute ethanol	
95% ethyl alcohol	
GelRed Nucleic Acid Stain	Biotium, Fremont, CA, USA

3.2.3 Restriction enzymes and DNA molecular weight marker

Table 3.2: Restriction enzymes *Sma*I and *Xba*I for PFGE and BenchTop 100bp DNA Ladder as DNA molecular weight marker.

Name	Source
*Sma*I • Buffer J, 10X • Bovine Serum Albumin (BSA) (Acetylated) • MULTI-CORE Buffer, 10X • *Sma*I restriction enzyme	Promega, Madison, WI, USA
*Xba*I • Buffer D, 10X • BSA • MULTI-CORE Buffer, 10X • *Xba*I restriction enzyme	
BenchTop 100bp DNA Ladder	

3.2.4 Primers

All commercially synthesized primers (Table 3.3) were purchased from Integrated DNA Technologies, USA.

Table 3.3: Details of the primers used in this study.

Name of the primer	Primer sequence (5' →3')	Specificity	Product length (bp)	References
SCC*mec* typing				
CIF2-F2	TTCGAGTTGCTGATGAAGAAGG	I	495bp	(Milheirico *et al.*, 2007b)
CIF2-R2	ATTTACCACAAGGACTACCAGC			
*ccr*C-F2	GTACTCGTTACAATGTTTGG	V	449bp	
*ccr*C-R2	ATAATGGCTTCATGCTTACC			
RIF5-F10	TTCTTAAGTACACGCTGAATCG	III	414bp	
RIF5-R13	ATGGAGATGAATTACAAGGG			
SCC*mec*-V-J1-F	TTCTCCATTCTTGTTCATCC	V	377bp	
SCC*mec*-V-J1-R	AGAGACTACTGACTTAAGTGG			
dcs-F2	CATCCTATGATAGCTTGGTC	I, II, IV, VI	342bp	
dcs-R1	CTAAATCATAGCCATGACCG			
*ccr*B2-F2	AGTTTCTCAGAATTCGAACG	II, IV	311bp	
*ccr*B2-R2	CCGATATAGAAWGGGTTAGC			
kdp-F1	AATCATCTGCCATTGGTGATGC	II	284bp	
kdp-R1	CGAATGAAGTGAAAGAAAGTGG			
SCC*mec*-III-J1-F	CATTTGTGAAACACAGTACG	III	243bp	
SCC*mec*-III-J1-R	GTTATTGAGACTCCTAAAGC			
*mec*I P2	ATCAAGACTTGCATTCAGGC	II, III	209bp	
*mec*I P3	GCGGTTTCAATTCACTTGTC			
*mec*A P4	TCCAGATTACAACTTCACCAGG	Internal control	162bp	
*mec*A P7	CCACTTCATATCTTGTAACG			

Table 3.3, continued

Name of the primer	Primer sequence (5' →3')	Specificity	Product length (bp)	References
4a1	TTTGAATGCCCTCCATGAATAAAAT	IVa	458bp	(Zhang *et al.*, 2005)
4a2	AGAAAAGATAGAAGTTCGAAAGA			
4b1	AGTACATTTTATCTTTGCGTA	IVb	994bp	
4b2	AGTCATCTTCAATATCGAGAAAGTA			
4c1	TCTATTCAATCGTTCTCGTATTT	IVc	678bp	
4c2	TCGTTGTCATTTAATTCTGAACT			
4d1	TTTGAGAGTCCGTCATTATTTCTT	IVd	1010bp	
4d2	AGAATGTGGTTATAAGATAGCTA			
PVL gene				
luk-PV-1	ATCATTAGGTAAAATGTCTGGACATGATCCA	PVL	433bp	(Lina *et al.*, 1999)
luk-PV-2	GCATCAASTGTATTGGATAGCAAAAGC			
MLST				
arc up	TTGATTCACCAGCGCGTATTGTC	arc	456bp	(Enright *et al.*, 2000)
arc dn	AGGTATCTGCTTCAATCAGCG			
aro up	ATCGGAAATCCTATTTCACATTC	aro	456bp	
aro dn	GGTGTTGTATTAATAACGATATC			
glp up	CTAGGAACTGCAATCTTAATCC	glp	465bp	
glp dn	TGGTAAAATCGCATGTCCAATTC			
gmk up	ATCGTTTTATCGGGACCATC	gmk	429bp	
gmk dn	TCATTAACTACAACGTAATCGTA			
pta up	GTTAAAATCGTATTACCTGAAGG	pta	474bp	
pta dn	GACCCTTTTGTTGAAAAGCTTAA			
tpi up	TCGTTCATTCTGAACGTCGTGAA	tpi	402bp	
tpi dn	TTTGCACCTTCTAACAATTGTAC			
ygi up	CAGCATACAGGACACCTATTGGC	ygi	516bp	
ygi dn	CGTTGAGGAATCGATACTGGAAC			

3.2.5 Commercial Kits for PCR typing and Purification of PCR products

Table 3.4: Kits used in this study for PCR and MLST.

Name	Source
GoTaq® Flexi DNA Polymerase • 5X Green and Colourless Buffer • $MgCI_2$ solution • Taq Polymerase • dNTP mix	Promega, Madison, WI, USA
MEGAquick-spin™ Plus Total Fragment DNA Purification Kit	Intron Biotechnology, Korea

3.2.6 Software

Table 3.5: List of software.

Software	Source
BioRad Quantity One Software	Bio-Rad, USA
BioNumerics 6.5	Applied Maths, Inc, USA
VassarStats	http://vassarstats.net/
MLST	http://saureus.beta.mlst.net/
eBURST algorithm	http://eburst.mlst.net/v3/instructions/version3.asp
APACHE II Calculator	http://clincalc.com/icumortality/apacheii.aspx
Charlson comorbidity index (CCI) calculator	https://www.thecalculator.co/health/Charlson-Comorbidity-Index-(CCI)-Calculator-765.html
Simpson's Index of diversity	http://www.comparingpartitions.info/index.php?link=Tool

3.3 Methods

3.3.1 Antimicrobial Susceptibility Testing

The vancomycin minimum inhibitory concentration (MIC) results were obtained from the hospital's microbiology diagnostic laboratory database and medical records unit. The test was done using E-test and the MIC results were interpreted according to the CLSI guidelines (Clinical and Laboratory Standard Institute, 2017).

The antimicrobial susceptibility pattern of 2013 strains (except for one strain) for penicillin (PEN), erythromycin (ERY), gentamicin (GEN), cefoxitin (FOX), rifampicin (RIF), fusidic acid (FUS), cotrimoxazole (CMX), ciprofloxacin (CIP), clindamycin (CLI), ceftriaxone (CRO), amoxicillin-clavulanate (AMC), colistin (CST), piperacillin-tazobactam (TZP), ceftazidime (CAZ) and ampicillin-sulbactam (SAM) were also collected from the hospital's microbiology diagnostic laboratory database. Unfortunately, the susceptibility pattern for all year 2011 and 2012 MRSA strains could not be retrieved from the hospital's database.

3.3.2 DNA Extraction

Crude genomic DNA was extracted by using simple boiling method. One loopful of bacterial colonies was picked and suspended in 100 µL of ddH_2O in a 1.5mL microcentrifuge tube. It was boiled at 99^oC for 10 minutes and snap-cooled on ice for 5 minutes. The cell lysates were centrifuged at 13,000 rpm for 10 minutes and stored in -20^oC. DNA purity and concentration were measured using NanoDrop™ spectrophotometer and approximately 20 ng of DNA were used for several PCR analyses.

3.3.3 SCC*mec* typing

SCC*mec* PCR typing was performed as described by Milheirico *et al.* (2007b) with slight modification and optimized by using positive control strains. The assay was performed in thermal cycler (Life Technologies, USA). The optimal cycling conditions for SCC*mec* typing were as followed; 94°C for 4 minutes, 30 cycles of 94°C for 30 seconds, 53°C for 30 seconds and 72°C for 1 minute and a final extension at 72°C for 4 minutes. The PCR mixtures with a final volume of 25 µL contained of 20 ng of template, 1X PCR buffer with 1.5mM $MgCI_2$, 40 µM dNTPs, 0.2 µM primers kdp F1 and kdp R1; 0.4 µM primers CIF2 F2, CIF2 R2, RIF5 F10, RIF5 R13, SCC*mec* III J1F, SCC*mec* III J1R, SCC*mec* V J1 F, and SCC*mec* V J1 R; 0.8 µM primers *mec*I P2, *mec*I P3, dcs F2, dcs R1, *mec*A P4, *mec*A P7, *ccr*B2 F2, *ccr*B2 R2, *ccr*C F2, and *ccr*C R2 and 1.0 U Taq DNA polymerase (Milheirico *et al.*, 2007b). The PCR products were analyzed in 2% agarose LE gel with SYBR safe gel stain in 0.5X Tris-borate-EDTA (TBE) buffer at 120V for 1 hour and visualized.

For SCC*mec* type IV subtyping, the optimal cycling conditions were as described by Milheirico *et al.* (2007a) as followed; 94°C for 4 minutes, 35 cycles of 94°C for 30 seconds, 48°C for 30 seconds and 72°C for 2 minutes and a final extension at 72°C for 4 minutes (Milheirico *et al.*, 2007a). The PCR mixtures were as described by Zhang *et al.* (2005) with a final volume of 25 µL containing 20 ng of template, 1X PCR buffer with 1.5mM $MgCI_2$, 40 µM dNTPs, 0.104 µM primers 4a1 and 4a2; 0.092 µM primers 4b1 and 4b2; 0.078 µM primers 4c1 and 4c2 and 0.28 µM 4d1 and 4d2 and 1.0 U Taq DNA polymerase (Zhang *et al.*, 2005).

3.3.4 PCR-based assay for PVL gene

The detection of PVL gene was performed as described by Holmes *et al.* (2005) with slight modification. Standardization of the PCR was done by the optimization of the following reaction components: the primers, $MgCI_2$ and the dNTPs. Once the PCR conditions were optimized, the assay was performed on all the strains. The optimal cycling conditions were as followed; 95°C for 5 minutes, 30 cycles of 95°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes and a final extension at 72°C for 5 minutes (Holmes *et al.*, 2005). The PCR mixture with a final volume of 25 µL contained of 20 ng of template, 1X PCR buffer with 1.5mM $MgCI_2$, 40 µM dNTPs, 0.08 µM primers *luk-PV-1* and *luk-PV-2* and 1.0 U Taq DNA polymerase.

3.3.5 Multilocus sequence typing (MLST)

MLST was performed as previously described by Enright *et al.* (2000). The optimal cycling conditions of 95°C for 5 minutes, followed by 30 cycles of 95°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute and a final extension at 72°C for 5 minutes were used (Enright *et al.*, 2000). The PCR mixture with a final volume of 25 µL contained of 20ng of template, 1X PCR buffer with 1.5mM $MgCI_2$, 40 µM dNTPs, 0.4 µM primers (as listed in Table 3.3) and 1.0 U Taq DNA polymerase.

The representative PCR products were purified by using MEGAquick-spin Total Fragment DNA Purification Kit and the purified products were sent for sequencing. The gene sequences were analyzed and the sequence types (STs) were assigned by comparing the sequences of each locus with those in the *S. aureus* MLST database (http://saureus.mlst.net). Examples of allelic profiles query for MLST and database query are shown in Appendix C and D, respectively. The list of STs for MRSA strains from blood in Malaysia is also described in Appendix E.

3.3.6 Pulsed-field gel electrophoresis (PFGE)

PFGE was performed according to the CDC PulseNet protocol with slight modification (Centers for Disease Control and Prevention, 2008). A single colony was streaked on TSA and incubated at 37ºC for overnight. The bacterial culture was transferred to 1mL cell suspension buffer (CSB) and turbidity was adjusted to OD = 0.73-0.87. An aliquot of 100µL of bacterial cell suspension was transferred to a 1.5mL microcentrifuge tube. Four microlitre of lysostaphin (1mg/mL) and 15µL of lysozyme (10mg/mL) were added into the suspension. The suspension was incubated at 37ºC for 30 minutes. Then, one microlitre of proteinase K (20mg/mL) was added into the suspension followed by 100µL of 1% Seakem Gold agarose. The cell suspension was

dispensed into the plug mold to form plugs. The plugs were added into cell lysis buffer (CLB) and incubated at 54°C for 3 hours to lyse the bacterial cells. The plug was washed with sterile ddH_2O (2 times) and sterile TE buffer (6 times). For digestion, 2mm slices of the agarose plugs were incubated overnight with *Sma*I enzyme supplemented with BSA at room temperature. As for the reference strain *Salmonella* serotype Braenderup, the plug slice was incubated overnight at 37°C with *Xba*I enzyme supplemented with BSA. The plug slices were loaded onto 1% Type 1 agarose gel and separated on CHEF MAPPER in 0.5X TBE as running buffer at 14°C. The electrophoresis conditions were as followed; 6V/cm gradient, a run time of 22 hours, an initial switch time of 5 seconds and a final switch time of 60 seconds. The gel was stained in GelRed dye for 30 minutes and visualized using BioRad GelDoc XR. BioNumerics 6.5 Software package was used for cluster analysis using UPGMA with a tolerance of 1.5% and optimization of 1%. The PFGE profile was assigned an arbitrary designation and the Dice coefficient of similarity, *F* defined the differences. A similarity cutoff of 80% in a dendrogram was commonly selected to define a PFGE cluster.

3.3.7 Clinical data collection

Patient's demographic and clinical data were extracted from the hospital's medical record unit. An example of patient's clinical data form is shown in Appendix F. The following information was of those that were included in the data extraction process. Patient's demographic information such as registration number (RN), date of birth, age, ethnicity, sex and profession. Clinical data consisting of admission and discharge date, ICU admission and discharge date (if present), date of positive MRSA culture, onset of infection (HA- or CA- MRSA), ward and risk factors (presence of medical devices, previous hospitalization, comorbidities and previous antibiotic exposure in the last 90 days). In addition, the severity of infection scores (Acute Physiology and Chronic Health

Evaluation (APACHE) II, Pitt bacteraemia and Charlson Comorbidity Index (CCI)), empirical antibiotic regimens, infection data (site of infection such as bacteraemia, SSTIs, surgical site infection or pneumonia; and type of source including pus, line, catheter or implant), treatment details (type of anti MRSA given and duration of treatment) and treatment outcome (status of bacteraemia and mortality status) were also collected. Clinical diagnosis of bacteraemia, skin and soft tissue infections, osteomyelitis, meningitis, pericarditis or other infection was assigned to each case according to the documentation of such diagnosis in the admission or discharge summary or based on the sterile site from which MRSA was isolated.

Based on the CDC definitions, HA-MRSA is defined as positive culture obtained more than 48 hours after admission, who had a history of hospitalization, dialysis, surgery or residence in a long-term healthcare facility within six months prior to the culture date and those with indwelling catheter or any medical device at the time culture was taken. CA-MRSA is defined as positive culture obtained within 48 hours of admission without HA risk factors (David & Daum, 2010; Gorwitz *et al.*, 2006; Huang *et al.*, 2006; Kumari *et al.*, 2016). For this study, MRSA infections were identified as HA- or CA- MRSA according to the information collected from patient's clinical notes and the Infection Control Department's database on multidrug-resistant organisms (MDROs). Based on these two sources, the risk factors for HA- and CA- MRSA infections were identified with reasonable accuracy.

Bacteremia is defined as at least one positive blood culture with or without systemic manifestations of infection (Ok *et al.*, 2013) and is designated as primary bacteremia when no infection focus is identified (Laupland, 2013). Catheter-related bloodstream infection is defined as semi-quantitative culture of the catheter tip that yielded more than 15 MRSA colony-forming units (Ok *et al.*, 2013) or growth of more than 10^2 MRSA colony forming units by quantitative culture of the peripheral vein blood sample drawn from a catheter hub (Mermel *et al.*, 2009). Patients with more than one infecting organisms isolated from blood were considered to have polymicrobial infection (Lenz *et al.*, 2012). Whereas, blood culture that yielded only MRSA is defined as mono infection. Persistent MRSA bacteraemia is defined as positive blood culture taken ≥ 7 days after the index positive blood culture (Ok *et al.*, 2013). On the other hand, recurrent bacteraemia refers to the return of MRSA bacteraemia two weeks after the report of negative blood cultures (Welsh *et al.*, 2011). The major patient outcome was all-cause mortality.

Heart rate, respiratory rate, blood pressure, body temperature, glasgow coma score, chemistry (arterial blood gases, sodium, potassium, baseline serum creatinine, estimated glomerular filtration rate and albumin) and blood (haematocrit, haemoglobin, WBC and platelet counts) tests results were recorded for the purpose of infection severity scores calculation. The purpose of calculating the APACHE II and CCI scores are to determine the prognosis or severity of the disease and to categorize the comorbidities, respectively. Both scores can be used to predict mortality (Naved *et al.*, 2011; Rhee *et al.*, 2009). Pitt bacteraemia score is used to evaluate the severity of illness in bacteraemic patient (Feldman *et al.*, 2009).

3.3.8 Statistical analysis

Statistical analysis was performed to correlate the phenotypic, molecular and clinical data as well as to identify the risk factors. Categorical variables were compared using Fisher's exact test or the Pearson's chi-square test, where appropriate. Logistic regression was used to calculate the odds ratio (OR) and 95% confidence interval (CI). All reported *p* values are two-tailed and analyses were performed using VassarStats software (http://vassarstats.net/). Variables with $p < 0.05$ were considered to be statistically significant.

CHAPTER 4: RESULTS

4.1 Bacterial strains

Two hundred seventy-eight MRSA strains were collected in this study (95 from year 2011, 114 from year 2012 and 69 from year 2013) (Table 4.1). The strains were isolated from various clinical specimens including blood (n = 158; 56.8%), tissues (n = 81; 29.1%), CSF (n = 3; 1.1%), pus, slough and abscess (n = 19; 6.8%), pericardial fluid (n =1; 0.4%), bullae fluid (n = 1; 0.4%), synovial fluid (n = 4; 1.4%) and bone (n = 11; 3.9%) (Figure 4.1).

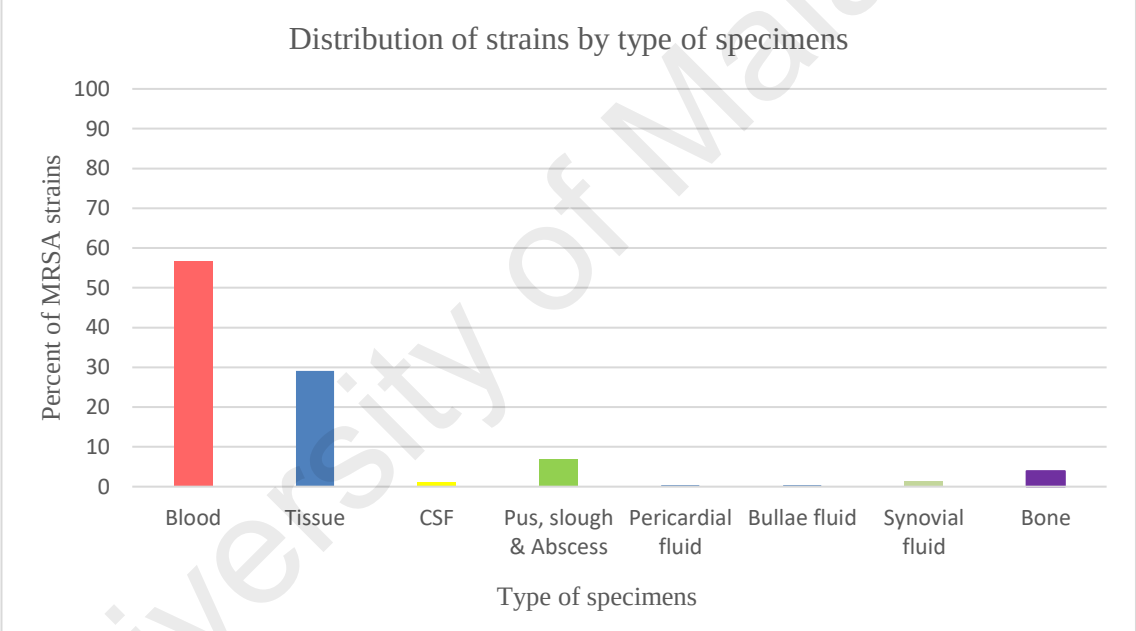

Figure 4.1: Distribution of the MRSA strains based on type of specimens.

The median age was 59 years old which ranged from 16 to 92 years old. A total of 172 (61.9%) strains were collected from male, while 102 (36.7%) were collected from female. MRSA infections were significantly decreased in patients ≤ 50 years old (41.1% in 2011 vs 23.7% in 2012 vs 20.3% in 2013) (p = 0.005) while an increased can be observed in patients > 50 years old (54.7% in 2011 vs 75.4% in 2012 vs 78.3% in 2013) (p = 0.001) during the 3-year study period. Patients between the age group of 51 to 92

years old ($p < 0.0001$) and male gender ($p < 0.0001$) were the significant risk factors in the acquisition of MRSA (Table 4.1).

Sixty-five percent (181 of 278) of MRSA strains were HA-MRSA while 26.9% (75 of 278) were CA-MRSA (Figure 4.2). HA-MRSA ($p < 0.0001$) infections were significantly increased while CA-MRSA ($p < 0.0001$) type infections significantly decreased from year 2011 to 2013 (Table 4.1). Some MRSA strains have incomplete clinical data including unknown age, gender, type of MRSA, comorbidities and clinical outcome as the patient's medical records could not be retrieved from the archived records; which is a limitation in this study.

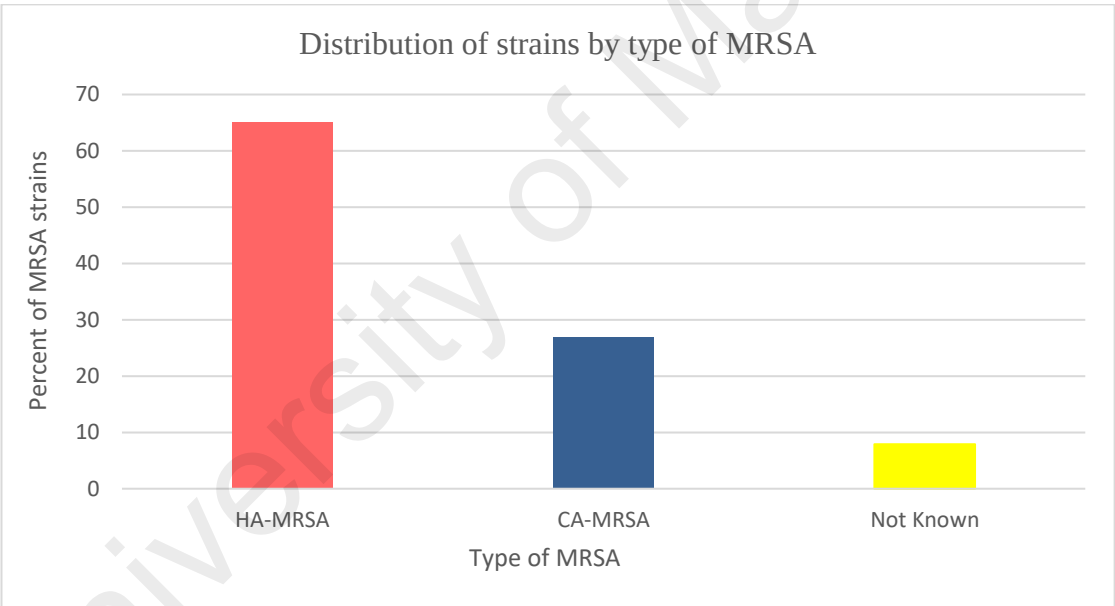

Figure 4.2: Distribution of the MRSA strains based on type of MRSA.

Table 4.1: Comparisons of patients' demographics, phenotypic and genotypic characteristics between MRSA strains from year 2011 to 2013.

	2011 N = 95 (%)	2012 N = 114 (%)	2013 N = 69 (%)	*P* value	Total N = 278 (%)	*P* value
Age						
≤ 50 years old	39 (41.1)	27 (23.7)	14 (20.3)	0.005*	80 (28.8)	< 0.0001*
> 50 years old	52 (54.7)	86 (75.4)	54 (78.3)	0.001*	192 (69.1)	
Not Known	4 (4.2)	1 (0.9)	1 (1.4)		6 (2.2)	
Gender						
Female	30 (31.6)	44 (38.6)	28 (40.6)	0.432	102 (36.7)	< 0.0001*
Male	62 (65.3)	70 (61.4)	40 (57.9)	0.645	172 (61.9)	
Not Known	3 (3.2)	0 (0)	1 (1.4)		4 (1.4)	
Clinical specimens						
Blood	40 (42.1)	51 (44.7)	67 (97.1)	< 0.0001*	158 (56.8)	
Tissue	37 (38.9)	42 (36.8)	2 (2.9)	< 0.0001*	81 (29.1)	
CSF	3 (3.2)	0 (0)	0 (0)	0.054	3 (1.1)	
Pus, slough & Abscess	8 (8.4)	11 (9.6)	0 (0)	0.014*	19 (6.8)	
Pericardial fluid	1 (1.1)	0 (0)	0 (0)	0.589	1 (0.4)	
Bullae fluid	0 (0)	1 (0.9)	0 (0)	0.999	1 (0.4)	
Synovial fluid	2 (2.1)	2 (1.8)	0 (0)	0.682	4 (1.4)	
Bone	4 (4.2)	7 (6.1)	0 (0)	0.101	11 (3.9)	
SCC*mec* types						
SCC*mec* I	0 (0)	0 (0)	1 (1.4)	0.248	1 (0.4)	
SCC*mec* II	2 (2.1)	0 (0)	0 (0)	0.177	2 (0.7)	
SCC*mec* III	61 (64.2)	78 (68.4)	38 (55.1)	0.189	177 (63.7)	
SCC*mec* IV	25 (26.3)	34 (29.8)	20 (28.9)	0.848	79 (28.4)	
SCC*mec* IVa	5 (5.3)	4 (3.5)	0 (0)		9 (3.2)	
SCC*mec* IVb	5 (5.3)	0 (0)	0 (0)		5 (1.8)	
Novel subtypes	15 (15.8)	30 (26.3)	20 (28.9)		65 (23.4)	
SCC*mec* V	6 (6.3)	1 (0.9)	9 (13)	0.001*	16 (5.8)	
Untypeable	1 (1.1)	1 (0.9)	1 (1.4)	0.999	3 (1.1)	

Table 4.1, continued

	2011 N = 95 (%)	2012 N = 114 (%)	2013 N = 69 (%)	*P* value	Total N = 278 (%)	*P* value
Type of MRSA						
HA-MRSA	44 (46.3)	79 (69.3)	58 (84.1)	< 0.0001*	181 (65.1)	
SCC*mec* I	0 (0)	0 (0)	1 (1.7)	0.564	1 (0.6)	
SCC*mec* II	2 (4.5)	0 (0)	0 (0)	0.058	2 (1.1)	
SCC*mec* III	35 (79.5)	56 (70.9)	34 (58.6)	0.073	125 (69.1)	
SCC*mec* IV	7 (15.9)	22 (27.8)	16 (27.6)	0.304	45 (24.9)	
SCC*mec* IVa	1 (2.3)	3 (3.8)	0 (0)		4 (2.2)	
SCC*mec* IVb	3 (6.8)	0 (0)	0 (0)		3 (1.7)	
Novel subtypes	3 (6.8)	19 (24.1)	16 (27.6)		38 (20.9)	
SCC*mec* V	0 (0)	1 (1.3)	6 (10.3)	0.009*	7 (3.9)	<0.0001*
Untypeable	0 (0)	0 (0)	1 (1.7)	0.564	1 (0.6)	
CA-MRSA	41 (43.2)	24 (21.1)	10 (14.5)	<0.0001*	75 (26.9)	
SCC*mec* III	23 (56.1)	13 (54.2)	3 (30)	0.356	39 (52)	
SCC*mec* IV	13 (31.7)	11 (45.8)	4 (40)	0.564	28 (37.3)	
SCC*mec* IVa	4 (9.8)	1 (4.2)	0 (0)		5 (6.7)	
SCC*mec* IVb	2 (4.9)	0 (0)	0 (0)		2 (2.7)	
Novel subtypes	7 (17.1)	10 (41.7)	4 (40)		21 (28)	
SCC*mec* V	4 (9.8)	0 (0)	3 (30)	0.021*	7 (9.3)	
Untypeable	1 (2.4)	0 (0)	0 (0)	0.999	1 (1.3)	
Not Known	10 (10.5)	11 (9.6)	1 (1.4)		22 (7.9)	
SCC*mec* III	3 (30)	9 (81.8)	1 (100)		13 (59.1)	
SCC*mec* IV	5 (50)	1 (9.1)	0 (0)		6 (27.3)	
SCC*mec* V	2 (20)	0 (0)	0 (0)		2 (9.1)	
Untypeable	0 (0)	1 (9.1)	0 (0)		1 (4.5)	

Table 4.1, continued

	2011 N = 95 (%)	2012 N = 114 (%)	2013 N = 69 (%)	*P* value	Total N = 278 (%)	*P* value
PVL gene	5 (5.3)	0 (0)	0 (0)	0.005*	5 (1.8)	
Vancomycin MIC						
< 1.5µg/mL	58 (61.1)	48 (42.1)	29 (42)	0.011*	135 (48.6)	0.611
≥ 1.5µg/mL	37 (38.9)	66 (57.9)	39 (56.5)	0.014*	142 (51.1)	
Not Known	0 (0)	0 (0)	1 (1.4)		1 (0.4)	

N: number of strains; %: percent; ≤: less than or equal to; >: more than; <: less than; ≥: more than or equal to; *p* value of < 0.05 was considered to be statistical significant and indicated by asterisk (*) symbol.

4.2 Antimicrobial Susceptibility Testing

The antimicrobial susceptibility pattern for year 2013 MRSA strains is shown in Table 4.2. Low resistance rates were noted for rifampicin (2.9%) and fusidic acid (5.9%). On the contrary, resistance to penicillin (100%), cefoxitin (100%), erythromycin (88.2%), ciprofloxacin (83.8%), clindamycin (77.9%) and gentamicin (60.3%) were high. All MRSA strains from year 2013 were multidrug-resistant (MDR) in which they were resistant to more than two classes of antimicrobial agents. Fifty-six strains were resistant to five and above antimicrobial agents including penicillin, cefoxitin, ciprofloxacin, clindamycin, ceftriaxone, gentamicin, erythromycin, ceftazidime, rifampicin, fusidic acid, cotrimaxozole, colistin, amoxicillin-clavulanate and piperacillin-tazobactam (Table 4.3).

All the MRSA strains (isolated from year 2011, 2012 and 2013) were sensitive to vancomycin according to the CLSI 2017 guidelines which defined breakpoints for vancomycin MIC as follows; VSSA = ≤ 2µg/mL, VISA = 4-8 µg/mL and VRSA = ≥ 16 µg/mL (Clinical and Laboratory Standard Institute, 2017). The MIC for vancomycin ranged from 0.38 to 2 µg/mL. Out of 278 MRSA strains, 48.6% of the strains showed low vancomycin MIC (< 1.5 µg/mL) while 51.1% had high MIC (≥ 1.5 µg/mL) (Table 4.1). The MRSA strains with vancomycin MIC < 1.5 µg/mL trend appeared to decline between 2011 and 2012 (61.1% in 2011 vs 42.1% in 2012) and reached a plateau between 2012 and 2013 (42.1% in 2012 vs 42% in 2013). On the other hand, the rate of MRSA strains with vancomycin MIC ≥ 1.5µg/mL was seen to fluctuate from year to year (38.9% in 2011 vs 57.9% in 2012 vs 56.5% in 2013). The comparisons of patients' demographics, clinical diagnosis and molecular characteristics between low and high vancomycin MIC groups are shown in Table 4.4. There was no significant difference between the two vancomycin MIC groups in terms of age, gender, diagnosis, presence of comorbidities,

polymicrobial infections and type of MRSA. However, SCC*mec* type III was found to be strongly associated with high vancomycin MIC ($p < 0.0001$) while SCC*mec* type IV was strongly associated with low vancomycin MIC ($p < 0.0001$) (Table 4.4).

Table 4.2: Antimicrobial susceptibility pattern for year 2013 MRSA strains.

Antimicrobial agent	2013		
	N = 68 (%)		
	No of sensitive strains (%)	No of intermediate strains (%)	No of resistant strains (%)
Penicillin	0 (0)	0 (0)	68 (100)
Erythromycin	8 (11.8)	0 (0)	60 (88.2)
Gentamicin	23 (33.8)	4 (5.9)	41 (60.3)
Cefoxitin	0 (0)	0 (0)	68 (100)
Rifampicin	65 (95.6)	1 (1.5)	2 (2.9)
Fusidic Acid	32 (47.1)	11 (16.2)	4 (5.9)
Cotrimoxazole	11 (16.2)	0 (0)	7 (10.3)
Ciprofloxacin	10 (14.7)	1 (1.5)	57 (83.8)
Clindamycin	15 (22.1)	0 (0)	53 (77.9)
Ceftriaxone	0 (0)	0 (0)	4 (5.9)
Amoxicillin-clavulanate	0 (0)	0 (0)	2 (2.9)
Colistin	0 (0)	0 (0)	1 (1.5)
Piperacillin Tazobactam	2 (2.9)	0 (0)	11 (16.2)
Ceftazidime	0 (0)	0 (0)	2 (2.9)
Ampicillin-sulbactam	1 (1.5)	0 (0)	0 (0)

Table 4.3: Resistance profiles, clinical, phenotypic and genotypic characteristics of year 2013 MRSA strains.

Strains No	MRSA types	Van MIC	Resistance profiles	SCC*mec* types	MLST	Pulsotypes
ST/1301-28	HA-MRSA	1	CIP, PEN, FOX	IV	ST22	A10
ST/1307-5	CA-MRSA	0.75	ERY, CIP, CLI, PEN, FOX	IV	ST22	A10
ST/1312-23	HA-MRSA	< 0.5	ERY, CIP, CLI, PEN, FOX	IV	ST22	A12
ST/1304-34	HA-MRSA	1.5	ERY, CIP, CLI, PEN, FOX	IV	ST22	A14
ST/1309-15	HA-MRSA	< 0.5	ERY, CIP, CLI, PEN, FOX	IV	ST22	A14
ST/1302-28	HA-MRSA	1	CIP, PEN, FOX	IV	ST22	A15
ST/1306-20	HA-MRSA	0.5	ERY, RIF, CIP, CLI, PEN, FOX	IV	ST22	A2
ST/1306-21	HA-MRSA	1	ERY, CIP, PEN, FOX	IV	ST22	A5
ST/1308-32	HA-MRSA	< 0.5	ERY, CIP, CLI, PEN, FOX	IV	ST22	A7
ST/1303-4	HA-MRSA	< 0.5	ERY, GEN, CIP, CLI, AMC, PEN, FOX	V	ST45	B1
ST/1312-3	HA-MRSA	1	ERY, CIP, CLI, PEN, FOX	V	ST45	B2
ST/1310-14	HA-MRSA	< 0.5	CMX, PEN, FOX	I	ST152	C
ST/1301-23			NA	III	ST239	E1
ST/1304-5	HA-MRSA	2	ERY, GEN, FUS, CIP, CLI, PEN, FOX	III	ST239	E1
ST/1303-46	HA-MRSA	1.5	ERY, GEN, CIP, CLI, PEN, FOX	III	ST239	E1
ST/1302-6	HA-MRSA	2	ERY, GEN, CIP, CLI, PEN, FOX, TZP	III	ST239	E1
ST/1301-25	HA-MRSA	1.5	ERY, GEN, CIP, CLI, PEN, FOX, TZP	III	ST239	E1
ST/1307-21	HA-MRSA	2	ERY, CIP, CLI, PEN, FOX	III	ST239	E1
ST/1304-35	HA-MRSA	1.5	ERY, GEN, CIP, CLI, PEN, FOX	III	ST239	E11
ST/1303-39	HA-MRSA	1.5	ERY, GEN, CIP, CLI, PEN, FOX	III	ST239	E16
ST/1306-13	HA-MRSA	0.5	ERY, GEN, CIP, CLI, PEN, FOX, CAZ	III	ST239	E16
ST/1306-3	CA-MRSA	1.5	ERY, GEN, CIP, CLI, PEN, FOX	III	ST239	E16
ST/1308-25	HA-MRSA	2	ERY, GEN, CMX, CIP, CLI, PEN, FOX	III	ST239	E16
ST/1308-24	HA-MRSA	2	ERY, CIP, CLI, PEN, FOX, TZP	III	ST239	E16
ST/1312-27	HA-MRSA	1	ERY, GEN, CMX, CIP, CLI, PEN, FOX	III	ST239	E16
ST/1309-24	HA-MRSA	1.5	ERY, GEN, CIP, CLI, PEN, FOX, TZP	III	ST239	E18

Table 4.3, continued

Strains No	MRSA types	Van MIC	Resistance profiles	SCC*mec* types	MLST	Pulsotypes
ST/1309-22	HA-MRSA	2	ERY, GEN, CIP, CLI, PEN, FOX	III	ST239	E18
ST/1305-12	HA-MRSA	1.5	ERY, GEN, CIP, CLI, AMC, PEN, FOX	III	ST239	E19
ST/1312-22	CA-MRSA	2	ERY, GEN, CMX, CIP, CLI, PEN, FOX	III	ST239	E19
ST/1305-6	HA-MRSA	2	ERY, GEN, CIP, CLI, PEN, FOX	III	ST239	E2
ST/1310-10	HA-MRSA	< 0.5	ERY, GEN, CIP, CLI, PEN, FOX	III	ST239	E2
ST/1310-27	HA-MRSA	1	ERY, CMX, CIP, CLI, PEN, FOX	III	ST239	E2
ST/1310-24	HA-MRSA	1	ERY, CMX, CIP, CLI, PEN, FOX	III	ST239	E2
ST/1310-21	HA-MRSA	< 0.5	ERY, GEN, CIP, CLI, PEN, FOX	III	ST239	E2
ST/1312-12	HA-MRSA	1	ERY, GEN, FUS, CMX, CIP, CLI, PEN, FOX	III	ST239	E2
ST/1308-29	HA-MRSA	1.5	ERY, GEN, CIP, CLI, PEN, FOX	III	ST239	E2
ST/1308-26	CA-MRSA	2	ERY, GEN, CIP, CLI, PEN, FOX	III	ST239	E29
ST/1302-5	HA-MRSA	1.5	ERY, GEN, CIP, CLI, PEN, FOX, TZP	III	ST239	E3
ST/1301-21	HA-MRSA	1.5	ERY, GEN, CIP, CLI, PEN, FOX	III	ST239	E3
ST/1304-21	HA-MRSA	2	ERY, GEN, CIP, CLI, PEN, FOX, TZP	III	ST239	E30
ST/1311-5	HA-MRSA	1	ERY, GEN, CIP, CLI, PEN, FOX	III	ST239	E31
ST/1311-23	HA-MRSA	< 0.5	ERY, CLI, PEN, FOX	V	ST951	E32
ST/1305-24	HA-MRSA	1.5	ERY, GEN, CIP, CLI, CRO, PEN, FOX	III	ST239	E4
ST/1306-1	HA-MRSA	0.5	ERY, CIP, CLI, PEN, FOX	IV	ST22	E4
ST/1306-4	HA-MRSA	1.5	ERY, GEN, RIF, CIP, CLI, PEN, FOX	III	ST239	E4
ST/1303-30	HA-MRSA	1.5	ERY, GEN, CIP, CLI, PEN, FOX, CST	III	ST239	E7
ST/1303-20	HA-MRSA	2	ERY, GEN, CIP, CRO, PEN, FOX	III	ST239	E8
ST/1305-16	HA-MRSA	1.5	ERY, GEN, CIP, CLI, PEN, FOX	III	ST239	E8
ST/1304-2	HA-MRSA	2	ERY, GEN, CIP, CLI, PEN, FOX	III	ST239	E9
ST/1303-40	HA-MRSA	1.5	ERY, GEN, CIP, CLI, PEN, FOX, TZP	III	ST239	E9
ST/1305-3	HA-MRSA	1.5	ERY, GEN, CIP, CLI, PEN, FOX	V	ST772	F1
ST/1305-11	CA-MRSA	2	ERY, GEN, CIP, PEN, FOX	V	ST772	F1
ST/1303-29	CA-MRSA	1.5	ERY, GEN, CIP, PEN, FOX	V	ST772	F1

Table 4.3, continued

Strains No	MRSA types	Van MIC	Resistance profiles	SCC*mec* types	MLST	Pulsotypes
ST/1311-2	HA-MRSA	1	ERY, GEN, CIP, CLI, PEN, FOX	Untypeable	ST5	G1
ST/1308-13	HA-MRSA	1	ERY, GEN, FUS, CLI, PEN, FOX, TZP	V	ST1	H1
ST/1307-23	HA-MRSA	1	ERY, GEN, CLI, PEN, FOX	V	ST1	H2
ST/1312-28	HA-MRSA	< 0.5	ERY, CLI, PEN, FOX, CAZ	IV	ST6	I1
ST/1306-22	HA-MRSA	1.5	ERY, CLI, PEN, FOX, TZP	IV	ST6	I3
ST/1303-12	HA-MRSA	1.5	ERY, CLI, PEN, FOX	IV	ST6	I3
ST/1312-16	HA-MRSA	0.5	PEN, FOX	IV	ST6	I4
ST/1305-2	CA-MRSA	1.5	PEN, FOX	IV	ST6	I7
ST/1309-11	CA-MRSA	1.5	CRO, PEN, FOX	IV	ST6	I8
ST/1303-14	HA-MRSA	1.5	PEN, FOX, TZP	IV	ST1179	I9
ST/1303-3	HA-MRSA	< 0.5	ERY, GEN, CIP, CLI, PEN, FOX	III	ST239	I9
ST/1302-14	CA-MRSA	1.5	CIP, CRO, PEN, FOX	IV	ST30	K1
ST/1306-12	HA-MRSA	1	ERY, CIP, PEN, FOX, TZP	IV	ST30	K2
ST/1308-5	HA-MRSA	2	ERY, CIP, PEN, FOX	IV	ST30	K3
ST/1307-22	HA-MRSA	< 0.5	ERY, GEN, FUS, CIP, CLI, PEN, FOX	III	NA	NA
ST/1302-4	CA-MRSA	1.5	ERY, GEN, CIP, PEN, FOX	V	NA	NA

No: Number; Van: Vancomycin; MIC: Minimum inhibitory concentration; NA: not available.

Table 4.4: Comparisons between low and high vancomycin MIC in terms of patients' demographics, clinical data and genotypic characteristics of MRSA strains.

	Vancomycin MIC		*P* value
	< 1.5µg/mL	≥ 1.5µg/mL	
	Total N = 135 (%)	Total N = 142 (%)	
Age			
≤ 50 years old	42 (31.1)	38 (26.8)	0.430
> 50 years old	90 (66.7)	102 (71.8)	0.365
Not Known	3 (2.2)	2 (1.4)	
Gender			
Female	48 (35.6)	54 (38)	0.709
Male	85 (62.9)	87 (61.3)	0.805
Not Known	2 (1.5)	1 (0.7)	
Clinical diagnosis			
Bacteraemia	77 (57)	80 (56.3)	1.000
Skin and soft tissue infections	47 (34.8)	52 (36.6)	0.802
Osteomyelitis or septic arthritis	6 (4.4)	9 (6.3)	0.599
Meningitis	3 (2.2)	1 (0.7)	0.359
Pericarditis	2 (1.5)	0 (0)	0.237
Comorbidities			
Diabetes mellitus	52 (38.5)	59 (41.5)	0.626
Hypoglycaemia	1 (0.7)	0 (0)	0.487
Hypertension	50 (37)	56 (39.4)	0.712
Obesity	1 (0.7)	0 (0)	0.487
Chronic kidney disease	40 (29.6)	52 (36.6)	0.251
Cancer	18 (13.3)	12 (8.5)	0.246
Head injury	21 (15.6)	26 (18.3)	0.631
Liver disease	7 (5.2)	4 (2.8)	0.368
Respiratory disease	27 (20)	25 (17.6)	0.646
Cardiovascular disease	24 (17.8)	17 (11.9)	0.181
Gastrointestinal disease	7 (5.2)	6 (4.2)	0.781
Autoimmune disease	2 (1.5)	2 (1.4)	1.000
Bone and joint disorder	12 (8.9)	7 (4.9)	0.237
Endocrine disorder	3 (2.2)	1 (0.7)	0.359
Blood disorder	7 (5.2)	7 (4.9)	1.000
Viral disease	2 (1.5)	0 (0)	0.237
Skin disease	2 (1.5)	7 (4.9)	0.174
None	7 (5.2)	4 (2.8)	
Not Known	26 (19.3)	20 (14.1)	

Table 4.4, continued

	Vancomycin MIC		*P* value
	< 1.5µg/mL	≥ 1.5µg/mL	
	Total N = 135 (%)	Total N = 142 (%)	
Polymicrobial infections			
MSSA	0 (0)	1 (0.7)	1.000
Acinetobacter spp	1 (0.7)	2 (1.4)	1.000
CONS	3 (2.2)	3 (2.1)	1.000
Enterococcus spp	6 (4.4)	5 (3.5)	0.765
Klebsiella pneumoniae	1 (0.7)	1 (0.7)	1.000
Escherichia coli	1 (0.7)	0 (0)	0.487
Enterobacter spp	1 (0.7)	0 (0)	0.487
Corynebacterium striatum	0 (0)	1 (0.7)	1.000
Pseudomonas aeruginosa	5 (3.7)	3 (2.1)	0.492
Candida spp	2 (1.5)	0 (0)	0.237
More than one organisms	4 (2.9)	5 (3.5)	1.000
Type of MRSA			
HA-MRSA	87 (64.4)	94 (66.2)	0.801
CA-MRSA	37 (27.4)	38 (26.8)	1.000
Not Known	11 (8.1)	10 (7)	
SCC*mec* types			
SCC*mec* I	1 (0.7)	0 (0)	0.487
SCCmec II	0 (0)	2 (1.4)	0.499
SCC*mec* III	63 (46.7)	113 (79.6)	< 0.0001*
SCC*mec* IV	60 (44.4)	19 (13.4)	< 0.0001*
SCC*mec* IVa	7 (5.2)	2 (1.4)	
SCC*mec* IVb	5 (3.7)	0 (0)	
Novel subtypes	48 (35.6)	17 (11.9)	
SCC*mec* V	9 (6.7)	7 (4.9)	0.612
Untypeable	2 (1.5)	1 (0.7)	0.614

Cancer includes: Prostate cancer, lung cancer, breast cancer, cervical cancer, rectal cancer, bladder cancer and rectosigmoid cancer. Head injury includes: stroke basal ganglia bleed and subdural hematoma. Liver disease includes: alcoholic liver disease and liver cirrhosis. Respiratory disease includes: pneumonia, acute pulmonary haemorrhage, pulmonary embolism, chronic obstructive pulmonary disease and acute respiratory distress syndrome. Cardiovascular disease includes: atrial fibrillation, mitral valve regurgitation, ischaemic heart disease, acute coronary syndrome and congestive cardiac failure. Gastrointestinal disease includes: acute gastroenteritis, Crohn's disease, gastric antral vascular ectasia, perforated diverticular disease and intestinal obstruction. Autoimmune disease includes: systemic lupus erythematosus. Endocrine disorder includes: thyroid disease and primary hypothyroidism. Bone and joint disorders include: infected distal femur locking plate, right knee osteoarthritis, rheumatoid arthritis and right femur osteomyelitis. Blood disorder includes: myelofibrosis, multiple myeloma, chronic lymphocytic leukaemia and anaemia. Viral disease includes: human immunodeficiency virus and cytomegalovirus. Skin disease includes: Stevens Johnson Syndrome, bullous pemphigoid, exfoliative dermatitis and pemphigus vulgaris.

4.3 SCC*mec* types

The predominant SCC*mec* type in this hospital from year 2011 to 2013 was SCC*mec* type III (64.2% in 2011, 68.4% in 2012 and 55.1% in 2013), high rates were reported as compared to other SCC*mec* types. For year 2011 MRSA strains, 25 SCC*mec* type IV strains were further subtyped as SCC*mec* type IVa (n = 5; 5.3%) and SCC*mec* type IVb (n = 5; 5.3%). The remaining 15 MRSA strains were known as novel SCC*mec* type IV subtypes as they could not be subtyped. There were also two (2.1%) and six (6.3%) MRSA strains typed as SCC*mec* type II and SCC*mec* type V, respectively. Apart from SCC*mec* type III in year 2012 MRSA strains, SCC*mec* types IV (n = 34; 29.8%) and V (n = 1; 0.9%) were also observed. SCC*mec* type IV strains were further subtyped as SCC*mec* type IVa (n = 4; 3.5%) and novel type IV SCC*mec* subtypes (n = 30; 26.3%). SCC*mec* types I (n = 1; 1.4%), IV (n = 20; 28.9%) and V (n = 9; 13%) were present among year 2013 MRSA strains and the SCC*mec* type IV strains were further subtyped as novel type IV SCC*mec* subtypes (n = 20; 28.9%). There were three untypeable MRSA strains which harboured *mec*A gene present in each year. The number of MRSA strains with SCC*mec* type V appeared to fluctuate from year 2011 and 2013 ($p = 0.001$). In this study, HA-MRSA strains carried SCC*mec* types I, (n = 1; 0.6%), II (n = 2; 1.1%), III (n = 125; 69.1%), IV (n = 45; 24.9%) and V (n = 7; 3.9%) while CA-MRSA strains carried SCC*mec* types III (n = 39; 52%), IV (n = 28; 37.3%) and V (n = 7; 9.3%) (Table 4.1).

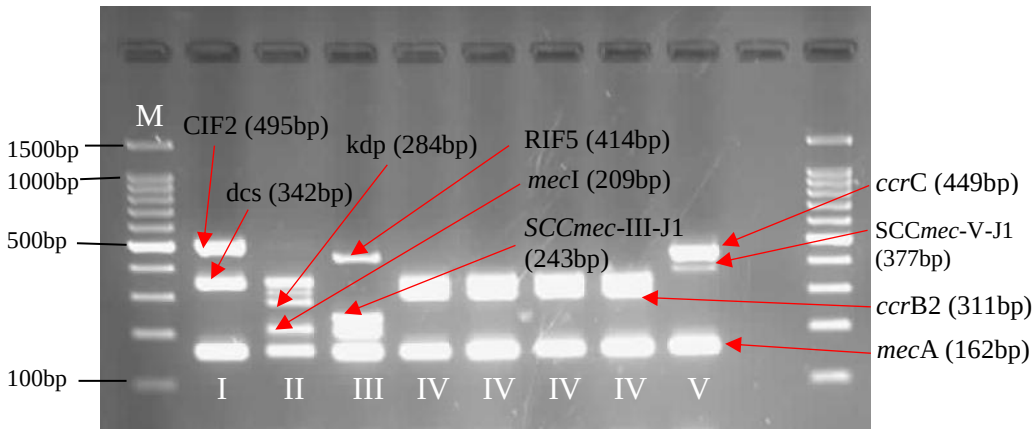

Figure 4.3: Representative agarose gel that illustrated different banding patterns of SCC*mec* types. Sterile Ultrapure Water was used as negative control.

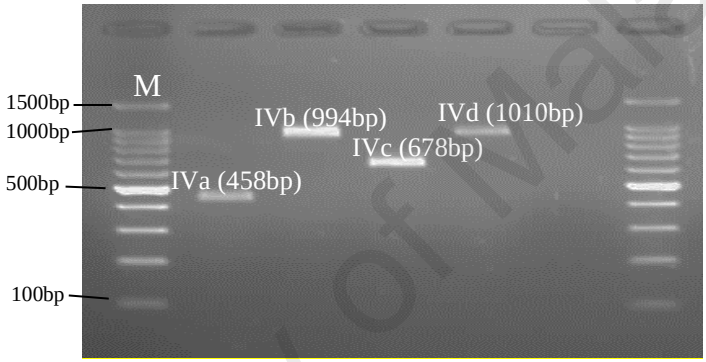

Figure 4.4: Representative agarose gel that illustrated different banding patterns of SCC*mec* type IV subtypes. Sterile Ultrapure Water was used as negative control.

4.4 The prevalence of PVL gene

The presence of PVL gene was determined by PCR amplification. PVL gene was present in five (5.3%) MRSA strains from year 2011 and these strains were isolated from tissue, pus and abscess. All PVL-positive strains were CA-MRSA and carried both SCC*mec* types IV and V. There was significant difference in the prevalence of PVL gene from 2011 to 2013 strains (p = 0.005) as no PVL gene was detected in 2012 and 2013 (Table 4.1).

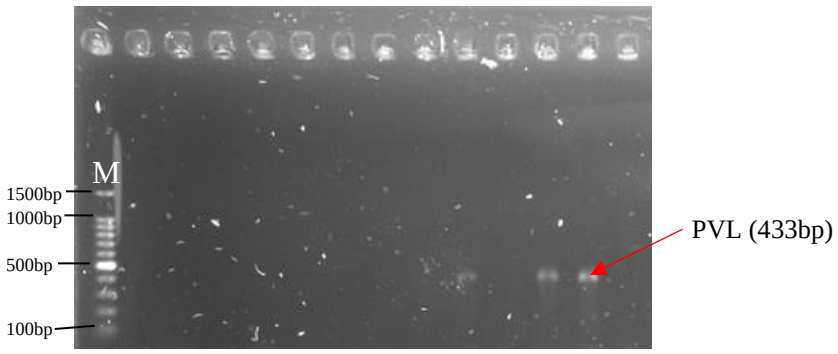

Figure 4.5: Representative agarose gel of PCR amplification of PVL gene. Sterile Ultrapure Water was used as negative control.

4.5 Clinical characteristics and molecular diversity of MRSA strains from blood

4.5.1 Clinical characteristics of MRSA bacteraemia

The incidence of MRSA bacteraemia in this study were significantly increased from year 2011 (n = 40; 42.1%), 2012 (n = 51; 44.7%) and 2013 (n = 67; 97.1%) ($p < 0.0001$) (Table 4.1). Of 158 MRSA bacteraemia episodes, the majority occurred in patients > 50 years old (n = 127; 80.4%) and were caused by HA-MRSA (n = 108; 68.4%). In addition, the rate of bacteraemia in male gender (n = 87; 55.1%) was higher compared to female (n = 68; 43%). The most commonly identified sources of bacteraemia were primary bacteraemia (n = 75; 47.5%) followed by catheter-related (n = 32; 20.3%) and pneumonia (n = 21; 13.3%). Diabetes mellitus (n = 66; 41.8%), hypertension (n = 70; 44.3%) and chronic kidney disease (CKD) (n = 69; 43.7%) were the major comorbidities found among MRSA bacteraemia patients. There was no significant difference between the SCC*mec* types in terms of age, gender, comorbidities and type of MRSA. However, MRSA bacteraemia patients with SCC*mec* type IV strains were found to be significantly associated with cardiovascular diseases including mitral valve regurgitation, ischaemic heart disease, acute coronary syndrome and congestive cardiac failure ($p = 0.034$). On the contrary, SCC*mec* type V strains were significantly associated with implant-related bacteraemia ($p = 0.027$) (Table 4.5).

Table 4.5: Comparisons between SCC*mec* types in terms of patients' demographics, clinical data and molecular characteristics of MRSA bacteraemia.

	SCC*mec* I N = 1 (%)	SCC*mec* III N = 97 (%)	SCC*mec* IV N = 47 (%)	SCC*mec* V N = 10 (%)	Untypeable N = 3 (%)	*P* value	Total N = 158 (%)	*P* value
Age								
≤ 50 years old	0 (0)	20 (20.6)	7 (14.9)	0 (0)	1 (33.3)	0.449	28 (17.7)	< 0.0001*
> 50 years old	1 (100)	75 (77.3)	39 (82.9)	10 (100)	2 (66.7)	0.431	127 (80.4)	
Not Known	0 (0)	2 (2.1)	1 (2.1)	0 (0)	0 (0)		3 (1.9)	
Gender								
Female	1 (100)	46 (47.4)	16 (34)	5 (50)	0 (0)	0.192	68 (43)	0.043*
Male	0 (0)	49 (50.5)	30 (63.8)	5 (50)	3 (100)	0.195	87 (55.1)	
Not Known	0 (0)	2 (2.1)	1 (2.1)	0 (0)	0 (0)		3 (1.9)	
Comorbidities								
Diabetes mellitus	1 (100)	39 (40.2)	20 (42.6)	6 (60)	0 (0)	0.285	66 (41.8)	
Hypertension	1 (100)	43 (44.3)	21 (44.7)	5 (50)	0 (0)	0.437	70 (44.3)	
Obesity	0 (0)	0 (0)	1 (2.1)	0 (0)	0 (0)	0.666	1 (0.6)	
Chronic kidney disease	0 (0)	42 (43.3)	22 (46.8)	4 (40)	1 (33.3)	0.886	69 (43.7)	
Cancer	0 (0)	16 (16.5)	4 (8.5)	1 (10)	0 (0)	0.645	21 (13.3)	
Head injury	1 (100)	18 (18.6)	12 (25.5)	3 (30)	2 (66.7)	0.086	36 (22.8)	
Liver disease	0 (0)	6 (6.2)	3 (6.4)	0 (0)	0 (0)	0.920	9 (5.7)	
Respiratory disease	1 (100)	21 (21.6)	13 (27.7)	4 (40)	0 (0)	0.199	39 (24.7)	
Cardiovascular disease	1 (100)	12 (12.4)	13 (27.7)	2 (20)	0 (0)	0.034*	28 (17.7)	
Gastrointestinal disease	0 (0)	6 (6.2)	4 (8.5)	1 (10)	0 (0)	0.950	11 (6.9)	
Autoimmune disease	0 (0)	3 (3.1)	1 (2.1)	0 (0)	0 (0)	0.972	4 (2.5)	
Bone and joint disorder	0 (0)	5 (5.2)	3 (6.4)	2 (20)	0 (0)	0.455	10 (6.3)	
Endocrine disorder	0 (0)	0 (0)	2 (4.3)	1 (10)	0 (0)	0.142	3 (1.9)	
Blood disorder	0 (0)	9 (9.3)	2 (4.3)	1 (10)	0 (0)	0.818	12 (7.6)	
Viral disease	0 (0)	1 (1)	1 (2.1)	0 (0)	0 (0)	0.974	2 (1.3)	
Skin disease	0 (0)	7 (7.2)	0 (0)	1 (10)	0 (0)	0.385	8 (5.1)	
None	0 (0)	1 (1)	0 (0)	0 (0)	0 (0)		1 (0.6)	
Not known	0 (0)	7 (7.2)	5 (10.6)	0 (0)	1 (33.3)		13 (8.2)	

Table 4.5, continued

	SCC*mec* I N = 1 (%)	SCC*mec* III N = 97 (%)	SCC*mec* IV N = 47 (%)	SCC*mec* V N = 10 (%)	Untypeable N = 3 (%)	*P* value	Total N = 158 (%)	*P* value
Source of bacteraemia								
Primary bacteraemia	0 (0)	45 (46.4)	23 (48.9)	4 (40)	3 (100)	0.339	75 (47.5)	
Catheter-related	0 (0)	19 (19.6)	11 (23.4)	2 (20)	0 (0)	0.856	32 (20.3)	
Skin and soft tissue infection	0 (0)	9 (9.3)	2 (4.3)	0 (0)	0 (0)	0.666	11 (6.9)	
Pneumonia	1 (100)	11 (11.3)	7 (14.9)	2 (20)	0 (0)	0.099	21 (13.3)	
Surgical site infection	0 (0)	3 (3.1)	1 (2.1)	0 (0)	0 (0)	0.972	4 (2.5)	
Implant-related infection	0 (0)	1 (1)	2 (4.3)	2 (20)	0 (0)	0.027*	5 (3.2)	
Prosthetic valve endocarditis	0 (0)	1 (1)	0 (0)	0 (0)	0 (0)	0.959	1 (0.6)	
Septic arthritis	0 (0)	0 (0)	1 (2.1)	0 (0)	0 (0)	0.666	1 (0.6)	
More than one source	0 (0)	7 (7.2)	0 (0)	0 (0)	0 (0)	0.329	7 (4.4)	
Not Known	0 (0)	1 (1)	0 (0)	0 (0)	0 (0)		1 (0.6)	
Type of MRSA								
HA-MRSA	1 (100)	69 (71.1)	31 (65.9)	6 (60)	1 (33.3)	0.565	108 (68.4)	< 0.0001*
CA-MRSA	0 (0)	18 (18.6)	13 (27.7)	4 (40)	1 (33.3)	0.435	36 (22.8)	
Not Known	0 (0)	10 (10.3)	3 (6.4)	0 (0)	1 (33.3)		14 (8.9)	

4.5.2 Genotyping by PFGE

PFGE of *Sma*I-digested chromosomal DNA of 158 MRSA strains associated with bacteraemia have generated 12 major pulsotypes (Pulsotype A to L) comprising of 13 to 20 restriction fragments that ranged in size from approximately 20 kb to 700 kb and with a Dice coefficient, *F* ranging from 0.5 to 1.0. Based on 80% similarity, eight clusters were observed, namely Cluster I to Cluster VIII (Figure 4.7). The majority of the strains were clustered in Cluster III which consists of 97 strains (61.4%), followed by Clusters I, VII and IV which contained 29 strains (18.4%), 14 strains (8.9%) and four strains (2.5%), respectively. The remaining Clusters VIII, II, V and VI contained only a few strains. Clusters I, III and VII comprised of MRSA strains from year 2011, 2012 and 2013 whereas MRSA strains within Clusters IV and V were from year 2011 and 2013. Clusters II, VI and VIII consist of MRSA strains from year 2013 only. SCC*mec* type IV strains were grouped in Clusters I, VII and VIII while SCC*mec* type V strains in Clusters II, IV, V and VI. SCC*mec* type III strains were mainly grouped in Cluster III.

Pulsotype E in Cluster III was seen with multiple subtypes (E1 to E33), followed by Pulsotype A in Cluster I with subtypes A1 to A16 and Pulsotype I in Cluster VII with subtypes I1 to I9. Pulsotype K in Cluster VIII was less common with fewer subtypes (K1 to K3), followed by Pulsotype B in Cluster II with subtypes B1 to B2, Pulsotype F in Cluster IV with subtypes F1 to F2, Pulsotype G in Cluster V with subtypes G1 to G2 and Pulsotype H in Cluster VI with subtypes H1 to H2. Four MRSA strains were not assigned to any clusters and appeared as singletons exhibiting Pulsotypes C, D, J and L.

Pulsotype E in Cluster III was the predominant pulsotype which indicated increased nosocomial transmission of the MRSA strains with this pulsotype within the hospital. Twenty-five MRSA strains from year 2011 to 2013 belonged to the major subtype E2 within Pulsotype E. They were indistinguishable or clonally related (sharing 100% similarity) despite being isolated from different wards and years.

Based on Figure 4.7, the presence of clonally and closely related MRSA strains although they were from different years and wards, suggested the persistence of the MRSA clones and were transmitted between wards. In addition, there were also possibility of the spread of MRSA strains between hospital and community.

The relationships between MRSA types, vancomycin MICs, resistance profiles and genotypic characteristics for year 2013 MRSA strains is shown in Table 4.3. Strains exhibiting pulsotype A shared the same characteristics in which they were resistant to ciprofloxacin and carried SCC*mec* type IV-ST22. On the contrary, MRSA strains with pulsotype B were HA-MRSA, resistant to erythromycin, ciprofloxacin and clindamycin and carried SCC*mec* type V-ST45. MRSA strains with pulsotype E were mostly SCC*mec* type III-ST239 and resistant to erythromycin. Those strains exhibiting pulsotype F have high vancomycin MIC, resistant to erythromycin, gentamicin and ciprofloxacin and carried SCC*mec* type V-ST772. Strains with pulsotype H were HA-MRSA, carried SCC*mec* type V-ST1 and were resistant to erythromycin, gentamicin and clindamycin. MRSA strains exhibiting pulsotype K belonged to SCC*mec* type IV-ST30 and resistant to ciprofloxacin.

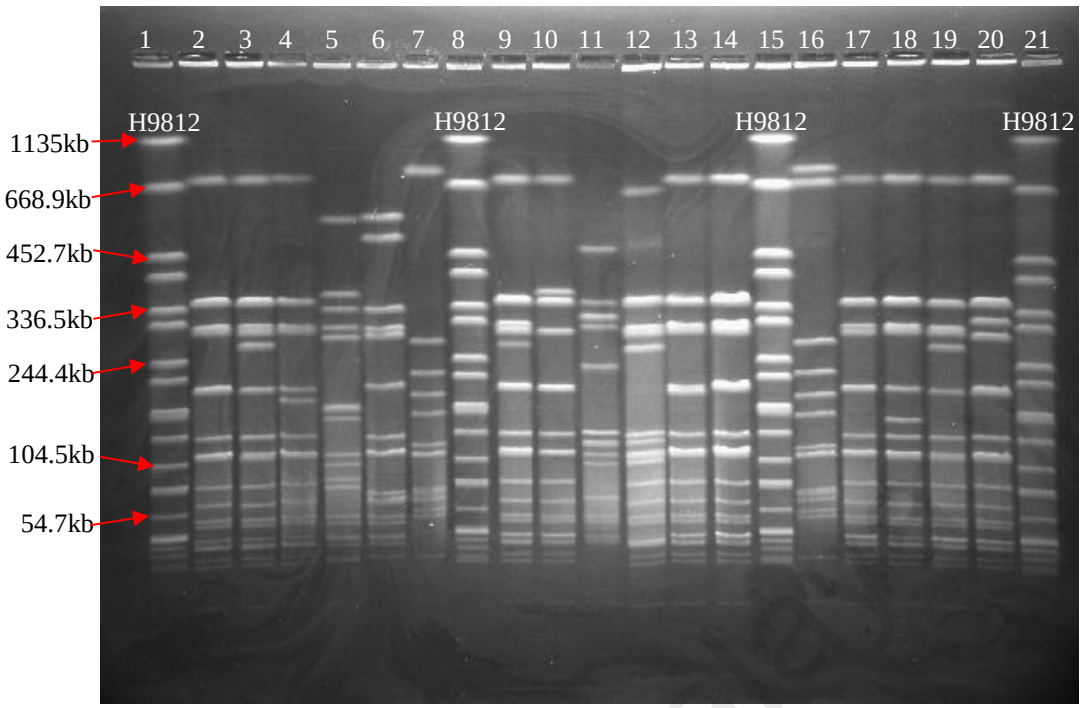

Figure 4.6: Representative PFGE gel that illustrated different restriction fragments of MRSA strains. The strains are situated on lanes 2-7, 9-14 and 16-20. H9812 was used as reference strain and positioned on lanes 1, 8, 15 and 21.

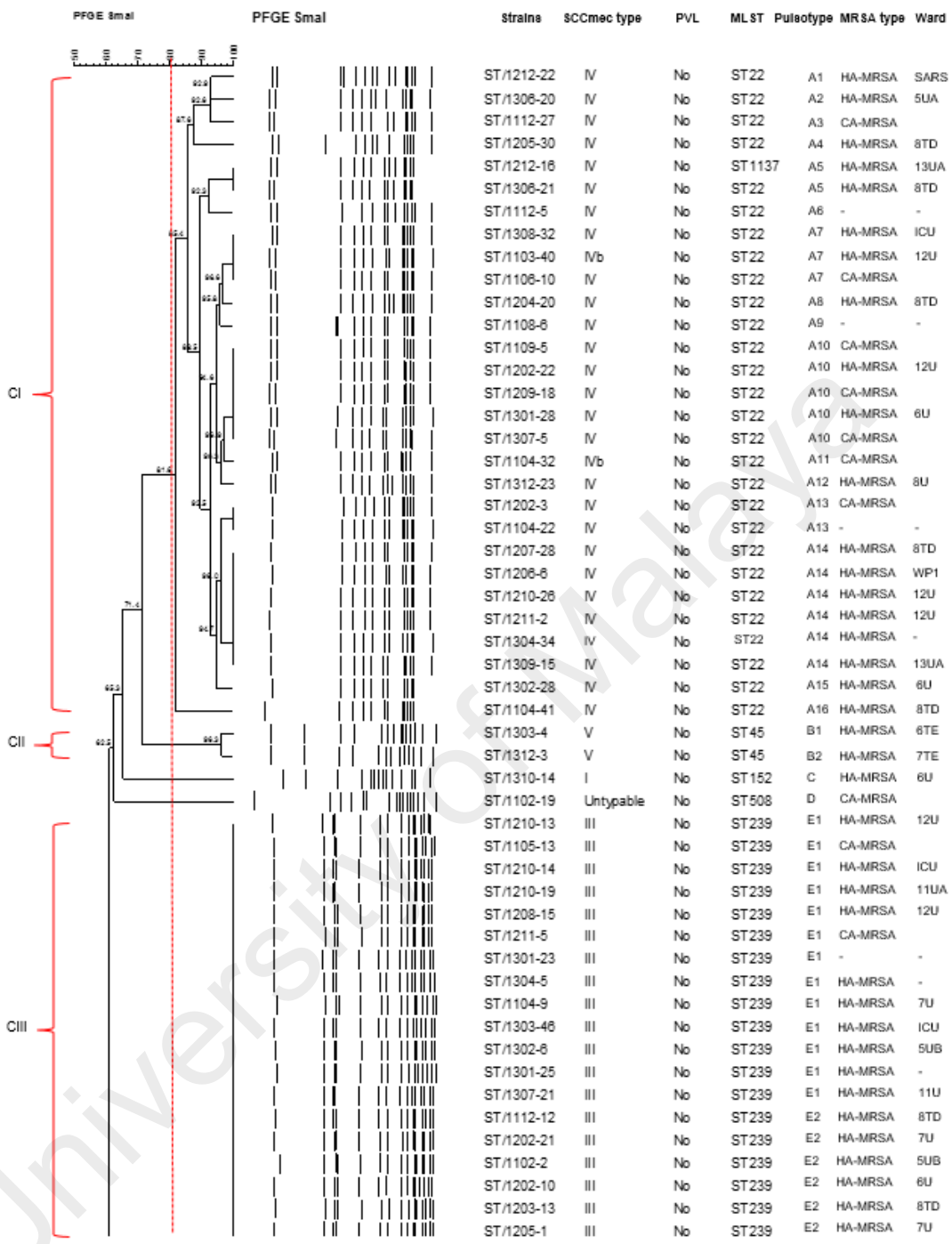

Figure 4.7: Dendrogram of MRSA strains. The dotted vertical line represents 80% similarity level.

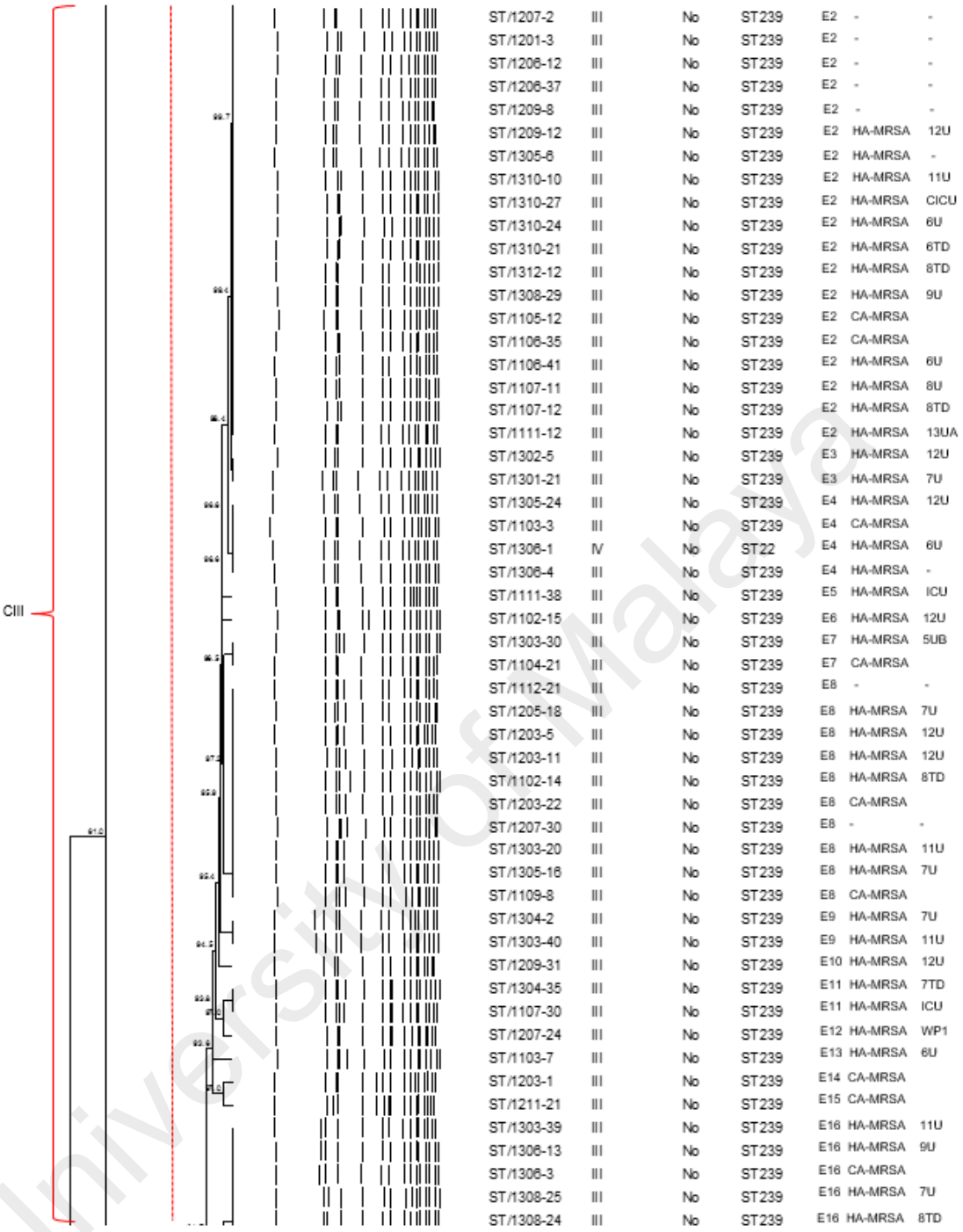

Figure 4.7, continued

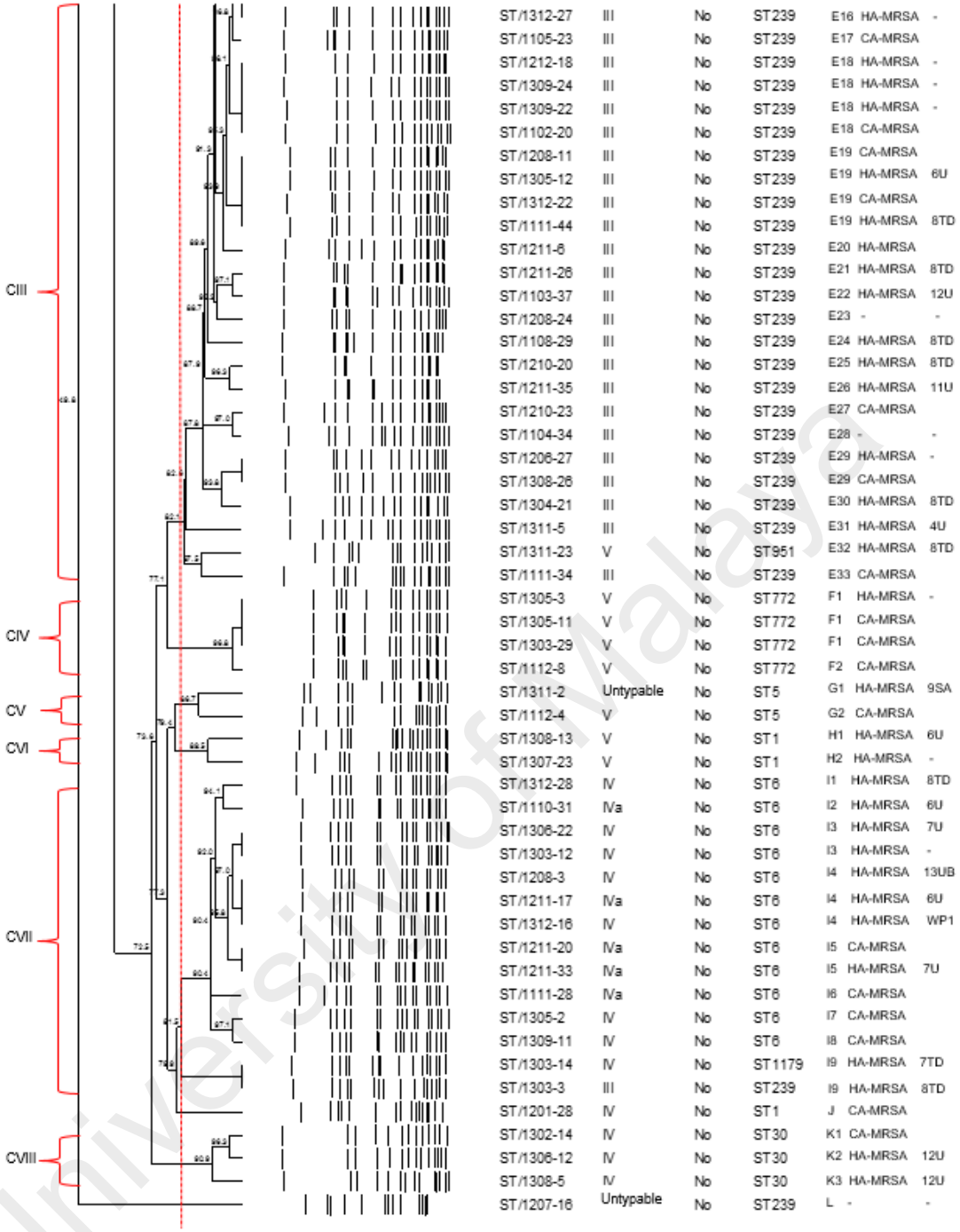

Figure 4.7, continued

4.5.3 Genotyping by MLST

MLST analysis on 158 MRSA strains revealed 13 different sequence types (STs) which consist of ST1 (n = 3), ST5 (n = 2), ST6 (n = 12), ST22 (n = 29), ST30 (n = 3), ST45 (n = 2), ST152 (n = 1), ST239 (n = 98), ST508 (n = 1), ST772 (n = 4), ST951 (n = 1), ST1137 (n = 1) and ST1179 (n = 1) (Table 4.6). ST5, ST6, ST22, ST239, ST508 and ST772 were present among 2011 MRSA strains. Whereas, ST1, ST6, ST22, ST239 and ST1137 were observed in MRSA strains from year 2012. Eleven STs were observed among 2013 MRSA strains; namely ST1, ST5, ST6, ST22, ST30, ST45, ST152, ST239, ST772, ST951 and ST1137.

MRSA strains were clustered into nine clonal complexes (CCs) based on the similarity between STs in six of seven loci (Figure 4.9). The predominant ST in this study was ST239 which is a single-locus variant (SLV) of ST8, belonging to CC8. ST1, ST5, ST6, ST22, ST30 and ST45 are the primary founders of CC1, CC5, CC6, CC22, CC30 and CC45, respectively. ST5 (CC5), ST45 (CC45), ST152 (singleton), ST508 (CC45), ST951 (CC59) and ST1137 (CC22) were the STs reported for the first time in Malaysia. The STs found in this study including ST1 (CC1), ST6 (CC6), ST22 (CC22), ST30 (CC30), ST239 (CC8), ST772 (CC1) and ST1179 (CC97), were also reported in previous studies in Malaysian hospitals.

SCC*mec* type I strain belonged to ST152 while SCC*mec* type III strains belonged to ST239. SCC*mec* type IV strains were associated with ST1, ST6, ST22, ST30, ST1137 and ST1179. On the contrary, SCC*mec* type V strains were seen to harbour ST1, ST5, ST45, ST772 and ST951. Strains with untypeable SCC*mec* were represented by three STs (ST5, ST239 and ST508). The presence of same ST in different SCC*mec* type such as ST1 in SCC*mec* types IV and V may indicate this MRSA clone arise at several occasion

in the same genetic background. High genetic diversities in SCC*mec* types IV and V strains were observed by the presence of various STs within the types. This also indicated their capabilites to transfer between different genetic clones of *Staphylococcus aureus.*

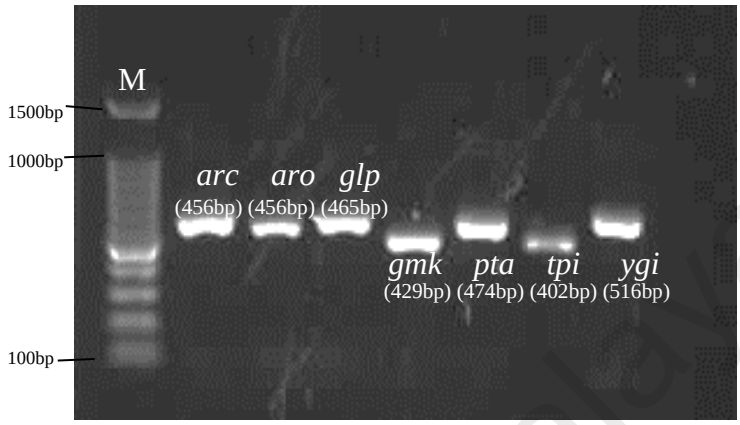

Figure 4.8: Representative agarose gel that illustrated different banding patterns of seven housekeeping genes (*arc, aro, glp, gmk, pta, tpi and ygi*).

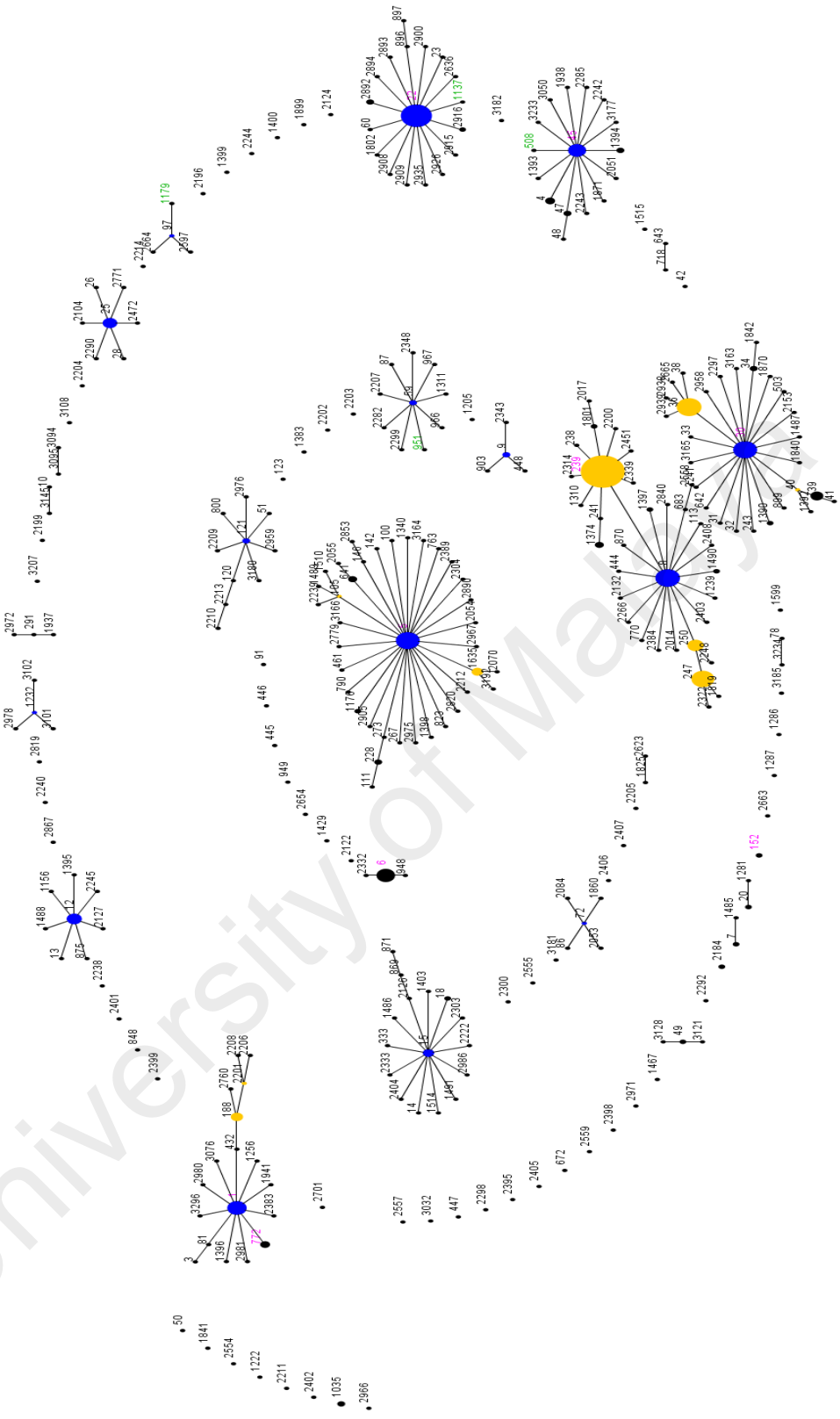

Figure 4.9: Population snapshot of MRSA bacteraemia in the MLST database. Individual ST highlighted in black is the ST present in the database, green is the ST found in this study and pink is the ST present in both the database and in this study. Each ST is represented by the circle and the size of the circle indicates the frequency of

a particular ST. The colour of the circle represents founders and subgroup founders. Blue circles are ‘founders’ whereas yellow circle are ‘subgroup founders’.

Table 4.6: Allelic profiles for MLST.

ST	**CC**	**No of MRSA strains**	***arc***	***aro***	***glp***	***gmk***	***pta***	***tpi***	***ygi***
1	1	3	1	1	1	1	1	1	1
5	5	2	1	4	1	4	12	1	10
6	6	12	12	4	1	4	12	1	3
22	22	29	7	6	1	5	8	8	6
30	30	3	2	2	2	2	6	3	2
45	45	2	10	14	8	6	10	3	2
152	SINGLETON	1	46	75	49	44	13	68	60
239	8	98	2	3	1	1	4	4	3
508	45	1	10	40	8	6	10	3	2
772	1	4	1	1	1	1	22	1	1
951	59	1	19	23	15	89	19	20	15
1137	22	1	143	6	1	5	8	8	6
1179	97	1	144	1	1	1	1	5	3

4.6 Association between clinical, phenotypic and molecular variables with the patient's outcome

Variables of clinical importance were included in a logistic regression model as shown in Table 4.7 to identify the risk factors associated with mortality. In univariate analysis, patients ≤ 50 years old (OR = 0.15; 95% CI = 0.06-0.34; $p < 0.0001$), male gender (OR = 0.52; 95% CI = 0.30-0.89; $p = 0.019$) and SSTIs as diagnosis (OR = 0.34; 95% CI = 0.18-0.63; $p = 0.0006$) were associated with lower mortality rate.

On the contrary, age > 50 years old (OR = 6.86; 95% CI = 2.97-15.87; $p < 0.0001$), female gender (OR = 1.93; 95% CI = 1.13-3.32; $p = 0.019$), bacteraemia as diagnosis (OR = 2.87; 95% CI = 1.59-5.14; $p = 0.0003$) and respiratory diseases as comorbidities (OR = 2.94; 95% CI = 1.55-5.56; $p = 0.0009$) were significantly associated with higher mortality rate.

The mortality rate due to MRSA bacteraemia was 75.6% (n = 65). In terms of infection severity, the APACHE II, Pitt bacteraemia and CCI scores were found to be higher in death compared to survived patients. There was no significant difference between patients who survived and those who died in terms of presence of polymicrobial infections, SCC*mec* types, MRSA types and vancomycin MIC status. (Table 4.7).

Table 4.7: Determination of possible risk factors associated with patient's mortality.

	Outcome		OR	95% CI	*P* value
	Survival N =156 (%)	**Death N = 86 (%)**			
Age					
≤ 50 years old	59 (37.8)	7 (8.1)	0.15	0.06-0.34	<0.0001*
> 50 years old	97 (62.2)	79 (91.9)	6.86	2.97-15.87	<0.0001*
Gender					
Female	50 (32.1)	41 (47.7)	1.93	1.13-3.32	0.019*
Male	106 (67.9)	45 (52.3)	0.52	0.30-0.89	0.019*
Clinical diagnosis					
Bacteraemia	81 (51.9)	65 (75.6)	2.87	1.59-5.14	0.0003*
Skin and soft tissue infections	63 (40.4)	16 (18.6)	0.34	0.18-0.63	0.0006*
Osteomyelitis or septic arthritis	9 (5.8)	3 (3.5)	0.59	0.16-2.24	0.547
Meningitis	2 (1.3)	1 (1.2)	0.91	0.08-10.14	1.000
Pericarditis	1 (0.6)	1 (1.2)	1.82	0.11-29.52	1.000
Comorbidities					
Diabetes mellitus	72 (46.2)	34 (39.5)	0.76	0.45-1.30	0.346
Hypoglycaemia	0 (0)	1 (1.2)	NA	NA	0.355
Hypertension	72 (46.2)	31 (36)	0.66	0.38-1.13	0.137
Obesity	1 (0.6)	0 (0)	NA	NA	1.000
Chronic kidney disease	52 (33.3)	38 (44.2)	1.58	0.92-2.72	0.098
Cancer	16 (10.3)	11 (12.8)	1.28	0.57-2.91	0.670
Head injury	25 (16)	21 (24.4)	1.69	0.88-3.25	0.125
Liver disease	5 (3.2)	6 (6.9)	2.27	0.67-7.65	0.205
Respiratory disease	22 (14.1)	28 (32.6)	2.94	1.55-5.56	0.0009*
Cardiovascular disease	21 (13.5)	18 (20.9)	1.70	0.85-3.41	0.146
Gastrointestinal disease	5 (3.2)	6 (6.9)	2.27	0.67-7.65	0.205
Autoimmune disease	3 (1.9)	0 (0)	NA	NA	0.309
Bone and joint disorder	12 (7.7)	4 (4.7)	0.59	0.18-1.87	0.429
Endocrine disorder	2 (1.3)	2 (2.3)	1.83	0.25-13.25	0.617
Blood disorder	5 (3.2)	8 (9.3)	3.09	0.98-9.79	0.069
Viral disease	2 (1.3)	0 (0)	NA	NA	0.539
Skin disease	5 (3.2)	2 (2.3)	0.72	0.14-3.79	1.000
None	11 (7.1)	0 (0)			
Not Known	19 (12.2)	7 (8.1)			

Table 4.7, continued

	Outcome		OR	95% CI	*P* value
	Survival N =156 (%)	Death N = 86 (%)			
Polymicrobial infections					
MSSA	1 (0.6)	0 (0)	NA	NA	1.000
Acinetobacter spp	3 (1.9)	0 (0)	NA	NA	0.309
CONS	3 (1.9)	3 (3.5)	1.84	0.36-9.34	0.669
Enterococcus spp	6 (3.8)	5 (5.8)	1.54	0.46-5.21	0.527
Klebsiella pneumoniae	2 (1.3)	0 (0)	NA	NA	0.539
Escherichia coli	1 (0.6)	0 (0)	NA	NA	1.000
Enterobacter spp	1 (0.6)	0 (0)	NA	NA	1.000
Corynebacterium striatum	1 (0.6)	0 (0)	NA	NA	1.000
Pseudomonas aeruginosa	6 (3.8)	2 (2.3)	0.59	0.12-3.02	0.715
Candida spp	1 (0.6)	1 (1.2)	1.82	0.11-29.52	1.000
More than one organisms	6 (3.8)	3 (3.5)	0.90	0.22-3.71	1.000
Type of MRSA					
HA-MRSA	110 (70.5)	55 (63.9)	0.74	0.42-1.29	0.315
CA-MRSA	42 (26.9)	21 (24.4)	0.88	0.48-1.61	0.760
Not Known	4 (2.6)	10 (11.6)			
SCC*mec* types					
SCC*mec* I	0 (0)	1 (1.2)	NA	NA	0.355
SCCmec II	2 (1.3)	0 (0)	NA	NA	0.539
SCC*mec* III	99 (63.5)	51 (59.3)	0.84	0.49-1.44	0.581
SCC*mec* IV					
SCC*mec* IVa	6 (3.8)	2 (2.3)	0.59	0.12-3.02	0.715
SCC*mec* IVb	4 (2.6)	0 (0)	NA	NA	0.300
Novel subtypes	34 (21.8)	27 (31.4)	1.64	0.91-2.97	0.122
SCC*mec* V	10 (6.4)	4 (4.7)	0.71	0.22-2.34	0.775
Untypeable	1 (0.6)	1 (1.2)	1.82	0.11-29.52	1.000
Vancomycin MIC					
< 1.5µg/mL	76 (48.7)	41 (47.7)	0.96	0.57-1.62	0.894
≥ 1.5µg/mL	80 (51.3)	45 (52.3)	1.04	0.62-1.77	0.894
Severity of infection					
APACHE II score	15.69±4.70	20.6±8.06			
Pitt bacteraemia score	0.39±0.88	2.5±2.55			
Charlson Comorbidity Index	4.28±2.76	5.5±2.55			

OR: odds ratio; 95% CI: 95% confidence interval.

CHAPTER 5: DISCUSSION

This study is a single-centre retrospective cohort study from year 2011 to 2013 MRSA strains. The rates of MRSA infections were higher in male gender and patients aged > 50 years compared to their respective groups. Elderly are more vulnerable to serious infectious diseases compared to younger adults, possibly due to age-related immune changes, particularly cell-mediated immunity (Yoshikawa, 2000). Males have higher MRSA infection rates compared to female probably due to hand-hygiene practices. Studies in New Zealand and the US reported that males had significantly lower frequency of hand hygiene than females. In addition, profession as well as other physiological and immunological factors might also be the contributory factors in gender differences in acquiring MRSA infections (Humphreys *et al.*, 2015).

This study demonstrates that high proportions (65.1%) of MRSA patients have HA-MRSA infections ($p < 0.0001$). The rate of HA-MRSA infection was significantly increased and caused by SCC*mec* types I to V strains. SCC*mec* types IV and V which are predominantly known to be carried by CA-MRSA strains, were observed among the HA-MRSA strains, indicating that these strains have invaded into this hospital. Similar findings were also reported in the US and Brazil, where SCC*mec* type IV isolates caused nosocomial outbreaks, especially bloodstream infections (Seybold *et al.*, 2006; Trindade *et al.*, 2005). In Malaysia, minority of SCC*mec* type IV-ST30 were present in hospital isolates (Ahmad *et al.*, 2009) and in Taiwan, the prevalence of SCC*mec* type IV among HA-MRSA isolates increased from 20% in 1999 to 43% in 2005 (Huang *et al.*, 2007). The presence of SCC*mec* type IV among HA-MRSA strains is worrying as they may replace the SCC*mec* type III as the predominant SCC*mec* type in the hospital in the future (D'Souza *et al.*, 2010). SCC*mec* type IV which is much smaller in size compared to other

SCC*mec* types, can facilitate horizontal transfer on a bacteriophage or plasmid to a distantly related *S. aureus* clones (Berglund *et al.*, 2005; Daum *et al.*, 2002). In addition, SCC*mec* type IV strains may acquire additional resistant genes within this hospital as they now coexist with the typical nosocomial strains (Simons & Alcabes, 2008).

CA-MRSA infections were seen to be significantly decreasing from year to year and are caused by SCC*mec* types III, IV and V strains. The high rates of SCC*mec* type III among CA-MRSA strains might indicate the spreading of this hospital strains to the community. Previous international surveillance study on the CA-MRSA epidemiology in Asian countries including Taiwan, Hong Kong, Korea, Thailand, Philippines and Vietnam has reported the spreading of HA-MRSA isolates to the community based on the presence of SCC*mec* types I, II and III among CA-MRSA isolates (Song *et al.*, 2011).

SCC*mec* type III was the predominant SCC*mec* type in this hospital from year 2011 to 2013, which was consistent with the previous studies done in Malaysian hospitals by Lim *et al.* (2013), Ghaznavi-Rad *et al.* (2010a) and Samat Muttaqillah *et al.* (2015) (Ghaznavi-Rad *et al.*, 2010a; Lim *et al.*, 2013; Samat Muttaqillah *et al.*, 2015). SCC*mec* type III is also common in Asian countries including Singapore, Indonesia, Thailand and Taiwan (Chen & Huang, 2014). Other SCC*mec* types were also present where SCC*mec* types I and II were seen among year 2013 and 2011 MRSA strains, respectively and SCC*mec* types IV and V strains were seen throughout the study period. SCC*mec* type II was reported as the predominant SCC*mec* type in Japan and Korea (Chen & Huang, 2014).

PVL gene is known to cause leucocyte destruction and is often carried by CA-MRSA strains (Gordon & Lowy, 2008) was detected in five MRSA strains. All PVL-positive strains were from year 2011 CA-MRSA strains isolated from pus, tissue and abscess, carried SCC*mec* types IV and V and caused SSTIs. The prevalence of PVL gene among MRSA strains in this hospital was 1.8%. Previous study in 2003 and 2008 MRSA strains by Lim *et al.* (2013) reported that 2% of MRSA strains from 2008 were PVL-positive (Lim *et al.*, 2013). This showed that the prevalence of PVL gene in this hospital remained less than 5% despite a few years gap between the study periods.

All MRSA strains from 2013 were multidrug-resistant as they were resistant to more than two classes of antimicrobial agents. Overall, the rates of rifampicin and fusidic acid resistance in this hospital remained low, which was in agreement with our National Antibiotic Guideline 2014 that reported on the antibiotic resistance trend for MRSA (Ministry of Health Malaysia, 2014). The reason for low rifampicin resistance in this hospital might be due to limited usage of this drug in this hospital. In addition, rifampicin is reported to be used in combination with vancomycin or fusidic acid to treat MRSA infections which minimize the emergence of rifampicin-resistant MRSA (Tang *et al.*, 2013; University Malaya Medical Centre, 2014).

Of concern, the MRSA strains showed highest resistance to erythromycin, ciprofloxacin, clindamycin and gentamicin in this study. These findings were consistent with the previous studies which reported high resistances of MRSA strains to erythromycin, gentamicin and ciprofloxacin in UMMC from year 2003 and 2008 (Lim *et al.*, 2013; Thong *et al.*, 2009) as well as in Hospital Universiti Sains Malaysia (HUSM) from 2002 to 2007 (Hassanain *et al.*, 2010). The high resistances to the antimicrobials mentioned above were also reported in the National Antibiotic Guideline 2014 where

MRSA strains from 37 Malaysian hospitals were tested (Ministry of Health Malaysia, 2014). Erythromycin, ciprofloxacin and clindamycin are used to treat respiratory tract infections and other MRSA infections such as osteomyelitis, skin and soft tissue infections in both primary care and hospital. Gentamicin is used in combination with cloxacillin, vancomycin or rifampicin for endocarditis (University Malaya Medical Centre, 2014).

HA-MRSA is often multidrug-resistant towards erythromycin, ciprofloxacin, clindamycin and gentamicin because of the presence of multiple antimicrobial resistance genes in the SCC*mec* gene cassette (David & Daum, 2010; Thong *et al.*, 2009). Traditionally, CA-MRSA is known to be susceptible to most non-β-lactams antibiotics (Weigelt, 2016). However, since 2003, studies have reported the increasing non-β-lactams resistances in CA-MRSA isolates (Wang *et al.*, 2012). For example, in the US, clindamycin resistance rates among CA-MRSA isolates were 3 to 24% (Hulten *et al.*, 2006). Wang *et al.* (2012) have reported resistances rates of CA-MRSA to erythromycin, ciprofloxacin, clindamycin and gentamicin resistance were 85.9%, 19%, 92% and 14.7% (Wang *et al.*, 2012). In published studies in India, CA-MRSA was found to be resistant to clindamycin, ciprofloxacin, erythromycin and gentamicin (Alvarez-Uria & Reddy, 2012; Indian Network for Surveillance of Antimicrobial Resistance group *et al.*, 2013; Kali *et al.*, 2013).

According to our data, the rates of erythromycin, ciprofloxacin, clindamycin and gentamicin resistances among CA-MRSA strains are 11.7%, 14%, 7.5% and 14.6%, respectively. Whereas, the resistances rates of erythromycin, ciprofloxacin, clindamycin and gentamicin among HA-MRSA strains are 88.3%, 85.9%, 92.5% and 85.4%, respectively. Hence, high resistances to these antimicrobials which can be observed from

this study is not surprising. Strategic plan such as lowering the selective pressure by limiting the use of antimicrobials should be implemented to prevent further resistance to more antimicrobials (Holmes *et al.*, 2015).

Based on the vancomycin susceptibility testing results, all MRSA strains from year 2011 to 2013 were sensitive to vancomycin. There was significant fluctuation in the rate of MRSA strains with vancomycin MIC ≥ 1.5μg/mL (p = 0.014). This MIC trend appeared to significantly increased between 2011 and 2012, but was slightly decreased in 2013 which might be due to the large variability among the MRSA strains each year (Sancak *et al.*, 2013) or might be the result from the decreased in the number of particular factor associated with high vancomycin MIC. Based on the statistical analysis, SCC*mec* type III was significantly associated high vancomycin MIC whereas SCC*mec* type IV was associated with low vancomycin MIC. This was supported by studies done by Jang *et al.* (2012) and Holmes *et al.* (2014) where both reported similar findings (Holmes *et al.*, 2014; Jang *et al.*, 2012). The decreased in the number of MRSA strains carrying SCC*mec* type III from year 2011 (n = 61; 64.2%) to 2013 (n = 38; 55.1%) might be the cause of decreased in the number of MRSA strains with high vancomycin MIC in 2013. The rate of MRSA strains with vancomycin MIC < 1.5 μg/mL was significantly decreased from 2011 to 2013 (p = 0.011). Overall, no vancomycin MIC creep was observed over a 3-year period. Nonetheless, the use of vancomycin as MRSA treatment should be monitored by long-term studies as vancomycin MIC creep phenomenon is possible to occur in the future and might lead to the emergence of VISA and VRSA strains.

Bacteraemia is the third most common nosocomial infection, following urinary tract infection and pneumonia (Weigelt, 2016). Nosocomial bacteraemia cases that were caused by coagulase-negative staphylococci (CONS), MRSA and enterococci are common in hospitals worldwide leading to high morbidity and mortality as well as treatment failure (Bamberger, 2007; Gopal Katherason *et al.*, 2010; Karchmer, 2000; Ok *et al.*, 2013; van Hal *et al.*, 2012). In Malaysia, 21% of bacteraemia cases were caused by MRSA (Ahmad *et al.*, 2010). The incidence of MRSA bacteraemia in this study showed significant increase from year 2011 and 2013. Furthermore, MRSA bacteraemia has an attributable mortality rate of 75.6%. Therefore, in order to determine the molecular characteristics of MRSA bacteraemia, 56.8% of MRSA strains isolated from blood were further typed by PFGE and MLST.

Genotyping by PFGE and MLST showed that the MRSA strains were genetically diverse with the presence of various MRSA clones transferred between wards and circulating in this hospital. Based on the PFGE dendrogram, 29 MRSA strains within Cluster I shared the similar characteristics of having SCC*mec* type IV and MLST ST22. SCC*mec* type IV-ST1137, also belonging to the same clonal complex (CC) as ST22, was also grouped in the same cluster (Cluster I). MRSA strains with the same MRSA clones SCC*mec* type IV-ST6 and SCC*mec* type IV-ST30 were assigned in Clusters VII and VIII, respectively.

Two MRSA strains with SCC*mec* type V-ST45, four SCC*mec* type V-ST772 strains, one SCC*mec* type V-ST5 strain and two SCC*mec* type V-ST1 strains were clustered in Clusters II, IV, V and VI, respectively. The untypeable strains with ST239 and ST508 as well as MRSA clones SCC*mec* type I-ST152 and SCC*mec* type IV-ST1 were not assigned to any clusters and appeared as singleton. The presence of SCC*mec*

type IV-ST22 and SCC*mec* type V-ST951 strains in Cluster III, untypeable-ST5 strain in Cluster V as well as SCC*mec* type III-ST239 and SCC*mec* type IV-ST1179 strains in Cluster VII showed possible genetic linkages with the other strains in their respective clusters.

In addition, the similar PFGE patterns between HA- and CA-MRSA strains indicated the possibility of spread of MRSA strains between the hospital and community. For instance, SCC*mec* type III-ST239 strains in the hospital and community shared more than 80% similarity in their PFGE patterns. The same were also observed among the HA- and CA-SCC*mec* type IV and V strains.

The majority of MRSA strains causing bacteraemia throughout the study belonged to SCC*mec* type III-ST239 in Cluster III and exhibited the major pulsotype E. The predominance of this pulsotype might be due to the increase in nosocomial transmission within this hospital. The strains of this pulsotype remained persistent in this hospital and identified as the endemic strain.

SCC*mec* type III-ST239, SCC*mec* type IV-ST6, ST22, ST30, ST1179 and SCC*mec* type V-ST1, ST772 were observed among MRSA strains causing bacteraemia from year 2011 to 2013. These findings concurred with those of Sam *et al.* (2008), Ahmad *et al.* (2009), Ghaznavi-Rad *et al.* (2010a), Lim *et al.* (2013) and Samat Muttaqillah *et al.* (2015) which reported the presence of HA-MRSA clone SCC*mec* type III-ST239 as well as CA-MRSA clones SCC*mec* type IV-ST6, ST22, ST30, ST1178, ST1179 and SCC*mec* type V-ST1, ST772 in Malaysian hospitals including UMMC, Universiti Kebangsaan Malaysia (UKM) and HKL (Ahmad *et al.*, 2009; Ghaznavi-Rad *et al.*, 2010a; Lim *et al.*, 2013; Sam *et al.*, 2008; Samat Muttaqillah *et al.*, 2015).

As observed from this study, the presence of HA-MRSA clones SCC*mec* type I-ST152, SCC*mec* type V-ST45, ST951 and SCC*mec* type IV-ST1137; as well as CA-MRSA clones SCC*mec* type V-ST5, untypeable-ST508 and SCC*mec* type IV-ST1 were described for the first time in Malaysia. These events proved that new strains being introduced and spread into this hospital. Therefore, it is essential to re-strategize the infection control measures for preventing the further spread of these variants of MRSA strains in this hospital.

Several studies have documented the existence of the above mentioned MRSA clones. For instance, ST152 was reported to cause PVL-positive CA-MRSA infections in Central Europe (Basanisi *et al.*, 2017; Ruimy *et al.*, 2008) and also known as HA-MRSA strain responsible for enhanced severity of infective endocarditis in Italy (Campanile *et al.*, 2012). CA-MRSA clone SCC*mec* type IV-ST1 was first reported in the 1980s among the aborigines in Australia and the US; and also has emerged in Rio de Janeiro, causing nosocomial bloodstream infections (Ferreira *et al.*, 2013). In addition, this strain was known to cause infection among the homeless and drug users in South London (Otter & French, 2008). SCC*mec* type V-ST5 strain was found among Japanese CA-MRSA isolates (Urushibara *et al.*, 2012) whereas ST951 was reportedly seen in healthy university student athletes (Champion *et al.*, 2014). A study in Urban Detroit revealed that ST45 strain causes MRSA bloodstream infections and was identified as the first endemic strain established in Germany, Netherlands and Canada (Moore *et al.*, 2010). ST508 isolate was found to cause community–acquired infections in Ghana and harboured SC*Cmec* type V (Egyir *et al.*, 2014). This isolate was also reported among the community settings in Taiwan and carried SCC*mec* type IV (Wang *et al.*, 2009). To our knowledge, there have been no study reporting the presence of SCC*mec* type IV-ST1137.

High genetic diversities among SCC*mec* types IV and V strains were observed based on the occurrence of various STs (ST1, ST5, ST6, ST22, ST30, ST45, ST772, ST951, ST1137 and ST1179). This indicated their abilities to transfer the genetic elements between different genetic clones of *S. aureus.* Based on PFGE, MRSA strains had greater diversity with Simpson's index of diversity, D of 0.960 (C.I.N.A. 0.942-0.977). On the contrary, MRSA strains were less diverse based on MLST with Simpson's index of diversity, D of 0.578 (C.I.N.A. 0.497-0.658). This showed PFGE is more discriminatory than MLST in typing the MRSA strains.

According to the data from this study, MRSA bacteraemia accounted for 56.8% of all infections. The incidence of MRSA bacteraemia was found to be significantly associated with old age ($p < 0.0001$), male gender ($p = 0.043$) and were caused by HA-MRSA ($p < 0.0001$). In addition, the cases were mostly primary bacteraemia accompanied by the presence of severe underlying diseases such as diabetes mellitus, hypertension and chronic kidney disease. Weigelt (2016) had stated the risk factors for MRSA bacteraemia include increased age, male gender and severe underlying diseases (diabetes and renal failure) (Weigelt, 2016). MRSA bacteraemia patients with SCC*mec* type IV strains were significantly correlated with cardiovascular diseases which was similar to the findings by Huang *et al.* (2007) (Huang *et al.*, 2007). Compared with other SCC*mec* types, SCC*mec* type V strain was significantly associated with implant-related bacteraemia. This finding was supported by a recent report in India by Prakash *et al.* (2016), that stated high prevalence of SCC*mec* types IV and V were present in device-associated infections (Prakash *et al.*, 2016).

The association between clinical, phenotypic and molecular variables was studied in order to determine the risk factors associated with patient's mortality. The overall mortality rate was 30.9%, with 63.9% of them were caused by HA-infections. No significant difference was found regarding vancomycin MIC status, MRSA types, presence of polymicrobial infections and SCC*mec* types among the deceased patients compared to those who survived. Old age, female gender, bacteraemia as diagnosis, respiratory diseases (including pneumonia, acute pulmonary haemorrhage, pulmonary embolism, chronic obstructive pulmonary disease and acute respiratory distress syndrome) as comorbidities and high APACHE II, Pitt bacteraemia and CCI scores were significantly associated with a trend towards increased mortality. On the contrary, age ≤ 50 years old, male gender and SSTIs as diagnosis were associated with a trend toward reduced mortality. These findings are consistent with studies done by van Hal *et al.* (2012), Pastagia *et al.* (2012) and Yahav *et al.* (2016) where they discussed predictors for mortality include older adults, female gender (despite the incidence of bacteremia is generally higher in males compared to females) and high severity scores (Pastagia *et al.*, 2012; van Hal *et al.*, 2012; Yahav *et al.*, 2016). The high mortality occurred among elderly patients probably because of several factors such as age-related reduced physiologic reserve capacity, decreased host resistance, presence of chronic underlying diseases, delays in diagnosis and therapy, poor tolerance to invasive diagnostic and therapeutic procedures, delayed or poor response to antimicrobial therapy and higher rates of adverse reactions to antibiotics (Yoshikawa, 2000). Poor management of MRSA bacteraemia such as inadequate or delayed antimicrobial therapy (empiric antibiotic therapy) may be one of the factors causing bacteraemia-associated mortality (Weigelt, 2016).

CHAPTER 6: CONCLUSION

This study demonstrated that increased age and male gender were the significant patient-associated risk factors for MRSA acquisition. Most of the infections were caused by HA-MRSA strains which carried SCC*mec* types I, II, III, IV and V, while the rest were CA-MRSA strains carrying SCC*mec* types III, IV and V. The occurrence of SCC*mec* types IV and V among hospital strains as well as SCC*mec* type III among CA-MRSA strains are alarming.

MRSA strains belonging to SCC*mec* type III remained predominant in this hospital during the three-year study period and the prevalence of PVL gene among MRSA strains was 1.8%. Based on the antimicrobial susceptibility pattern, all year 2013 MRSA strains were multidrug-resistant in which they were resistant to more than two classes of antimicrobial agents including penicillin, cefoxitin, ciprofloxacin, clindamycin, ceftriaxone, gentamicin, erythromycin, ceftazidime, rifampicin, fusidic acid, cotrimaxozole, colistin, amoxicillin-clavulanate and piperacillin-tazobactam. In addition, the erythromycin, ciprofloxacin, clindamycin and gentamicin resistance rates remained high while rifampicin and fusidic acid resistance rates remained low.

All MRSA strains in this study were sensitive to vancomycin. There was no vancomycin MIC creep observed during this three-year period based on the significant fluctuation in the rate of MRSA with vancomycin MIC $\geq$ 1.5µg/mL. However, the trend of vancomycin MIC requires continuous monitoring in the subsequent years. Furthermore, SCC*mec* type III was found to be significantly associated with high vancomycin MIC whereas SCC*mec* type IV was associated with low vancomycin MIC.

The characteristics of MRSA strains causing bacteraemia were studied as MRSA bacteraemia was known to cause significant morbidity and mortality as well as treatment failure. In this study, the number of MRSA bacteraemia cases were significantly increased from year 2011 to 2013 and were mostly primary bacteraemia. The important predictors for MRSA bacteraemia were identified including male gender, increased age, severe underlying medical conditions (diabetes mellitus, hypertension and chronic kidney disease) and origin of infection (HA-MRSA). Genotyping by PFGE and MLST showed that the MRSA strains were genetically diverse based on the presence of various MRSA clones transmitted between wards and circulating in this hospital in which a majority belonged to SCC*mec* type III with MLST ST239 and exhibited pulsotype E.

Prior to this study, there has yet to be any published reports in Malaysia on MRSA clones such as SCC*mec* type I-ST152, SCC*mec* type IV-ST1, SCC*mec* type IV-ST1137, untypeable-ST508, SCC*mec* type V-ST5, SCC*mec* type V-ST45 and SCC*mec* type V-ST951. This suggested that new strains were continuously introduced into this hospital which requires proper monitoring. Further studies should be conducted on the new MRSA clones as they may have epidemic and pathogenic potentials that could pose serious threat to public health.

Age, gender, diagnosis and presence of comorbidities were the identified as critical prognostic factors for patient's mortality. In addition, infection severity scores were also found to provide with excellent predictions of mortality. However, variables such as presence of polymicrobial infections, SCC*mec* types, MRSA types and vancomycin MIC status were not significant in our study.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

PUBLICATIONS

Sit, P. S., Teh, C. S. J., Idris, N., Kamarulzaman, A., Sam, I. C., Syed Omar, S. F., Sulaiman, H., Thong, K. L. and Ponnampalavanar, S. (2017). Prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) infection and the molecular characteristics of MRSA bacteraemia over a two-year period in a tertiary teaching hospital in Malaysia. *BMC Infectious Diseases*, 17(274), 1-14. doi:10.1186/s12879-017-2384-y.

Sit, P. S., Teh, C. S. J., Idris, N. and Ponnampalavanar, S. (2018). Methicillin-resistant *Staphylococcus aureus* (MRSA) bacteremia: Correlations between clinical, phenotypic, genotypic characteristics and mortality in a tertiary teaching hospital in Malaysia. *Infection, Genetics and Evolution*, 59C (2018), 132-141. doi:10.1016/j.meegid.2018.01.031.

PRESENTATIONS

Sit, P. S., Teh, C. S. J., Idris, N., Kamarulzaman, A., Sam, J. I. C., Syed Omar, S. F., Sulaiman, H., Thong, K. L. and Ponnampalavanar, S. 2016. SCC*mec* typing and PVL detection of Methicillin-Resistant *Staphylococcus aureus* (MRSA) strains isolated from a tertiary teaching hospital. International Postgraduate Research Awards Seminar, University of Malaya, 7- 8th March 2016 (Poster Presentation).

Sit, P. S., Teh, C. S. J., Idris, N., Kamarulzaman, A., Sam, J. I. C., Syed Omar, S. F., Sulaiman, H., Thong, K. L. and Ponnampalavanar, S. 2016. Molecular Characterization of Methicillin-Resistant *Staphylococcus aureus* (MRSA) strains isolated from a tertiary teaching hospital over a three-year period. Malaysian Society for Microbiology Postgraduate Seminar, University Kebangsaan Malaysia, 24th August 2016 (Poster Presentation).