MOLECULAR CHARACTERIZATION OF METHICILLIN-RESISTANT *Staphylococcus aureus* (MRSA) AND FACTORS ASSOCIATED WITH CLINICAL OUTCOMES IN MRSA-INFECTED PATIENTS AT THAMMASAT UNIVERSITY HOSPITAL

BY

MISS PIMONWAN PHOKHAPHAN

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most common nosocomial pathogens. Clinical outcomes by this pathogen ranges from skin and soft tissue infection, musculoskeleton infection, respiratory tract infection, and bloodstream infection. Treatment failure of MRSA infections have been increasingly common, leading to rising numbers of mortality and morbidity cases. Factors, either clinical factors or microbiological factors, which may be responsible for the treatment failure have not been investigated in Thammasat University Hospital. The aim of this study was to find the factors contributing to the outcomes of treatment with vancomycin in the patients infected by MRSA. MRSA strains were characterized by using several methods including broth micro-dilution method, E-test method, Population Analysis Profile (PAP), Polymerase chain reaction (PCR) technique, SCC*mec* typing and Pulsed field gel electrophoresis (PFGE). Regression analysis

method was used to analyze the association of clinical and microbiological factors with the treatment outcomes. A total 101 MRSA isolates were collected at Thammasat University Hospital during August 2012 to July 2015. Although vancomycin, teicoplanin and linezolid, still remained as effective antibiotics for treatment of all MRSA infections, it was found that the vancomycin MICs creep rate was 31%. There were no significant differences in terms of clinical failure rate between patients with MRSA isolates with vancomycin MICs creep or non-creep. By using univariate regression analysis, heterogeneous vancomycin intermediate *S. aureus* (hVISA) strains were found to be significantly associated with the treatment failure in patients with MRSA infections (p =0.03). The multivariate regression analysis indicated that chronic hemodialysis (OR=5.77; 95% CI: 1.7-19.49; p = 0.00), multiple sites infection (OR=3.71; 95% CI: 1.08-12.77; p =0.03), co-microbe resistant strains (OR=3.68; 95% CI: 1.16-11.65; p =0.02) were significant factors associated with clinical failure in MRSA-infected patients. All MRSA isolates were positive for *mecA*, *femA* and *icaA* genes, whereas all isolates were negative for *sec*, *sed, eta, etb, tst*, and *pvl* genes. Furthermore, *sea, seb,* and *see* genes were found in 43%, 38% and 43% of all isolates. Six different virulence genes patterns designated type I (40%), II (37%), III (19%), IV (2%), V (2%) and VI (1%) were exhibited. The isolates were classified as SCC*mec* type I (36%), type II (41%) and type III (24%). PFGE differentiated all MRSA isolates into 17 patterns, designated as types A-Q. The majority of isolates were type A (16%). There were no significant associations between the specific virulence gene pattern, SCC*mec*-type, PFGE pattern and clinical outcomes. In conclusion, the findings of this study showed the factors that were associated with the treatment failure among patients infected with MRSA. Along with providing standard medical treatment, closely monitoring and controlling these factors may improve the overall outcomes of patients suffered from the MRSA infections.

Keywords: Methicillin-resistant *Staphylococcus aureus* (MRSA), Vancomycin, Minimum inhibitory concentration creep (MIC creep), Pulse field gel electrophoresis, Virulence genes, Antibiogram, Clinical outcome

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Miss Pimonwan Phokhaphan

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LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
A	adenine
C	cytosine
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleotide-5'-triphosphate
EDTA	ethelenediaminetetra-acetic acid
e.g.	for example
G	guanine
hr	hour (s)
M	molar
µl	microlitre (s)
µM	micromolar
µg	microgram (s)
mg	milligram (s)
$MgCl_2$	magnesium chloride
min	minute (s)
ml	millilitre (s)
mM	millimolar
NaCl	sodium chloride
ng	nanogram (s)
PCR	polymerase chain reaction
RNase	ribonuclease
rpm	revolutions per minute
sec	second (s)
T	thymine
UV	ultraviolet

CHAPTER 1
INTRODUCTION

1.1 Introduction

Staphylococcus aureus is a major cause of several infections, and treatment mainly relies on the use of antibiotics (Chen & Huang, 2014; Ogston, 1984). Methicillin is an antibiotic that was first used to treat *S. aureus* infections in 1959. However, two years later, the first reported case of methicillin-resistant *S. aureus* (MRSA) occurred in the United Kingdom (Moellering, 2012). Since then, MRSA, and its expanding resistance to multiple drugs, has proliferated throughout the world (Chambers & Deleo, 2009; Falagas, Karageorgopoulos, Leptidis, & Korbila, 2013).

Several reports have revealed that MRSA is one of the main causes of hospital-acquired (HA) and community-acquired (CA) infections (Boucher, Miller, & Razonable, 2010; Deresinski, 2005). These HA infections were mostly found in blood, urinary tract, respiratory tract, and surgical sites (Bouza et al., 2012; Lawes et al., 2015). Transmission usually takes place during direct contact with a person who had an active infection, a carrier of the infection, or a contaminated object (Kelly & Monson, 2012).

Although the prevalence of HA-MRSA infection is now decreasing in European countries, the proportion of MRSA isolates is very high in East Asia, particularly in Sri Lanka, South Korea, Vietnam, Taiwan, and Thailand (Stefani *et al*., 2012). The Asian Network for Surveillance of Resistant Pathogens (ANSORP) study revealed MRSA accounted for 67.4% of HA *S. aureus* infections and 25.5% of CA *S. aureus* infections among Asian countries (Song *et al*., 2011) In Thailand, it was found that MRSA was responsible for 57% of HA *S. aureus* infections while the MRSA proportion among CA *S. aureus* infection was only 2.5% (Song *et al*., 2011). The mortality rate of HA-MRSA infections is higher than that of CA-MRSA infections, mainly

due to hospital interventions, more frequent uses of devices, and the virulence nature of causative strains (Groeneveld, 2009). Thus, it is important to conduct surveillance monitoring of the spread of MRSA both within the healthcare setting and communities.

Resistance to methicillin in *S. aureus* is conferred by the structural changes of penicillin-binding protein, the target of beta-lactam. As a result, MRSA strains are resistant to nearly all beta-lactam antibiotics. Vancomycin, a glycopeptide antibiotic, has generally been considered the drug of choice for MRSA infections for over 50 years (Levine, 2006). Nevertheless, there have been reports on the increase in vancomycin MICs (MIC creeps) of MRSA isolates (Chang *et al*., 2015; Dhand & Sakoulas, 2012). Despite being in the MIC range that is considered susceptible, MRSA strains with vancomycin MIC ≥1.5-2 ug/ml are more likely associated with treatment failure (Lodise *et al*., 2008) and higher mortality rates (van Hal, Lodise, & Paterson, 2012; Wang, Wang, Sheng, Chen, & Chang, 2010).

Previous studies have shown that certain clinical and microbiological factors have been associated with vancomycin treatment failure in MRSA infections. Risk associated with failure was first indicated by the increasing vancomycin MIC (Sakoulas *et al*., 2004). Endocarditis and vancomycin trough levels were predictors related with microbiological eradication failure (Forstner *et al*., 2013). In addition, the independent predictors of mortality associated with MRSA infection included receipt of inappropriate empirical therapy, increasing age, use of corticosteroids, fatal underlying diseases, high-risk and intermediate-risk sources of bacteremia, shock, high MIC isolates (2 μg/ml), pneumonia, post-cardiothoracic surgery, and a high Charlson Comorbidity Index (Soriano *et al*., 2008; Wang *et al*., 2010). In our study, all factors mentioned will be collected from medical records to determine the reason for the vancomycin treatment failure.

In addition, heteroresistant vancomycin-intermediate *S. aureus* (hVISA) strains were reported to be significantly related to vancomycin failure treatment (Casapao et al., 2013; Charles, Ward, Johnson, Howden, & Grayson, 2004) and high mortality rate compared to vancomycin-susceptible *S. aureus* (VSSA) (H. C. Hu et al., 2015). It is thus necessary for clinical laboratories to screen for the strain. These strains were largely found in the Spain (73.7%), Australia (37.6%) and Turkey (18%) but were low in Thailand (2.9%), Italy (1.1%), Korea (0.6%) and Japan (0.5%)(Panomket, Thirat, Wanram, & Sranujit, 2014; van Hal & Paterson, 2011). The screening methods for detecting hVISA included standard E-test, macrodilution E-test, glycopeptides resistance detection (GRD) and agar screening. Because hVISA is a heterogeneous and small subpopulation, these methods have variable sensitivity and specificity and poor accuracy when using a single method (Howden, Davies, Johnson, Stinear, & Grayson, 2010; Sader, Jones, Rossi, & Rybak, 2009; Wootton, MacGowan, Walsh, & Howe, 2007). It is known that the modified population analysis profile (PAP) has the highest accuracy; hence, it is the gold standard confirmation of hVISA (Khatib et al., 2015; Wootton et al., 2001). However, this method is time consuming, labor-intensive and cumbersome. Currently, there are no consistently accurate molecular markers to detect hVISA.

Furthermore, MRSA strains produce several virulent factors including toxic shock syndrome toxin-1, staphylococcal enterotoxin, panton-valentine leukocidin, and polysaccharide intercellular adhesion (Gillet et al., 2007; Gordon & Lowy, 2008; Prindeze et al., 2014). Therefore, it is of utmost importance to investigate the possibility of an association between these microbiological factors and clinical outcomes of patients infected with MRSA at Thammasat University Hospital.

Various strains of MRSA have been isolated globally. It is necessary to characterize the isolates for epidemiological study to monitor incidence trends, distribution changes, and emerging problems (Bonita, 2006). The characterization of

MRSA at the molecular level is significantly useful for insightful information to identify its origin, surveillance, and ultimately control the spread.

At the present time, there are several molecular tools for genotyping. For example, pulsed-field gel electrophoresis (PFGE), considered the gold standard for genotyping, has a high discriminatory power for MRSA characterization; however, this method is technically difficult and time consuming (Szabo, 2014). Multilocus sequence typing (MLST) is based on single nucleotide variation of housekeeping genes; the allelic profile can easily be compared in a central database via the internet but is costly (Chambers & Deleo, 2009; Ibarz Pavon & Maiden, 2009). *Spa* typing is based on variable number tandem repeats in the gene encoding protein A; accordingly, this rapid technique is suitable for the investigation of local/global *S. aureus* outbreak (Chambers & Deleo, 2009).

Staphylococcal cassette chromosome *mec* (SCC*mec*) typing is demonstrated by multiplex PCR. SCC*mec* is a mobile chromosomal element composite of the *mec* and *ccr* gene, which encode the methicillin resistance complex and the recombinases responsible for the mobility of SCC*mec*, respectively (Turlej, Hryniewicz, & Empel, 2011). SCC*mec* type I, II, and III are usually found in HA-MRSA strains, while SCC*mec* type IV and V are commonly detected in CA-MRSA strains (Hanssen & Ericson Sollid, 2006; Wilailuckana et al., 2006). Thus, SCC*mec* typing is the recommended method to distinguish between the HA-MRSA and CA-MRSA strains (Szabo, 2014).

Infectious diseases due to drug-resistant microbes are often severe and fatal. Common pathogens include MRSA, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Stenotrophomas maltophilia*, *Klebsiella* spp., *Escherichia coli* and *Enterococcus* spp. (Caini, Hajdu, Kurcz, & Borocz, 2013; Cardoso et al., 2014). These infections erode quality of life, with long hospitalization and a high treatment costs. In Thailand, the prevalence and mortality of patients with HA infection due to

multidrug resistant (MDR) pathogens is high (Lim et al., 2016). *S. aureus* is the second most common pathogen of bacteremia (19%), and nearly all MDR *S. aureus* are MRSA (92%) (Lim et al., 2016). As mentioned before, MRSA with vancomycin MIC creep or unresponsive strains have become more common worldwide. This challenge of incurable MRSA infection must be contemplated by all medical sectors, public health authorities, and the government.

At Thammasat University Hospital, there have been only limited data on the MRSA strains and their antibiotic profiles, especially the vancomycin MIC and hVISA. Data on the determinants correlated with vancomycin treatment failure and genotypic characteristics of MRSA strains are also needed. Therefore, this study will explore clinical and microbiological factors involved in vancomycin efficacy of treatment. In brief, the clinical data collected from medical records including basic demographics of patients, infection sites, underlying diseases, current medications, and type of infections will be examined. In addition, microbiological data including vancomycin MIC, antimicrobial susceptibility testing pattern (antibiogram), detection of hVISA by PAP, and virulence genes by polymerase chain reaction (PCR) will be studied. The isolates will be characterized at the molecular level using PFGE technique and SCC*mec* typing. It is hoped that this study will generate detailed information as a basis for future research in the assessment of medical treatment, monitoring and surveillance of drug-resistant bacteria spread.

1.1 Objectives

1.1.1 Overall objective

This study aims to determine clinical and microbiological factors associated with the treatment outcomes of MRSA-infected patients and molecular characterization of MRSA isolates from Thammasat University Hospital.

1.1.2 Specific objectives

1.1.2.1 To analyze the clinical and microbiological factors associated with clinical outcomes in MRSA-infected patients.

1.1.2.2 To investigate vancomycin MIC creep.

1.1.2.3 To determine the antimicrobial susceptibility of MRSA.

1.1.2.4 To detect hVISA strain of MRSA.

1.1.2.5 To detect virulence genes of MRSA.

1.1.2.6 To characterize and compare MRSA by molecular tools including pulse field gel electrophoresis (PFGE) and SCC*mec* typing.

1.2 Research problem

Treatment failures of MRSA infection have been widespread with unclear reasons. The association of clinical factors and/or microbiological characteristics with treatment outcomes was reported in previous studies (Forstner *et al*., 2013; Soriano *et al*., 2008; Wang *et al*., 2010). These clinical factors were associated with higher mortality rate, including receipt of inappropriate empirical therapy, high vancomycin MIC (2 μg/ml), pneumonia, post-cardiothoracic surgery and a high Charlson Comorbidity Index. Moreover, endocarditis and vancomycin trough levels were correlated with microbiological eradication failure. The clinical outcomes of MRSA infected patients at Thammasat University Hospital have not been analyzed for detailed and insightful information to help physicians overcome the infections. Therefore, the MRSA isolates from Thammasat University Hospital will be scrutinized at molecular level and examined for possible clinical characteristics involving in MRSA treatment failure.

1.2.1 Which clinical and/or microbiological factors relate to clinical outcomes including successful and/or failure outcomes?

1.2.1 Which microbiological factors of MRSA strains are associated with clinical outcomes?

1.2.2 Which molecular typing tools used in this study generate diversity with high discriminative power to investigate origins or spread of MRSA infections?

1.3 Outcomes and benefits

The obtained results of specific factors associated clinical outcomes will be used for the planning of efficient treatment.

1.3.1 The antimicrobial susceptibility patterns will provide useful data for treatment with the appropriate drugs of choice.

1.3.2 Epidemiology data will be meaningful for future research in various aspects of prevention and control the spread of MRSA.

CHAPTER 2
REVIEW OF LITERATURE

2.1 Historical background of *S. aureus*

S. aureus was discovered in 1880 by Alexander Ogston, a Scottish surgeon. It was found in a knee joint surgical abscess (Licitra, 2013). *S. aureus* is gram-positive cocci, with a 1 um diameter, classified in the family Micrococcaceae. They usually form grape-like clusters, are non-motile and non-spore forming, facultative anaerobic, coagulase positive, and present in golden colonies. The nasal passage and axillae are a natural habitat of *S. aureus* (Foster, 1996; Lan, Cheng, Dunman, Missiakas, & He, 2010).

2.2 Pathogenesis and virulence factors of *S. aureus*

Staphylococcal infection showed various clinical manifestations with mild to severe symptoms such as superficial skin lesions, localized abscesses, osteomyelitis, endocarditis, and serious skin infections. *S. aureus* has been reported as a major causes of hospital-acquired infections, particularly associated with indwelling medical devices, are *S. aureus* and *S. epidermidis* (Chessa et al., 2016). Moreover, its enterotoxins were detected in contaminated food, resulting in food poisoning; these same enterotoxins are also responsible for toxic shock syndrome, due to bloodstream super antigens. *S. saprophiticus* is frequently found in urinary tract infections. Other coagulase-negative strains include *S. lugdunensis, S. haemolyticus, S. warneri, S. schleiferi* and *S. intermedius*, but these are not commonly pathogenic. *S. aureus* is the most common cause of staphylococcus infection in people (Tong, Davis, Eichenberger, Holland, & Fowler, 2015).

Direct invasion and epithelial cell damage caused by staphylococci are important pathogenesis mechanisms. Several potential virulence factors include surface proteins which enhance host tissue colonization, capsules and immunoglobulin-binding protein A promoting phagocytosis inhibition, and toxins causing cell and tissue damage. In addition, some enzymes, including coagulase, catalase, hyaluronidase, staphylokinase, lipase and *β*-lactamase, are associated with disease pathogenesis caused by *S. aureus* (Foster, 1996).

The expression in coagulase-negative staphylococci is normally less severe (Baron, 1996). The detected *S. aureus* virulence factors are shown in Table 1.

2.2.1 Cell structures

The cell wall obviously plays an important role for *S. aureus* pathogenesis and anti-immune response system (Gordon & Lowy, 2008). The capsule is another factor promoting the inhibition of phagocytosis and adhesion to medical devices, such as catheters and prosthetic devices (Foster, 1996; Nanra et al., 2013), leading to the collection and increase of the disease-causing bacterial cells. Protein A, found in the *S. aureus* cell wall, is connected to peptidoglycan and adheres to the Fc receptor of immunoglobulin G (IgG), resulting in phagocytosis inhibition (Foster, 1996). Moreover, many proteins on the cell membrane, including collagen, fibronectin and fibrinogen, so-called “clumping factors”, adhere to host tissues (Gordon & Lowy, 2008); consequently, *S. aureus* is able to colonize and proliferate in infected patients.

Table 1: Virulence factors associated with pathogenesis of *S. aureus* infection

Factors	**Roles**
Capsule	Inhibition of phagocytosis and adherence to medical devices
Coagulase	Transfer of fibrinogen to fibrin, gathering of bacterial cells that is surrounded by fibrin
Exfoliative toxin (ET)	Staphylococcal scalded skin syndrome (SSSS)
Hyaluronidase	Degradation of hyaluronic acid tissue component
Hemolysin	Degradation of red blood cell
Leucosidin	Cytoplasmic membrane leakage of white blood cell
Lipase	Degradation of lipids on skin
Protein A	Binding with Fc receptor of antibody and inhibition of phagocytosis
Toxic shock syndrome toxin (TSST)	Food poisoning

2.2.2 Enzymes

The cell wall obviously plays an important role for *S. aureus* pathogenesis and anti-immune response system (Gordon & Lowy, 2008). The capsule is another factor promoting the inhibition of phagocytosis and adhesion to medical devices, such as catheters and prosthetic devices (Foster, 1996; Nanra et al., 2013), leading to the collection and increase of the disease-causing bacterial cells. Protein A, found in the *S. aureus* cell wall, is connected to peptidoglycan and adheres to the Fc receptor of immunoglobulin G (IgG), resulting in phagocytosis inhibition (Foster, 1996). Moreover, many proteins on the cell membrane, including collagen, fibronectin and fibrinogen, so-called "clumping factors", adhere to host tissues (Gordon & Lowy, 2008); consequently, *S. aureus* is able to colonize and proliferate in infected patients.

2.2.2.1 Coagulase

S. aureus can produce coagulase (*Coa*) and the Von-Willebrand factor binding protein (*vWbp*), which are not found in other species. The enzymes activate prothrombin in order to convert fibrinogen to fibrin, which promotes plasma or blood clots. Therefore, phagocytosis is inhibited; subsequently, the bacterial cells are protected (Cheng et al., 2010).

2.2.2.2 Catalase

Catalase is able to disintegrate hydrogen peroxide into water and oxygen in order to protect bacterial cells from white blood cells. White blood cells are responsible for producing hydrogen peroxide and free radicals, causing cell damage (Das & Bishayi, 2010).

2.2.2.3 Hyaluronidase

This enzyme can break up hyaluronic acid, a component of connective tissue, resulting in bacterial spread and tissue infection, the so-called "spreading factor" (Ibberson et al., 2014).

2.2.2.4 Staphylokinase

Staphylokinase converts plasminogen to plasmin and disintegrates fibrin on the infection site, leading to rapid host tissue infection. The majority of *S. aureus* can produce this enzyme are a common cause of skin and soft tissue infections (Wieckowska-Szakiel, Sadowska, & Rozalska, 2007).

2.2.2.5 Lipase

Lipase plays an important role in skin and soft tissue infection. Both types of tissue contain gland lipid and adipose tissue; lipase mechanism degrades lipids (C. Hu, Xiong, Zhang, Rayner, & Chen, 2012).

2.2.2.6 B-lactamase

Resistance to antibiotics is due to B-lactamase produced by *S.aureus* (C. C. Fuda, Fisher, & Mobashery, 2005).

2.2.3 Toxins

2.2.3.1 Cytotoxins

Cytotoxins, or hemolysins, cause lysis of red blood cells. There are four different forms of cytotoxins: alpha, beta, delta and gamma. Each has a particular role in host cell damage. Alpha-toxin causes leakage of host cell membranes, unbalancing of substance, and osmotic pressure, all activating the stress-signaling pathways of cell death (Bien, Sokolova, & Bozko, 2011; Foster, 1996). Beta-toxin hydrolyzes sphingomyelin, which makes up the host cell plasma membrane, causing cell lysis and death (Bien et al., 2011; Otto, 2014). Gamma-toxin combines with leucocidin, causing an imbalance in osmotic pressure and eventually leads to cell lysis and death.

Panton valentine leukocidin (PVL) is an important attribute in necrotizing skin infections (Foster, 1996).

2.2.3.2 Exfoliative toxins

Human-infected *S. aureus* strains produce mainly exfoliative toxin A (ET-A) and B (ET-B). These toxins are the leading cause of staphylococcal scalded skin syndrome (SSSS)(Bukowski, Wladyka, & Dubin, 2010).

2.2.3.3 Toxic shock syndrome toxin-1(TSST-1)

TSST-1 causes toxic shock syndrome. It is a super antigen, causing non-specific immune responses and the over secretion of several cytokines (Todd, Fishaut, Kapral, & Welch, 1978)

2.2.3.4 Enterotoxin

Staphylococcal enterotoxins (SEs) are heat-stable and cause toxic shock-like syndromes and food poisoning. The enterotoxins designated Staphylococcal Enterotoxins A (SEA) to Staphylococcal Enterotoxins IV (SEIV), are also super antigens similar to TSST-1 (Ortega, Abriouel, Lucas, & Galvez, 2010; Schelin et al., 2011).

2.3 Diseases caused by *S. aureus* infection

2.3.1 Skin and soft tissue infections

Many skin infections, such as impetigo, furuncle, carbuncle, wound infection and staphylococcal scalded skin syndrome, have been reported (McCaig, McDonald, Mandal, & Jernigan, 2006; Patel & Finlay, 2003).

2.3.2 Bacteremia

The major sources of bacteremia are skin and soft tissue infections, followed by osteomyelitis or septic arthritis, pneumonia, urinary tract, IV catheter, and abscess (Paulsen et al., 2015).

2.3.3 Pneumonia

Three types of pneumonia are caused by *S. aureus*: hospital-acquired pneumonia (HAP), ventilator-associated pneumonia (VAP), and health care-associated pneumonia (HCAP) (American Thoracic & Infectious Diseases Society of, 2005). Risk factors are longer duration of ventilator support, chronic obstructive pulmonary disease (COPD), prior bronchoscopy, prior use of corticosteroids, and prior antibiotic exposure (Park, 2005). Patients can develop secondary pneumonia infection supporting hematogenous dissemination from another site, including soft tissue infection (Naraqi & McDonnell, 1981).

2.3.4 Necrotizing soft tissue infections

Leukocyte destruction and tissue necrosis due to Panton valentine leukocidin is associated with necrotizing pneumonia (Monaco, Antonucci, Palange, Venditti, & Pantosti, 2005).

2.3.5 Food poisoning

Staphylococcal enterotoxins, especially SEA, are a common cause of food poisoning (Pinchuk, Beswick, & Reyes, 2010). The main source of food contamination is improper handling. The toxins cannot be destroyed by cooking. Symptoms are of speedy onset, such as severe vomiting, nausea, with or without diarrhea (Argudin, Mendoza, & Rodicio, 2010).

2.3.6 Osteomyelitis

Hematogenous osteomyelitis or directly receiving of pathogen into bone from nearby chronic wound or skin and soft tissue infection was reported (Hatzenbuehler & Pulling, 2011).

2.4 Epidemiology of HA-MRSA and CA-MRSA infections

Previous studies revealed that prevalence rate of HA-MRSA in European countries has declined, while in Asian countries prevalence remains high (Chen & Huang, 2014; Stefani et al., 2012). The highest HA-MRSA rates were found in South American countries such as Peru and Chile (80%), followed by Asian nations such as Hong Kong (75%), Bangladesh (53.1%), North India (46%), Thailand (41.5%), Singapore (35%), Malaysia (26%) (Grema, 2015). The prevalence rate of HA-MRSA infections fluctuates due to distinct hospitals and differing time periods (Chen & Huang, 2014). Moreover, incidence rate of CA-MRSA infection was less frequent in Asia (23.1%) than in Europe (37.4%) and North America (47.4%)(S. Li et al., 2014).

The Asian Network for Surveillance of Resistant Pathogens showed predominant clones of HA-MRSA spreading in Asian countries include ST239 and ST5, while CA-MRSA clones are ST30, ST59 and ST72 (Song et al., 2011). The most common lineage of CA-MRSA in the USA, California and European countries like Austria, Denmark, Finland, Germany, Italy, Spain, Switzerland, and the UK was USA300 (H. Huang et al., 2006; Johnson, 2011; Nimmo, 2012). The prevalence rate of USA300 CA-MRSA in European countries is smaller than the United States (Diekema et al., 2014; Glaser et al., 2016; Toleman et al., 2016). Furthermore, USA300 strain was able to cause both community and hospital-acquired infections. In addition, predominant HA-MRSA in the United States was USA100 and also known as “New York/Japan” clone (Carrel, Perencevich, & David, 2015; Chambers & Deleo, 2009). However, some reports indicated

that the USA300 clone replaced the USA100 HA-MRSA as the predominant clone in Metropolitan New York and predominance all regions in the US (Diekema et al., 2014; Pardos de la Gandara et al., 2016).

Table 2: Prevalence rates of CA-MRSA and HA-MRSA infection

Country	Overall MRSA	CA-MRSA	HA-MRSA	Remark	References
Western United States (51 rural hospitals)	724	44%	56%	18.75% of VRE cases had no prior healthcare exposure.	(Stevenson, Searle, Stoddard, & Samore, 2005)
United States (The U.S. CF center: children with cystic fibrosis)	49%	32%	68%	83% of patients were hospitalized prior to acquisition of MRSA.	(Harik et al., 2016)
United States (143 medical centers)	8,437	84.3%	15.7%	Susceptibility rates were generally lower among HA-MRSA compared to CA-MRSA.	(Sader, Mendes, Jones, & Flamm, 2016)
California (The University of California, Davis, Medical Center)	283	44.9%	-	Injecting drug users could be a major reservoir for CA-MRSA transmission.	(H. Huang et al., 2006)

Table 2: Prevalence rates of CA-MRSA and HA-MRSA infection (Continue)

Country	Overall MRSA	CA-MRSA	HA-MRSA	Remark	References
Pakistan (The Dermatology Department of Combined Military Hospital)	44.8%	60%	40%	A pyogenic skin and soft tissue infection was high.	(M. K. Ahmad & Asrar, 2014)
Thailand (Siriraj Hospital)	41.5%	0.9%	-	These patients could probably be HA-MRSA infections.	(Mekviwattanawong, Srifuengfung, Chokepaibulkit, Lohsiriwat, & Thamlikitkul, 2006)
India (Tertiary-care hospital)	52.2%	60.4%	39.6%	ST217 (the first time in India)	(Bouchiat et al., 2015)
South India (Tertiary care hospitals)	210	49%	51%	CA-MRSA (SSTI) and HA-MRSA (bacteremia)	(Veni Emilda, 2016)

2.5 Epidemiology of hVISA, VISA and VRSA strains

2.5.1 Heteroresistant vancomycin-intermediate *S. aureus* (hVISA)

The hVISA strain Mu3, was first report in MRSA infected pneumonia patient from Japan in 1997 (Hiramatsu, Aritaka, et al., 1997). In Thailand, the first report of hVISA was identified from University hospital designated strain MR135, MR187 and MR209 in 1998, 1996 and 1999, respectively (Trakulsomboon et al., 2001). Later, the first pediatric case of hVISA, who had truncus arteriosus type IV and ventricular septal defect developed with MRSA bacteremia and endocarditis after operation at Siriraj Hospital, Thailand in 2002 (Phongsamart et al., 2005). All cases of these reviews showed that the patients did not respond to vancomycin or combination with any antibiotics, except MR209-infected patient was not treated with any glycopeptide.

The prevalence of hVISA identified in Thailand was found in Samprasitthiprasong Hospital (2.9%) (Panomket et al., 2014), King Chulalongkorn Memorial Hospital (6.35%) (Pongkumpai, 2010) and Srinagarind Hospital (2.2%) (Lulitanond et al., 2009). This strain is not frequently isolated in routine practice due to inefficient detection method. However, there were many reports of VISA strains described in research study, therefore the hVISA reports are rare.

In addition, the strain was reported in other Asian countries including South Korea (6.1%), India (6.3%), Japan (8.2%) (Song et al., 2004), China (13.1%) (Sun et al., 2009) Singapore (12.5%) (Fong, Low, Koh, & Kurup, 2009), Taiwan (8.1%) (Lin et al., 2012) and Vietnam (7%) (D. R. Chung et al., 2015). A systematic review in 1997 to 2014 demonstrated that the prevalence rate of hVISA strain was higher in Asian countries (6.81%) than Europe/America (5.60%) (S. Zhang, Sun, Chang, Dai, & Ma, 2015). The prevalence in each country was based on the genotype distribution of MRSA strains.

2.5.2 Vancomycin-intermediate *S. aureus* (VISA)

The VISA strain was discovered from Japan in 1997 (Hiramatsu, Hanaki, et al., 1997). It was the threat of treatment failure. A number of VISA strains were reported globally. These strains were evolved from MRSA infected patients as previously observed between VISA and preexisting MRSA by Pulse field gel electrophoresis patterns (Franchi et al., 1999, Smithet al., 1999). The first report of VISA in Thailand was isolated from Srinagarind Hospital in 2006-2007 (Lulitanond et al., 2009). The VISA strain is rare to in Thailand. The prevalence rate of the strain in other Asian countries showed in Japan (0.33%) (Hanaki et al., 2007), Korea (0.09%) (G. Chung et al., 2010) and Taiwan (2.7%)(S. H. Huang et al., 2016). A systematic review appeared that it has been increasing worldwide, especially during 2010-2014 (S. Zhang et al., 2015). Interestingly, the first report of VISA isolated from a patient who had never received vancomycin treatment, was found in China (Zhu et al., 2015).

2.5.3 Vancomycin-resistant *S. aureus* (VRSA)

The first case of VRSA infection was identified from the United States In 2002 (Centers for Disease & Prevention, 2002). Two cases were reported from Michigan in 2007 (Finks et al., 2009). In Europe, the strain was first isolated in Portugal (Melo-Cristino, Resina, Manuel, Lito, & Ramirez, 2013). The prevalence rate of VRSA occurred in Southern Iran (9.2%) and Egypt (14%) (Sarrafzadeh, 2016; Shindia, 2011). The VRSA infection remains rare, and not yet detected in Thailand.

2.6 Vancomycin MIC creep and association with outcomes

Several researches demonstrated that trend of vancomycin MIC concentration has been increasing worldwide. In Hong Kong, the MIC of 1μg/ml significantly increased from 10.4% (between 1997-1999) to 38.3% (between 2006-2008) (Ho et al., 2010). Additional study in North Dakota, USA showed the vancomycin MIC of ≥2 μg/ml was significantly increased from 1998-2000 (12%) to 2007-2008(57.8%), and this is called as vancomycin MIC creep event (Kincaid, 2011). A survey of the trend of vancomycin MIC creep has not been reported in Thailand.

However, some surveys did not find the vancomycin MIC creep event in their institutes (Bardossy, 2016; Joana, Pedro, Elsa, & Filomena, 2013). For example, in Portugal, the majority of MIC concentration was still 1.5 μg/ml (40.9%) in 2007-2009 (Joana et al., 2013), and in Michigan, USA, the mean MIC was 1.57± 0.26 μg/ml in 2005-2014 (Bardossy, 2016).

Nowadays, many reports have expressed their concerns over the vancomycin treatment failure in patients infected with MRSA, particularly those with bloodstream infection. Many studies have attempted to find the predictors associated with success or failure outcome of treatment. It has been shown that the predictors of mortality or outcome failure from MRSA infection included higher vancomycin MIC (2 μg/ml), source of bacteremia, hVISA strain, ICU admission, receipt of inappropriate empirical therapy, increasing age, use of corticosteroids, ultimately or rapidly fatal underlying disease, and shock (Casapao et al., 2013; Koh, Kim, Chang, & Yi, 2016; Moore et al., 2011; Soriano et al., 2008). In contrast, an infectious disease consultation was related with lower risk for death (Pastagia, Kleinman, Lacerda de la Cruz, & Jenkins, 2012).

Interestingly, even though there are many studies that demonstrated the correlation of high vancomycin MIC (≥1.5 to ≥2 μg/ml) and mortality, failure or poor clinical outcome (Hidayat, Hsu, Quist, Shriner, & Wong-Beringer, 2006; Lodise et al., 2008; Sakoulas et al., 2004), some researches indicated that vancomycin MIC value did not predict the outcome or mortality rate in patient with MRSA infection (Kalil, Van Schooneveld, Fey, & Rupp, 2014; Kincaid, 2011; Rojas et al., 2012).

2.7 Mechanism of drug resistant of *S. aureus*

2.7.1 B-lactam antibiotics resistance of *S. aureus*

The mechanism of resistance to beta-lactam antibiotics of MRSA is the alternation of penicillin-binding proteins (PBPs) into PBP2a, which has a low affinity for the antibiotics. This PBP2a is encoded by the *mecA* gene (C. Fuda, Suvorov, Vakulenko, & Mobashery, 2004). This gene is carried by the Staphylococcal cassette chromosome *mec* (SCC*mec*) genetic which is a mobile genetic element of *S. aureus* (Katayama, Ito, & Hiramatsu, 2000). The expression of *mecA* gene prevents the inhibition of cell wall synthesis, ultimately leading to replication of MRSA.

2.7.2 Heteroresistant vancomycin-intermediate *S. aureus* (hVISA)

The genetic mechanism responsible for the resistance of hVISA strain is unclear, but common phenotypic feature with resistance to vancomycin of the strain is similar to vancomycin intermediate *S. aureus* (VISA) (Devi, 2015).

2.7.3 Vancomycin intermediate *S. aureus* (VISA)

In VISA, bacterial cell walls become thicker than normal. An increase of D-alanyl-D-alanine, a target site of vancomycin, is observed. As a result, the drug is insufficient and cannot penetrate through a thick cell wall (Howden et al., 2010). Currently, there is no evidence of *Van* genes in VISA strains, and the molecular mechanism of resistance in this strain has not yet been cleared.

2.7.4 Vancomycin resistant *S. aureus* (VRSA)

The vancomycin binding site, D-alanyl-D-alanine, is modified to D-alanyl-D-lactate which has low affinity or cannot to bind vancomycin. The *VanA* gene regulates the enzyme for synthesizes the precursor of this VRSA binding site (Gardete & Tomasz, 2014; Perichon & Courvalin, 2009).

2.7.5 Vancomycin MIC creep

Vancomycin MIC creep is known as reduced susceptibility to vancomycin. The common phenotype of vancomycin MIC creep is cell wall thickness which results in high MICs (Cui et al., 2003; Falcon et al., 2016).

2.7.6 Gene associated with cell wall thickening

Genes or mutation points implicated with cell wall thickening such as *graR, graS, mgrA* (Cui, Lian, Neoh, Reyes, & Hiramatsu, 2005)*, cmk* (Matsuo, Cui, Kim, & Hiramatsu, 2013) and *rpoB* genes (Saito et al., 2014) are likely to be involved.

2.8 Antibiotics for MRSA infections

2.8.1 Vancomycin

Vancomycin is the drug of choice for the MRSA infections. It has bactericidal effect on gram-positive cocci. The mechanism of action is by permanently binding with the Acyl-D-alanyl-D-alanine (cell wall precursor), leading to inability of bacteria to synthesize cell wall (Tverdek, Crank, & Segreti, 2008).

The monitoring of vancomycin trough level should be performed in order to maintain the effective concentration in serum at 15-20 μg/ml (known as therapeutic target) (Tverdek et al., 2008). Although higher doses may help with better outcome, previous study indicated that trough concentration of ≥15 μg/ml led to increase the risk of nephrotoxicity (van Hal, Paterson, & Lodise, 2013). The drug can distribute to most tissues, but tissue penetration into the lung and cerebral spinal

fluid may be poor (Kollef, 2007; X. Li et al., 2015). Red man syndrome is the most common side effect (Tverdek et al., 2008).

2.8.2 Linezolid

Linezolid is an oxazolidinone antibiotic with bacteriostatic effect. It inhibits protein synthesis on gram-positive bacteria (Tverdek et al., 2008). This drug can be given by intravenous injection and oral form. Some researchers suggest that linezolid has more survival and clinical cure rates than vancomycin in patients with hospital-acquired pneumonia due to MRSA (Wunderink, Rello, Cammarata, Croos-Dabrera, & Kollef, 2003), and it is super eminent to vancomycin for treatment of complicated skin and soft tissue infections due to MRSA (Weigelt et al., 2005). Linezolid is effective, but it is very expensive. Therefore, the drug should be used in case of treatment failure or in patients infected with highly resistant strains such as hVISA (Rayner & Munckhof, 2005).

2.8.3 Daptomycin

Daptomycin is a cyclic lipopeptide antibiotic with bactericidal activity. It acts on cell membrane of MRSA. The drug is effective for the treatment of skin and skin structure infections and bloodstream infections (Tverdek et al., 2008). Researcher revealed that using daptomycin has better outcome compared with vancomycin for treatment of bacteremia caused by MRSA with higher vancomycin MICs (Moore et al., 2012).

2.8.4 Tigecycline

Tigecycline is a glycylcyclines antibiotic with bacteriostatic activity on gram-negative and gram–positive bacteria, including MRSA. The drug can be used for treatment in few severe of skin and skin-structure infections and intra-abdominal infections (Tverdek et al., 2008). However, this drug is associated with the increase of mortality rate in patient with hospital-acquired pneumonia when compare to

comparator drugs (Yahav, Lador, Paul, & Leibovici, 2011). Thus, tigecycline should be used as a last-resort drug.

2.8.5 Other drugs

Patients with non-serious infections caused by MRSA strains can be treated with clindamycin or sulfamethoxazole-trimethoprim when the pathogens are susceptible. Other antibiotics against MRSA infection include aminoglycosides, mupirocin, fusidic acid and fosfomycin.

2.9 Molecular epidemiology of MRSA infection surveillance

Molecular tools such as MLST, PFGE, spa typing, SCC*mec* typing and DNA sequencing may have roles in the surveillance and monitoring of drug-resistant strains in hospitals. For example, the most epidemic genotypes of hVISA/VISA strains in HA-MRSA infection are belonged to SCC*mec* II and III groups as classified by SCC*mec* typing, or they are ST239 and ST5 as genotyped by MLST typing (S. Zhang et al., 2015). Surveillance system using molecular typing generates reference strains at both local and regional levels. The epidemiologic data is useful for creation the guidance of prevention and reduction of MRSA infection and spread.

2.9.1 Multilocus sequence typing (MLST)

MLST identifies the variations of single nucleotide of 7 housekeeping genes, which are called alleles. The typing provides a discriminatory allelic profile known as sequence type (ST). The STs are grouped into clonal complexes (CC), which is generated by the consideration of closed relationship in genetic loci (Chambers & Deleo, 2009).

2.9.2 Pulsed-filed gel electrophoresis (PFGE)

Genomic DNA fragments are digested by SmaI enzyme. It is separated in an agarose gel by pulsed-filed gel electrophoresis. Closely strains are grouped according to an 80% similarity coefficient. The method is suitable for assessment of more recent evolution between groups of strains(Chambers & Deleo, 2009).

2.9.3 Staphylococcal chromosome cassette *mec* (SCC*mec*)

The SCC*mec* is mobile cassette element, which is integrated into *orfX* (location of horizontal gene transfer). There are eight SCC*mec* allotypes that is designated as type I trough VIII. *mecA* gene is located in this element. The recombination, excision and insertion sequence of all cassettes into *orfX* are

encoded by gene complexes, which include *mec* and cassette chromosome recombinase (*ccr*). Two genes complex are used to classify SCC*mec* types. The *mecA gene* complex is divided into 3 classes including class A, B and C *mec*. The *ccr* gene complex includes two adjoining genes *ccrA-ccrB* and *ccrA-ccrC*. SCC*mec* I-IV, VI and VIII consist of *ccrA* and *ccrB*, and SCC*mec* V and VII consist of *ccrA* and *ccrC* (Chambers & Deleo, 2009).

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Study design

A retrospective cohort study was conducted using chart review during July 2012 to August 2015 at Thammasat University Hospital (TUH). The samples were selected by purposive sampling method.

3.2 Inclusion criteria

The patients included in this study were those who were under vancomycin treatment for MRSA infection with complete treatment and record of admission at TUH; furthermore, the patient had to be alive at follow-up. In cases of multiple-site MRSA infections, only the first isolate was included.

3.3 Exclusion criteria

Patients who were transferred to another hospital and/or passed away before the end of treatment were excluded from this study.

3.4 Sample size calculation

According to Carmen & Betsy (2007), if a study has six or more independent variables, the following equation is valid for regression analysis, (Carmen & Betsy, 2007).

n = 10 x number of independent variables.

This study has approximately ten independent variables, thus the number of sample size (n) is 100 subjects.

3.5 Data collection

The factors associated with clinical and microbiological outcomes were designed as described in previous study (Casapao et al., 2013; Koh et al., 2016; Moore et al., 2011; Pastagia et al., 2012; Soriano et al., 2008). The data collection form was developed as follows:

Data collection form

ID Code.....

Clinical characteristics

- Sex ☐ Male ☐ Female
- Age .....years old
- Elderly (>65 years old) ☐ Yes ☐ No
- Admission date .....
- Discharge date .....
- Length of stay .....
- Culture positive date .....
- Culture negative date .....
- Days to negative culture .....

General

- Prior MRSA infection in previous 3 month ☐ Yes ☐ No
- Prior vancomycin exposure ☐ Yes ☐ No
- Hospitalization within 1 month of MRSA infection ☐ Yes ☐ No
- Hospitalization ≥ 3 months before MRSA infection ☐ Yes ☐ No
- ICU admission before MRSA infection ☐ Yes ☐ No
- Type of intensive care unit .....
- Length of Intensive care unit stay .....
- Infectious diseases consultation ☐ Yes ☐ No

Infection source

- Central venous catheter ☐ Yes ☐ No
- Pneumonia ☐ Yes ☐ No
- Endocarditis ☐ Yes ☐ No
- Wound/skin or soft tissue ☐ Yes ☐ No
- Bone/joint ☐ Yes ☐ No
- Vascular graft ☐ Yes ☐ No
- Surgical site ☐ Yes ☐ No
- Others (specify) .....

Concurrent conditions

- Chronic kidney disease ☐ Yes ☐ No
- Acute kidney injury ☐ Yes ☐ No
- Chronic hemodialysis ☐ Yes ☐ No
- Cerebrovascular disease ☐ Yes ☐ No
- Cardiovascular diseases ☐ Yes ☐ No
- Congestive heart failure ☐ Yes ☐ No
- Cirrhosis/ Liver cirrhosis ☐ Yes ☐ No
- Diabetes mellitus ☐ Yes ☐ No
- Cancer ☐ Yes ☐ No
- AIDS ☐ Yes ☐ No
- Recent surgery ☐ Yes ☐ No
- Recent cardio-thoracic surgery ☐ Yes ☐ No
- Transplant ☐ Yes ☐ No
- Immunosuppressant use ☐ Yes ☐ No
- Implanted device ☐ Yes ☐ No
- Bedridden ☐ Yes ☐ No
- Others (specify) .....

Severity of disease

- Charlson Comorbidity Index (CCI) .....

Drug

- Adequate glycopeptides use within 48 hours ☐ Yes ☐ No
- Inappropriate antimicrobial drug therapy ☐ Yes ☐ No
- Adjustment of vancomycin trough level ☐ Yes ☐ No
- Vancomycin trough level (mg/L) in first week .....
- Final vancomycin trough level (mg/L) .....
- Detail adverse reaction .....
- Others current medication
  - Carbapenem ☐ Yes ☐ No
  - Aminoglycoside ☐ Yes ☐ No
  - Cefalosporin ☐ Yes ☐ No
  - Penicillin ☐ Yes ☐ No
  - Quinolone ☐ Yes ☐ No
  - Colistin ☐ Yes ☐ No
  - B-Lactam/B-Lactam Inhibitor ☐ Yes ☐ No
  - Others .....

Type of infection

- Hospital-acquired infection (HA) ☐ Yes ☐ No
- Community-acquired infection (CA) ☐ Yes ☐ No

Clinical outcome

Success

- Cure ☐ Yes ☐ No
- Improvement ☐ Yes ☐ No

Failure

- Persistence ☐ Yes ☐ No
- Recurrence ☐ Yes ☐ No

Microbiological outcome

Success

- Eradication ☐ Yes ☐ No

Failure

- Persistence ☐ Yes ☐ No
- Resistance ☐ Yes ☐ No
- Not available ☐ Yes ☐ No

Discharge status ☐ Die ☐ Survive

Microbiological characteristics

- Vancomycin MIC creep (µg/mL) .....
- Others MIC (specify) .....
- MRSA strains
  - VSSA ☐ Yes ☐ No
  - hVISA ☐ Yes ☐ No
- Co-infection other microbes .....
- Virulence genes .....

Note: .....

.....

3.6 Definitions

3.6.1 Vancomycin MIC creep

MRSA strain is susceptible to vancomycin with a minimal inhibitory concentration (MIC) of ≤ 1.5 to 2 μg/ml.

3.6.2 Days to negative culture

Duration indicates that growth of microorganisms is not found in culture after patients treated with vancomycin.

3.6.3 Inappropriate antimicrobial drug therapy

Patients infected with MRSA who are not treated with vancomycin within 48 hours.

3.6.4 Clinical cure

Patients do not have clinical signs/symptoms or blood culture evidence of MRSA infection after <7 days of vancomycin treatment or a sterile site culture evidence <21 days of vancomycin treatment (Howden et al., 2004).

3.6.5 Clinical improvement

Patients have a decrease of clinical signs and symptoms of MRSA bloodstream infection after <7 days of vancomycin treatment but still present with blood culture. Another sterile site infection were considered at <21 days of vancomycin treatment. New signs and symptoms that are related with the original infection are not detected (Howden et al., 2004; Lau et al., 2006).

3.6.6 Death within 28 days

Patients die within 28 days due to MRSA infection. Death is considered to be related to infection if one of the following criteria are present: (1) a culture test is positive for MRSA at the time of death; (2) MRSA infection is specified as a cause of death on discharge summary; (3) death occurs before the end of signs and symptoms of MRSA infection (Forstner et al., 2013).

3.6.7 Clinical persistence

Symptoms of infection that have not improved or worsened after finishing the vancomycin treatment (Kullar, Davis, Levine, & Rybak, 2011).

3.6.8 Clinical recurrence

Patients present with new signs/symptoms associated with the original infection and culture evidence of MRSA infection after completion of vancomycin treatment.

3.6.9 Microbiological eradication

MRSA bloodstream infection is eliminated <7 days after patient treated with vancomycin or a sterile site infection is eliminated <21 days after patient treated with vancomycin (Howden et al., 2004).

3.6.10 Microbiological persistence

Blood culture is positive for MRSA ≥7 days after patient treated with vancomycin or sterile site culture is positive for MRSA after ≥21 days of vancomycin treatment (Howden et al., 2004).

3.6.11 Microbiological resistance

The MRSA strain develops vancomycin resistance after patient treated with vancomycin.

3.6.12 Microbiological non-available

Chart review shows no evidence of the MRSA infection after treatment finished.

3.6.13 Hospital-acquired infection

Hospital acquired infection, also known as nosocomial infection, is defined when the isolation of MRSA or signs of infection occur more than 48 hours after admission. Signs/symptoms were not present before admission (Garner, Jarvis, Emori, Horan, & Hughes, 1996). The symptoms of the infection may appear after hospital discharge. The incubation period of MRSA is 4 to 10 days after exposure (Hawker, Begg, Blair, Reintjes, & Weinberg, 2008).

3.6.14 Community-acquired infection

An MRSA culture or symptoms/signs of infection appear before admission or within 48 hours after admission. The treatment history may include using a medical device that was not present before admission(namely hospitalization or a long-term care in year before infection), presence of medical device, prior dialysis in previous year, prior MRSA infection or colonization, and surgery (Buck et al., 2005).

3.7 Clinical and microbiological outcome assessment

The outcomes were assessed at the end of vancomycin treatment using clinical and microbiological data. Clinical success is defined as the patient who has been cured or has improved, while clinical failure is defined as recurrence or persistence. In addition, microbiological success is defined as eradication; microbiological failure is defined as resistance or persistence.

This study aims to discover the factors associated with clinical and microbiological outcomes by logistic regression analysis. Patients are divided into two groups: Group 1 consists of patients with vancomycin MIC creep, and Group 2 consists of patients with non-vancomycin MICs creep.

Descriptive statistics were implemented to summarize patients' characteristics as frequency, proportion, mean and standard deviation (SD). For categorical data, the chi-square test was used to compare differences between the two groups. A p-value less than or equal to 0.05 was considered statistically significant.

Logistic regression analysis was performed to find the factors associated with outcomes. The predictive power of an independent variable was estimated with univariable analysis of odds ratio. A p-value less than or equal to 0.25 was selected for the multi-variable regression model, using forward stepwise regression. Co-variables in this model having a p-value less than 0.05 were correlated with outcomes. All data was analyzed using SPSS software.

3.8 Bacterial strains

A total of 101 MRSA strains were analyzed from 99 inpatients. Clinical specimens were obtained from Microbiology Laboratory at Thammasat University Hospital from August 2012 to July 2015. There were four specimen types including sputum, blood, pus, and urine. Nine standard strains were used in order to verify the accuracy of assays that shown in Table 3.

Table 3: *S. aureus* strains used as control in this study***

Standard strains	Target control
SA ATCC 700698*	Modified population analysis profile (PAP)
SA ATCC 29213**	Antimicrobial susceptibility and PAP
NCTC 10442	SCC*mec* type I
85/2082	SCC*mec* type III
MR108	SCC*mec* type IV
N315	SCC*mec* type II, *fem*A, and *mec*A genes
KKU-MS1	*seb* and *tst* genes
KKU-MS3	*sea* gene
KKU-MS14	*sec* and *pvl* genes

* Heteroresistant vancomycin-intermediate *S. aureus* (hVISA); also known as Mu3 strain,

** B-lactamase positive and Vancomycin-susceptible *S. aureus* (VSSA),

*** All *S. aureus* strains were donated by Assoc. Prof. Dr. Aroonlug Lulitanond, Faculty of Associated Medical Sciences, Khon Kaen University.

3.9 Bacterial storage

The strains were cultured overnight on nutrient agar (NA) (Difco, USA). The inoculum was kept in a 2 ml Sterile Cryogenic Vial (Corning®, Mexico) containing 1.5 ml of 15% (v/v) Glycerol solution. The vials were then stored at -80°C.

3.10 Preparation of vancomycin

Vancomycin hydrochloride from *Streptomyces orientalis* (Sigma-Aldrich, China) was used. The amount of powder for standard solutions was determined by the following formula (CLSI, 2012).

$$\text{Weight (mg)} = \frac{\text{Volume (ml)} \bullet \text{Concentration (µg/ml)}}{\text{Potency (µg/mg)}}$$

$$\text{Weight (mg)} = \frac{1 \text{ (ml)} \bullet 10{,}000 \text{ (µg/ml)}}{900 \text{ (µg/mg)}}$$

Weight (mg) = 11.1111 mg or 11,111 µg

Stock and working solution of vancomycin

Vancomycin powder of 11,111 µg was dissolved in 10 ml of sterile distilled water. A concentration of 1,000 µg/ml was used as a stock solution: CLSI recommends at least 1,000 µg/ml; 32 µg/ml of working solution was calculated by the following formula.

$$\text{Volume1 (ml)} = \frac{\text{Concentration2 (µg/ml)} \bullet \text{Volume2 (ml)}}{\text{Concentration1 (µg/ml)}}$$

$$\text{Volume1 (ml)} = \frac{32\ (\text{µg/ml}) \bullet 10\ (\text{ml})}{1{,}000\ (\text{µg/ml})}$$

Volume1 = 0.32 ml or 320 µl

Thus, a volume of 9,680 µl sterile distilled water should be added to 320 µl of stock solution. This working solution was aliquoted into 1 ml portions, and stored at -20 °C.

3.11 Antimicrobial susceptibility testing

Antimicrobial susceptibility tests were used to determine the susceptibility of microorganism to antimicrobial agent. The result was regularly reported as the minimal inhibitory concentration (MIC), which is the lowest concentration of antimicrobial agent that inhibits the growth of the microbes. Reports are commonly shown as quantitative results in terms of µg/ml, and as qualitative interpretation divided into susceptible (S), intermediate (I), and resistant (R). For this study, the MIC of vancomycin was determined using broth microdilution method in accordance with CLSI guidelines (CLSI, 2007 2012) and also E-test method according to the manufacturer's instructions. Vancomycin MIC is categorized as MIC creep or non-creep by E-test method with quantitative wide-scale MICs. However, this method produces one twofold dilution higher than the MIC determined by broth microdilution method (Prakash, 2008). Thus, the purpose of broth microdilution

method is to compare the MICs generated by E-test. The MICs of other antimicrobial agents were performed only by E-test method as shown in Table 4.

Table 4: Antimicrobial agent used for susceptibility test

Antimicrobial agent	Code	Concentration range (µg/ml)
Vancomycin	VAN	0.016-256
Trimethoprim-Sulfamethoxazole	SXT	0.002-32
Fusidic acid	FUS	0.016-256
Rifampicin	RIF	0.002-32
Trimethoprim	TMP	0.002-32
Cefpirome	CPR	0.016-256
Clindamycin	CLI	0.016-256
Mupirocin	MUP	0.064-1024
Linezolid	LZD	0.016-256
Teicoplanin	TEC	0.016-256

3.11.1 E-test

E-test is a quantitative assay for MIC of antimicrobial agents against microorganisms. The MIC Test Strip (Liofilchem®, Italy) is impregnated with concentration gradient of antimicrobial agents which presents MIC scale (µg/ml) (Table 4).

Using direct colony suspension method, the MRSA isolates (4-5 fresh colonies) of an overnight NA culture were inoculated into 3-5ml Mueller-Hinton Broth (MHB) (Difco, USA). The inoculum suspension was adjusted to 0.5 McFarland standard ($1x10^8$ CFU/ml) and then freshly performed within 15 minute after preparation. A total volume of 200 µl of inoculum was added to Mueller-Hinton Agar (MHA) (Oxoid, England) using sterile swab, and then a 10-15 minute wait to make sure the surface is completely dry. The MIC test strip was placed on the plate using sterile forceps, incubated at 37°C for 16-20 hours; 24 hours for vancomycin. The MIC value

is read from the scale or point of intersection of an elliptical zone of inhibition. The MICs breakpoints of antimicrobial agents are interpreted as shown (Table 5).

Table 5: MIC interpretive standards for *Staphylococcus* spp. (CLSI, 2007)

Antimicrobial agent	MIC breakpoints (µg/ml)		
	Resistant	Intermediate	Susceptible
Vancomycin [a]	≥16	4-8	≤2
Trimethoprim-Sulfamethoxazole	≥4	-	≤2
Fusidic acid [b]	>1	-	≤1
Rifampicin	≥4	2	≤1
Trimethoprim	≥16	-	≤8
Cefpirome [c]	≥32	16	≤8
Clindamycin	≥4	1-2	≤0.5
Mupirocin [d]	≥512	8-256	≤4
Linezolid	≥8	-	≤4
Teicoplanin	≥32	16	≤8

[a] Heteroresistant vancomycin-intermediate *S. aureus* (hVISA) may be found in vancomycin MIC of 1-2 µg/ml (CLSI, 2012), [b] Using the EUCAST standard (EUCAST, 2016), [c] Using the same criteria as cefepime (Ishii, Alba, Kimura, & Yamaguchi, 2006), [d] According to journal (de Oliveira, Cardozo, Marques Ede, dos Santos, & Giambiagi-deMarval, 2007)

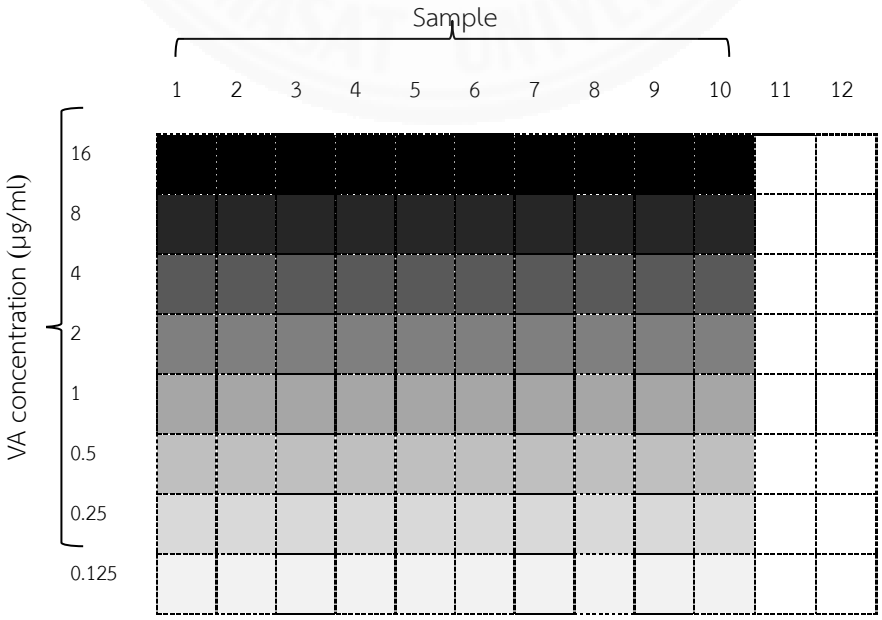
3.11.2 Broth microdilution method

It is the most common method for susceptibility testing of antimicrobial agents against microorganisms using sterile 96-well plate (Corning®, USA).

Sterile distilled water 50 µl was put into each well and 50 µl working solution of vancomycin was then added to the first row of the plates. This mixture was then transferred to the next well and continued serially in two-fold dilution.

Fresh inoculum ($1x10^8$ CFU/ml) was diluted 1:200 with sterile MHB (approximately $5x10^4$ CFU/ml), and 50 µl of this newly prepared inoculum was added into each well. The plate was incubated at 37°C for 24 hours on incubator shaker. The negative and positive controls were included. The positive control strain was *Staphylococcus aureus* (SA) ATCC 29213. The MIC value was read from a clear well. The acceptable control range for vancomycin MIC of SA ATCC 29213 is 0.5-2 µg/ml (CLSI, 2007).

Minimal bactericidal concentration (MBC), the lowest concentration of antimicrobial agents that kills microorganisms, is determined by subculturing the suspension from the well at MIC value onto an NA agar plate and then incubated at 37°C for 24 hours.



3.12 Modified population analysis profile (PAP) method (Modified method from Sirichoat et al., 2016)

Detection of heteroresistant vancomycin-intermediate Staphylococcus aureus (hVISA) was performed. Two-fold dilutions of bacterial suspension were plated onto the Brain-Heart Infusion (BHI) agar (Difco, USA) containing different concentrations of vancomycin. The colony counts of each isolate were plotted a graph. The area under the curve (AUC) was calculated. In this study, this method was modified from Sirichoat and colleagues (2016).

A fresh overnight MRSA culture was transferred into 5 ml BHI broth and, incubated at 37°C for 24 hours. Bacterial suspension was adjusted to the 0.5 McFarland standard, diluted in sterile saline from undiluted to 10-6. The diluted inoculum (100 µl) were then spread on each plate of BHA containing 0.125, 0.25, 0.5, 1, 2, 4 µg/ml of vancomycin, incubated at 37°C for 48 hours. Colonies (log10 number of CFU/ml) of each isolate were counted, and these were then plotted against the vancomycin concentration on semi-logarithmic graph paper. This graph was used to calculate the AUC. The ratio of the AUC of MRSA isolates divided by the AUC of the corresponding strain (Mu3) was calculated. The AUC ratios of <0.9, 0.9 to 1.3, and >1.3 were considered as VSSA, hVISA, and VISA strains, respectively.

3.13 DNA extraction

Total DNA was extracted by Genomic DNA Extraction Mini Kit (RBC Bioscience, Taiwan). Five steps were used to isolate and purify the DNA from MRSA strains, so called cell lysis, RNA degradation, DNA binding, washing, and DNA elution. The concentration and purity of DNA were then measured.

3.13.1 Cell lysis

The MRSA inoculum in a microcentrifuge tube was centrifuged for 1 minute at 13,000 rpm, and supernatant was discarded. Two hundred microliters of Lysozyme buffer were added and vortexed to re-suspend the cell pellet. Then this tube was incubated at room temperature for 10 minutes, and it was inverted every 2-3 minutes. Two hundred microliters of GB buffer were added to the sample and vortexed for 5 seconds to mix sample and incubated at 70°C for 10 minutes with inversion every three minutes until the sample lysate was clear.

3.13.2 RNA degradation

Following the step of cell lysis, five microliters of RNase A (10mg/ml) (Thermo Scientific, USA) were added to sample lysate and vortexed to mix sample and incubated at room temperature for 5 minutes.

3.13.3 DNA binding

Two hundred microliters of absolute ethanol were added to the sample lysate and vortexed to mix sample immediately for 10 seconds. Genomic DNA (GD) column was placed on a 2 ml collection tube. The mixture from previous step was added to the GD column, and the cap was closed. Then the tube was centrifuged at 13,000 rpm for 2 minutes. The flow-through was discarded, and the GD column was taken to the new collection tube for washing.

3.13.4 Washing

The GD columm was washed twice by using first washing with W1 buffer (400 µl) added to the GD column and then centrifuged at 13,000 rpm for 30 seconds. The flow-through was discarded, and the GD column was returned to the 2 ml collection tube. The second washing was performed by adding 600 µl of wash buffer containing ethanol to the GD column and centrifuged at 13,000 rpm for 30 seconds. At the end, the tube was centrifuged at full speed for 3 minutes to dry the column matrix.

3.13.5 DNA elution

The dried GD column was transferred into a clean 2 ml microcentrifuge tube. The elution buffer was preheated at 70°C before adding 100 µl of the preheated buffer to the centre of the column matrix. It was allowed to stand for 2 minutes to be absorbed by the matrix. Then the purified DNA was eluted by centrifugation at 13,000 rpm for 30 seconds. The DNA was measured for concentration (ng/µl) and purity of DNA by NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA).The ratio of absorption (260/280 nm) of pure DNA sample was 1.7-1.9. The pure DNA was then used as a template for PCR assay.

3.14 Identification of MRSA using PCR assay

Detection of *mecA* gene by PCR is a gold standard for identification of methicillin resistance of *S. aureus*. The amplified fragment size of the *mec* gene is 162 base pairs.

3.14.1 PCR master mix preparation

The PCR mixture consisted of 50 µl of a mixture of 1x PCR buffer: 2mM MgCl2, 200µM mixture of deoxynucleoside triphosphates (dATP, dGTP, dCTP, and dTTP) (Thermo Scientific, USA), 0.2 µM of each primer (Integrated DNA Technologies, Singapore), 1.25 U of Thermo Scientific Maxima Hot Stat Taq DNA

polymerase (Thermo Scientific, USA), and 20 ng of DNA template. The *mecA* gene was amplified using primers as previously described (Oliveira & de Lencastre, 2002). The primers are 5' -TCC AGA TTA CAA CTT CAC CAG G-3' and 5'-CCA CTT CAT ATC TTG TAA CG-3'.

3.14.2 PCR amplification conditions

The amplification was performed using a MyCycler™ Thermal Cycler (Bio-Rad, USA). Thermocycler conditions were as follows: initial denaturation: 94°C, 2 minutes; denaturation: 94°C, 30 seconds; annealing: 51°C, 30 seconds; extension: 72°C, 1 minute and final extension: 72°C, 5 minutes.

3.14.3 DNA fragments separation

The PCR products were mixed with 6x DNA Loading Dye (Thermo Scientific, USA), and 10 µl of mixtures were then loaded into a well of 1.5% (w/v) SeaGar® Agarose Gel (P2 Innovation Ltd., UK) in 1x TBE buffer containing GelStar™ Nucleic Acid Gel Stain (Lonza, USA). A GeneRuler 100 bp DNA ladder (Thermo Scientific, USA) was used as DNA marker. After running gel electrophoresis (Bio-RAD, USA) completely at 100 volts for 60 minutes, the gel was visualized under UV light on Gel Documentation (Alpha Innotech, USA).

3.15 Virulence genes detection using PCR assay

Detection of virulence genes was performed by using specific primers of *femA, icaA*, *sea*, *seb*, *sec*, *see*, *tst*, and *pvl* as shown Table 6. PCR master mix was performed as previously described.

3.15.1 PCR amplification conditions

Thermocycling conditions included initial denaturation step at 94°C for 2 minutes followed by 30 cycles of 94°C for 30 seconds, the annealing temperature based on the length and GC content of each primer (Table 6) for 30 s,

72°C for 1 minute, ending with a final extension step at 72°C for 7 minutes and a hold at 4°C.

3.15.2 DNA fragments separation

The amplified fragments were analyzed by 1.5% (w/v) agarose gel electrophoresis at 100 volts. The DNA was stained with GelStar™ Nucleic Acid Gel Stain and visualized under UV light on Gel Documentation. A 100 bp DNA ladder was used as a standard marker. Target DNA amplicon sizes of each virulence genes are shown in Table 6.

Table 6: Primers used for virulence gene detection

Primer*	Oligonucleotide sequence (5' -3')	Amplicon size (bp)	Anealing temp.(°C)	References
femA-F	AAA AAA GCA CAT AAC AAG CG	132	49	(Mehrotra, Wang, & Johnson, 2000)
femA-R	GAT AAA GAA GAA ACC AGC AG			
sea-F	GGT TAT CAA TGT GCG GGT GG	102	55	
sea-R	CGG CAC TTT TTT CTC TTC GG			
seb-F	GTA TGG TGG TGT AAC TGA GC	164	51	
seb-R	CCA AAT AGT GAC GAG TTA GG			
sec-F	AGA TGA AGT AGT TGA TGT GTA TGG	451	51	
sec-R	CAC ACT TTT AGA ATC AAC CG			
see-F	AGG TTT TTT CAC AGG TCA TCC	209	51	
see-R	CTT TTT TTT CTT CGG TCA ATC			
tst-F	ACC CCT GTT CCC TTA TCA TC	326	51	
tst-R	TTT TCA GTA TTT GTA ACG CC			
icaA-F	GAT TAT GTA ATG TGC TTG GA	770	56	(Kumar, Negi, Gaur, & Khanna, 2009)
icaA-R	ACT ACT GCT GCG TTA ATA AT			
pvl-F	ATC ATT AGG TAA AAT GTC TGG ACA TGA TCC A	433	56	(Lina et al., 1999)
pvl-R	GCA TCA AST GTA TTG GAT AGC AAA AGC			

3.16 SCC*mec* typing by multiplex PCR assay

A multiplex PCR assay was categorized SCC*mec* genes using multiple different DNA sequences simultaneously. Specific primers for SCC*mec* typing included type I, II, III, and IVc as shown Table 7. The PCR master mix was prepared as described previously.

3.16.1 PCR amplification conditions

Thermocycling conditions included initial denaturation at 94°C for 5 minutes followed by 10 cycles of 94°C for 45 seconds, 65°C for 45 seconds, and 72°C for 1.5 minutes. Further 25 cycles of 94°C for 45 seconds, 55°C for 45 seconds, 72°C for 1.5 minutes, continuing with a final extension step at 72°C for 10 minutes and holding the temperature at 4°C after PCR completion was used.

3.16.2 DNA fragments separation

The amplified fragment was detected by 1.5% (w/v) agarose gel electrophoresis as described previously. Target DNA amplicon sizes of SCC*mec* type are shown in Table 7.

.

Table 7: Primers used for SCC*mec* typing

Primer*	**Oligonucleotide sequence (5' -3')**	**Amplicon size (bp)**	**Specificity**	**Reference**
Type I-F	GCTTTAAAGAGTGTCGTTACAGG	613	SCC*mec* I	(K. Zhang, McClure, Elsayed, Louie, & Conly, 2005)
Type I-R	GTTCTCTCATAGTATGACGTCC			
Type II-F	CGTTGAAGATGATGAAGCG	398	SCC*mec* II	
Type II-R	CGAAATCAATGGTTAATGGACC			
Type III-F	CCATATTGTGTACGATGCG	280	SCC*mec* III	
Type III-R	CCTTAGTTGTCGTAACAGATCG			
Type IVc-F	ACAATATTTGTATTATCGGAGAGC	200	SCC*mec* IVc	
Type IVc-R	TTGGTATGAGGTATTGCTGG			

3.17 Modified Pulsed-field gel electrophoresis (PFGE) assay

PFGE is a gold standard technique in molecular epidemiological study of pathogenic organisms to separate very large DNA molecules (>20kb) with high discriminatory power (Schwartz & Cantor, 1984). In this study, the PFGE was modified from previous studies (McDougal et al., 2003; Norazah, Liew, Kamel, Koh, & Lim, 2001).

3.17.1 Pellet cell preparation and cell washing

Fresh colonies were inoculated to 5 ml of Brain Heart infusion (BHI) broth and incubated at 37°C for 16-18 hours. Two milliliters of bacterial suspension were centrifuged at 3,000 rpm for 10 minutes, and supernatant was then discarded. Cell washing step was continued by adding 1ml of 1x TE buffer (See appendix) to pellet cells and vortex to mix. The cell suspension was incubated at 37°C for 10 minutes by incubator shaker and then centrifuged at 3,000 rpm for 5 minutes. This step was repeated three times. The cell suspension was used in the next step.

3.17.2 DNA plug formation and plug cutting

Lysostaphin (0.5 U/µl) (Sigma-Aldrich, USA) were mixed with 300 µl cell suspension (from the previous step), and 300 µl of 1.8% (w/v) SeaKem® LE Agarose (Cambrex Bio Science Rockland, USA) were added in preheated TE buffer at 55°C The mixture was quickly mixed by pipetting up and down, then loaded into the wells of a clean Plug Mold (Bio-Rad, USA), leaving the gel to be set for approximately 10 to15 minutes. Finally, the agarose plug was cut into 3 mm wide pieces by sterile blade.

3.17.3 Cell lysis

The plugs were placed into a 1.5 ml microcentrifuge tube including with 1 ml of EDTA-Sarkosyl (ES) buffer containing 1 mg of Proteinase K (MP

Biomedicals, France) and incubated at 37°C for 24 hours with gentle shaking by incubator shaker.

3.17.4 Plug washing

After incubation, ES buffer was removed, and a plug was washed with 1 ml of TE buffer by gentle pipetting up and down several times and incubated at room temperature for 15 minutes. This step was repeated eight times. The plugs were stored in TE buffer at 4°C for 12 months with changing buffer monthly.

3.17.5 Endonuclease digestion

TE buffer was discarded after washing. Then 1 ml of sterile distilled water was added to a plug and soaked on ice for 20 minutes. This step was repeated two times. After that, distilled water was removed, and a plug was suspended into 1 ml of 1x Buffer Tango (Thermo Scientific, USA) and soaked on ice for 1 hour. The buffer was discarded and 300 µl of 1x restriction buffer containing 40U of *Sma*I restriction enzyme (Thermo Scientific, USA) was then added into a plug tube and incubated at room temperature for approximately 21-22 hours with agitation.

3.17.6 Agarose preparation

Preparation of 1% agarose gel was performed in 0.5x TBE buffer [final concentration, 1% (w/v)]. The mixture was dissolved by heating in microwave for 3 minutes. The molten agarose was kept in a water bath at 55°C and was aliquoted for next step. It was then poured into a pre-assembled clean gel casting mold with a twenty-lane well comb (Bio-Rad, USA). The gel was allowed to set at room temperature for 30-40 minutes. After completed digestion, the DNA plug was inserted into a single well using sterile spatula. The lambda ladder PFG marker (New England Biolabs, UK) was used as DNA marker. When all plugs were loaded, the aliquot of molten agarose was added over each plug until the wells were full by pipetting. It was ensured that no gaps or air bubbles were present between the plug and gel; 5-10 minutes were allowed to solidify.

3.17.7 Pulse Field Electrophoresis

CHEF-DR III Pulse Field Electrophoresis Systems CHEF-DR III Pulse Field Electrophoresis Systems (Contour-Clamped Homogeneous Electrophoresis Field) (Bio-Rad, USA) was cleaned with 70% ethanol before used. Two liters of distilled water were filled into the chamber and pumped for 15 minutes in order to clean the entire system. The water was removed, and 2 L of cold 0.5x TBE buffer was then poured into the chamber and pumped until temperature of the buffer was at 14°C. The gel was placed on the chamber to ensure the buffer was thoroughly covered, carefully that there was no the excess gel in the electrophoresis tank. The electrophoresis condition was set for electrophoresis as follows.

Table 8: Electrophoresis conditions setting for PFGE

Setting	Block 1	Block 2
Initial switch time (seconds)	5	15
Final switch time (seconds)	15	25
Voltage (V/cm)	6	6
Running time (hours)	8	10

A total duration of this running gel electrophoresis was 18 hours, and the direction running of DNA molecules (Figure 2). Prior to the running, the cooling temperature of system was rechecked at 14°C. After completely running, the buffer was drained, and 2 L distilled water were added to the chamber and pumped for 15 minutes. The chamber was washed three times.

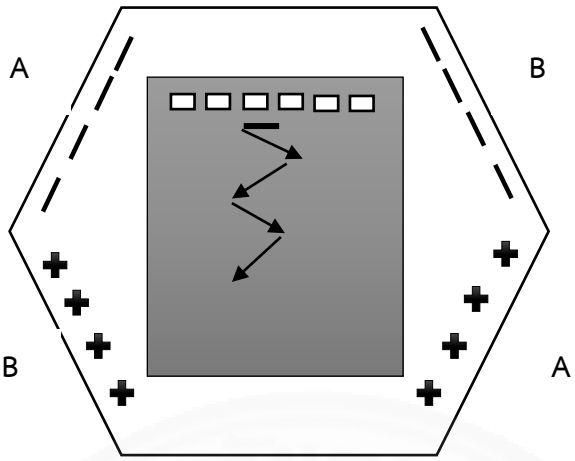


Figure 2 CHEF-DR III electric filed alternates 120° every 10 seconds for 18 hours at 14°C

3.17.8 Staining DNA restriction pattern and visualization

The gel was stained with 1.5 µg/ml of Ethidium bromide in 0.5x TBE for 20 minutes and followed by de-staining in distilled water for 30 minutes. The DNA fragment in gel was visualized and captured under UV light using Gel Documentation. All images were collected in TIFF format.

3.17.9 DNA banding pattern analyses

All images represent DNA fragment patterns. The similarity between banding patterns was analyzed by calculation of the Dice similarity coefficients with 2.0% band position tolerance, represented by the unweighted pair group method with arithmetic average (UPGMA) dendrogram using GeneTools and GeneDirectory program (SynGene, UK). Isolates were determined as the same PFGE type if the similarity coefficient was 71%. The dendrogram was rechecked using Tenover criteria (Table 9).

Table 9: The criteria for interpreting PFGE patterns developed by Tenover and colleagues (Tenover et al., 1995)

Category	Number of genetic differences	Typical number of DNA banding fragment differences
Indistinguishable	0	0
Closely related	1	2-3
Possibly related	2	4-6
Different	≥3	≥7

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Detection of *mecA* and *pvl* genes

A total of 101 MRSA isolates were tested for detection of methicillin resistance of *S. aureus*. All MRSA isolates harbored *mecA* (Figure 3). No MRSA isolate in this study carried *pvl* (Figure 4), and all inpatients with culture-positive MRSA were detected after 48 hours of admission. The results suggested that the tested strains in this study were HA-MRSA strains.

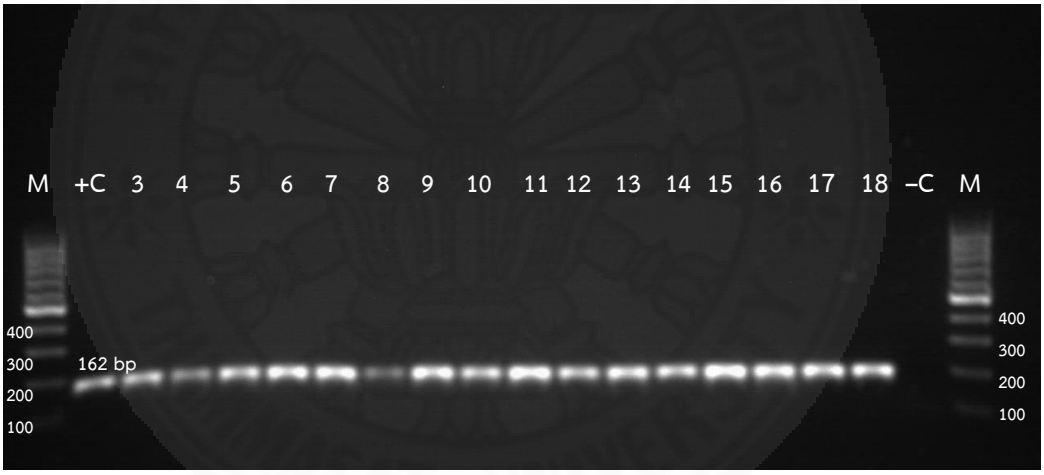


Figure 3 Detection of *mecA* gene in representative MRSA strains

M= Marker (100 bp DNA Ladder); -C = Negative control; +C= Positive Control (PCR product size=162 bp); Lane 3 -18, Representative of MRSA strains

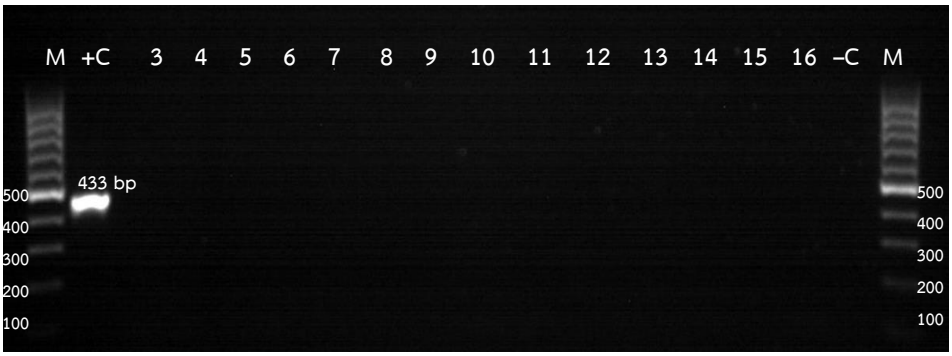


Figure 4 Detection of *pvl* gene in representative of MRSA strains

M= Marker (100 bp DNA Ladder); -C = Negative control; +C= Positive Control (PCR product size=433 bp); Lane 3-16, Representative of MRSA strains

4.2 Distribution of MRSA isolates

Distribution of MRSA isolates according specimen types were as follows: sputum (65%); blood (18%); pus (15%); and urine (2%). Among the clinical wards, highest number of MRSA isolates was from Internal Medicine Wards (76%), followed by Surgery (15%), Operation theaters (6%), and Pediatrics (3%).

Antibiogram pattern I (CPR^{R} CLI^{R} TMP^{S} SXT^{S} RIF^{S} MUP^{S} FUS^{S} VAN^{S} TEC^{S} LZD^{S}) was predominant in MRSA isolates from Internal Medicine Ward, Surgery and Operation Theater except the Pediatric ward, of which antibiogram pattern IV (CPR^{R} CLI^{R} TMP^{R} SXT^{S} RIF^{S} MUP^{S} FUS^{S} VAN^{S} TEC^{S} LZD^{S}) was obtained (Table 11).

The results mentioned above indicated the MRSA infection found in respiratory tract and in Internal Medicine Wards were most likely concerned for transmission of the MRSA strains. In addition, these isolates fell into antibiogram pattern I which was still susceptible to more antibiotics.

However, the transmission of multi-resistant MRSA strain is high potential to become widespread due to the resistance genes encoded by mobile genetic elements such as mupirocin, trimethoprim and trimethoprim-sulfamethoxazole (Dale et al., 1997; Engel, Soedirman, Rost, van Leeuwen, & van Embden, 1980). It is highly suggested that personnel involved in hospital infection control should be informed to be able to make an effective plan for surveillance of MRSA infection and prevent the spread in hospital.

4.3 MRSA with vancomycin MICs creep and non-creep

Using E-test and broth microdilution (BMD) method (Table 10), all of the isolates were susceptible to vancomycin. The vancomycin MICs creep (≥ 1.5-2 ug/ml) rate was 31%, and MRSA with vancomycin MIC creep strains were commonly found in Internal Medicine Ward (Figure 5). In this study, there were no significant differences in terms of clinical failure rate between patients with MRSA isolates with MICs ≥ 1.5-2 ug/ml or <1.5 ug/ml. Our finding is consistent with previous report (Yeh et al., 2012). However, it is essential that physicians should carefully consider MIC value for an appropriate selection of antimicrobial usage for treatment of MRSA infection.

The strains were found to be hVISA leading to unknown failure of treatment as the hVISA strains were unable to detect by routine laboratory method. Although E-test method can detect MRSA isolates with reduced susceptibility to vancomycin, the hetero-resistance strains of MRSA were not detected as mentioned. It is suggested that Population Analysis Profile (PAP) method would be possibly use for an alternative option to examine the hVISA. However, this method has been a tedious task to perform routinely. Therefore, further study for development of high throughput for hVISA detection is needed.

Table 10: Distribution of MRSA isolates by vancomycin MICs

Vancomycin MICs (ug/ml)	Number of isolates (%)	
	E-test	BMD*
0.38	1 (1)	-
0.5	3 (3)	19 (19)
0.75	27 (27)	-
1	39 (39)	68 (68)
1.5	25 (25)	-
2	6 (6)	14 (14)

* Vancomycin concentrations of 0.38, 0.75 and 1.5 ug/ml were not tested.

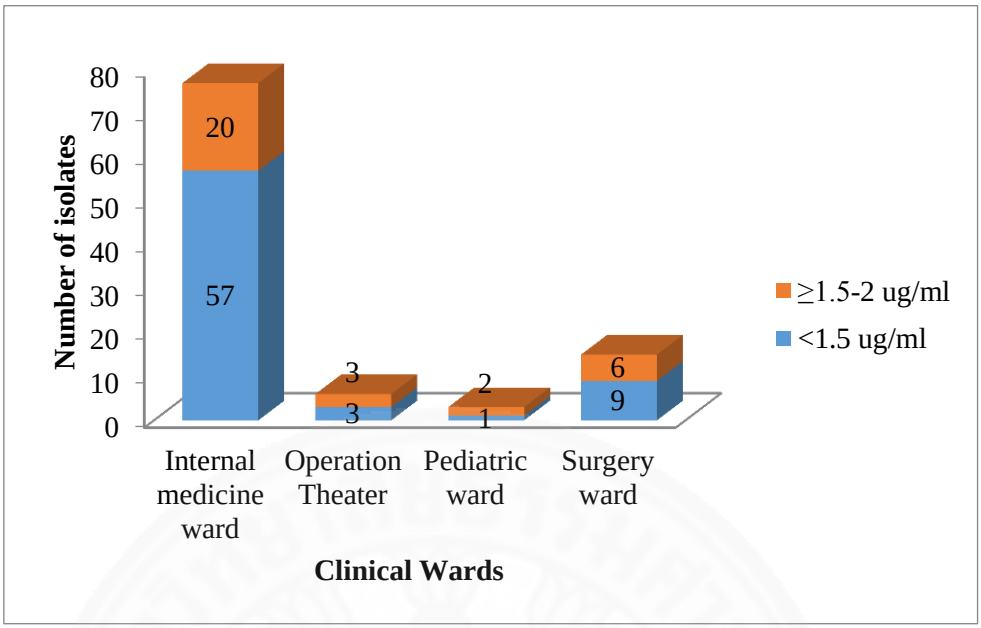


Figure 5 Distribution of MRSA isolates by type of clinical wards

4.4 Antibiotic susceptibility of MRSA isolates

According to Antibiogram profiles, the MRSA isolates can be classified into 12 patterns as shown in Table 11. Pattern I was the most common found isolated from Internal Medicine Ward, Surgery and Operation Theater except the Pediatric ward, belonging to Antibiogram pattern IV. There was no specific MRSA antibiogram pattern associated with specimen types (Table 12). This result revealed no effect for antibiotic usage in particular source of MRSA infections. However, clindamycin was nearly resistant to all MRSA strains apart from the cefpirome which completely resistant. This result suggested that physicians should avoid using the two mentioned antibiotics for MRSA infection treatment. Other antibiotics including vancomycin, teicloplanin and linezolid, still remained effective antibiotics for treatment of all MRSA infections. Resistance of trimethoprim, trimethoprim-sulfamethoxazole and rifampicin was found more frequent in pus specimen which would be a concern of selection for MRSA infection treatment.

Table 11: Antibacterial susceptibility patterns of MRSA isolates collected at Thammasat University Hospital, Thailand.

Pattern	CPR	CLI	TMP	SXT	RIF	MUP	FUS	VAN	TEC	LZD	Number of isolates (%)
I	R	R	S	S	S	S	S	S	S	S	76 (75)
II	R	S	S	S	S	S	S	S	S	S	8 (8)
III	R	R	R	R	R	S	S	S	S	S	4 (4)
IV	R	R	R	S	S	S	S	S	S	S	4 (4)
V	R	R	R	R	S	S	S	S	S	S	2 (2)
VI	R	R	R	R	R	R	R	S	S	S	1 (1)
VII	R	R	R	R	R	S	R	S	S	S	1 (1)
VIII	R	R	R	R	S	I	R	S	S	S	1 (1)
IX	R	R	R	S	R	S	S	S	S	S	1 (1)
X	R	R	S	S	R	S	S	S	S	S	1 (1)
XI	R	R	S	S	S	I	S	S	S	S	1 (1)
XII	R	S	S	S	S	I	S	S	S	S	1 (1)
Number of susceptible isolates (%)	0 (0%)	9 (9%)	87 (86%)	92 (91%)	93 (92%)	97 (96%)	98(97%)	101(100%)	101(100%)	101(100%)	

CPR, cefpirome (0.016-256 ug/ml); CLI, clindamycin (0.016-256 ug/ml); TMP, trimethoprim (0.002-32 ug/ml); SXT, trimethoprim-sulfamethoxazole (0.002-32 ug/ml); RIF, rifampicin (0.016-256 ug/ml); MUP, mupirocin (0.064-1024 ug/ml); FUS, fusidic acid (0.016-256 ug/ml); VAN, vancomycin (0.016-256 ug/ml); TEC, teicoplanin (0.016-256 ug/ml); LZD, linezolid (0.016-256 ug/ml); S, susceptible; I, intermediate; R, resistant.

Table 12: Antibiotic susceptibility of MRSA isolated from different specimen types, collected at Thammasat University Hospital, Thailand.

Specimen type	Total number of MRSA isolates	Number of MRSA susceptible isolates (%)[a]					
		CLI	TMP	SXT	RIF	MUP	FUS
Sputum	66	4 (6)	59 (89)	62 (94)	62 (94)	65 (98)	66 (100)
Blood	18	3 (17)	17 (94)	18 (100)	18 (100)	18 (100)	18 (100)
Pus	15	1 (7)	9 (60)	10 (67)	11 (73)	13 (87)	12 (80)
Urine	2	1 (50)	2 (100)	2 (100)	2 (100)	1 (50)	2 (100)

[a]All samples were susceptible to LZD, TEC and VAN, resistant to CPR.

Table 13: Antibiotic susceptibility of MRSA isolated from different clinical wards**,** collected at Thammasat University Hospital, Thailand.

Clinical ward type	Total number of MRSA isolates	Number of MRSA susceptible isolates (%)[a]					
		CLI	TMP	SXT	RIF	MUP	FUS
Internal medicine	77	7(9)	70(91)	72(94)	72(94)	74(96)	77(100)
Surgery ward	15	2(13)	14(93)	14(93)	14(93)	14(93)	14(93)
Operation theater	6	0(0)	3(50)	4(67)	4(67)	6(100)	5(83)
Pediatric ward	3	0(0)	0(0)	2(67)	3(100)	3(100)	3(100)

[a]All samples were susceptible to LZD, TEC and VAN, resistant to CPR.

4.5 Clinical and microbiological outcome

Of 92 patients infected with MRSA, MRSA failure treatment occurred in 21 patients (23%). These patients had significant association with the history of having chronic hemodialysis, hVISA strain infection, higher charlson comorbidity index and greater number of days to negative culture (all $p < 0.05$) compared to the patients with successful treatment (Table 14).

The proportion of MRSA isolates with vancomycin MICs creep was higher among patients failure outcome (42.9%) than patients with successful treatment (29.6%), but this result was no statistically significant. A previous report demonstrated that vancomycin MICs alone apart from clinical factors was not significantly associated with treatment outcome (Rojas et al., 2012).

Tested strains of MRSA were discovered vancomycin intermediate resistant population (MIC 4-8 ug/ml) so called hVISA strains. The finding that hVISA strains were significantly associated with the treatment failure in patients with MRSA infection was interesting (*p*-value=0.03) (Table 14). The similar finding was reported from USA (Casapao et al., 2014). In addition, infections caused by hVISA strains were associated with bacteremia for more than 7 days and pneumonia for more than 21 days after the initial of vancomycin treatment. Since hVISA strain cannot be detected by routine laboratory testing, the patients were more likely to have inappropriate doses. However, the effective therapeutic option for hVISA treatment remains uncertain.

Using multivariate regression analysis, chronic hemodialysis, multiple sites infection and co-microbe resistant strains, which were MRSA infection combined with other bacterial infection, were identified as independent predictors of vancomycin treatment failure (*p*-value < 0.05) (Table 15). However, when individual condition of having chronic hemodialysis was analyzed, the result revealed that chronic

hemodialysis factor was significantly related to clinical outcome (*p*-value <0.05) but not the other factors including multiple-sites infection and co-microbe resistant strains infection. Patients with chronic hemodialysis alone was significantly related to higher risk of MRSA bloodstream infection (Odds ratio = 5.7) compared the patients without chronic hemodialysis. However, the factor of co-microbes resistant strains including *Acinetobacter baumannii* (MDR), *Escherichia coli* (ESBL) and *Pseudomonas aeruginosa* (MDR) had no significant effect in clinical outcome (*p*-value = 0.05).

Table 14: Patient baseline, clinical and microbiological characteristics for patients with MRSA infection by clinical outcome

Characteristics	**Clinical failure n (%) (n=21)**	**Clinical success n (%) (n=71)**	***p*-value***
Clinical characteristics			
General			
- Male sex	13 (61.9)	35 (49.3)	0.33
- Age (year), mean ± SD	76 ± 16	69 ± 20	**0.18**
- Elderly (age >65 years old)	17 (81.0)	56 (78.9)	1.00
- Length of stay (day), mean ± SD	81 ± 86	57 ± 57	**0.14**
- Prior MRSA infection in previous 3 months	5 (23.8)	8 (11.3)	**0.16**
- Prior vancomycin exposure	5 (23.8)	10 (14.1)	0.32
- ICU admission before MRSA infection	6 (28.6)	19 (26.8)	1.00
Concurrent conditions			
- Chronic kidney disease	11 (52.4)	22 (31.0)	**0.11**
- Acute kidney injury	13 (61.9)	44 (62.0)	1.00
- Chronic hemodialysis	9 (42.9)	11 (15.5)	**0.01**
- Cerebrovascular disease	14 (66.7)	37 (52.1)	0.31
- Cardiovascular disease	17 (81.0)	52 (73.2)	0.57
- Diabetes mellitus	13 (61.9)	30 (42.3)	**0.13**
- Transplant	2 (9.5)	0 (0.0)	**0.05**
- Immunosuppressant use	8 (38.1)	26 (36.6)	1.00
- Bedridden	15 (71.4)	43 (60.6)	0.44
- Anemia	17 (81.0)	44 (62.0)	**0.12**
Severity of disease			
- Charlson comorbidity index, mean ± SD	8 ± 3	6 ± 3	**0.01**
Drug			
- Adequate glycopeptide use within 48 hours	8 (38.1)	31 (43.7)	0.80
- Adjustment of vancomycin trough level	17 (81.0)	56 (78.9)	1.00

**p*-value calculated using a Fisher's exact test

Table 14: Patient baseline, clinical and microbiological characteristics for patients with MRSA infection by clinical outcome (continue)

Characteristics	**Clinical failure n (%) (n=21)**	**Clinical success n (%) (n=71)**	***p*-value***
Infectious source			
- Pneumonia	11 (52.4)	50 (70.4)	**0.18**
- Wound/skin or soft tissue	1 (4.8)	2 (2.8)	0.54
- Bone/joint	1 (4.8)	3 (4.2)	1.00
- Bloodstream infection	6 (28.6)	12 (16.9)	0.34
- Surgical site	1 (4.8)	4 (5.6)	1.00
- Urinary tract infection	1 (4.8)	1 (1.4)	0.40
- Multiple sites infection	7 (33.3)	12 (16.9)	**0.12**
Microbiological characteristics			
- Vancomycin MICs value (1.5-2 ug/ml)	9 (42.9)	21 (29.6)	0.29
- hVISA strain infection**	6 (60.0)	4 (18.2)	**0.03**
- Co-microbe resistant strains infection	13 (61.9)	27 (38.0)	**0.07**
- Days to negative culture, mean ± SD	19 ± 11	7 ± 5	**0.00**

*p-value calculated using a Fisher's exact test

** Only 32 MRSA isolates, which have vancomycin MICs value of 1.5-2 ug/ml, were tested by PAP method.

Table 15: Univariable and multivariable analysis of factors associated with clinical failure in patients with MRSA infection

Characteristics	Univariate OR	95% CI	*p*-value[a]	Multivariate OR	95% CI	*p*-value[b]
Age >65 years old	1.13	0.33-3.89	0.83	-	-	ns
Prior MRSA infection in previous 3	2.46	0.70-8.54	**0.15**	-	-	ns
Chronic kidney disease	2.45	0.90-6.61	**0.07**	-	-	ns
Chronic hemodialysis	4.09	1.39-12.01	**0.01**	5.77	1.70-19.49	0.00
Diabetes mellitus	2.22	0.81-6.02	**0.11**	-	-	ns
Anemia	2.60	0.79-8.57	**0.11**	-	-	ns
Multiple sites infection	2.45	0.81-7.38	**0.10**	3.71	1.08-12.77	0.03
Co-microbe resistant strains infection	2.64	0.97-7.21	**0.05**	3.68	1.16-11.65	0.02
Vancomycin MIC Creep	1.78	0.65-4.87	0.25	-	-	ns

ns = not significant

[a] Statistically significant in univariate logistic regression analysis, using stepwise backward method (*p*-value <0.2), except age >65 years old and vancomycin MIC creep were included to model for independent variables control.

[b] Statistically significant in multivariate logistic regression analysis, using stepwise backward method (*p*-value <0.05).

Table 16: Patient baseline, clinical and microbiological characteristics for patients with MRSA infection by microbiological outcome

Characteristics	Microbiological failure n (%) (n=14)	Microbiological success n (%) (n=46)	*p*-value*
Clinical characteristics			
General			
- Male sex	10 (71)	19 (41)	**0.06**
- Elderly (age >65 years old)	10 (71)	37 (80)	0.47
- Prior MRSA infection in previous 3 months	3 (21)	5 (11)	0.37
- Prior vancomycin exposure	2 (14)	6 (13)	1.00
- ICU admission before MRSA infection	2 (14)	7 (15)	1.00
- Recent surgery	9 (64)	25 (54)	0.55
- Hospitalization within 3 months of MRSA infection	4 (29)	2 (4)	**0.02**
Concurrent conditions			
- Chronic kidney disease	7 (50)	15 (33)	0.34
- Acute kidney injury	9 (64)	29 (63)	1.00
- Chronic hemodialysis	5 (36)	8 (17)	**0.15**
- Cerebrovascular disease	10 (71)	23 (50)	0.22
- Cardiovascular disease	9 (64)	34 (74)	0.51
- Diabetes mellitus	8 (57)	19 (41)	0.36
- Transplant	1 (7)	0 (0.0)	0.23
- Immunosuppressant use	5 (36)	19 (41)	0.76
- Bedridden	9 (64)	30 (65)	1.00
- Anemia	11 (79)	31 (67)	0.52
Drug			
- Adequate glycopeptide use within 48 hours	3 (21)	22 (48)	**0.12**
- Adjustment of vancomycin trough level	12 (86)	39 (85)	1.00

**p*-value calculated using a Fisher's exact test

Table 16: Patient baseline, clinical and microbiological characteristics for patients with MRSA infection by microbiological outcome (continue)

Characteristics	Microbiological failure n (%) (n=14)	Microbiological success n (%) (n=46)	*p*-value*
Infectious source			
- Pneumonia	6 (43)	33 (72)	0.06
- Wound/skin or soft tissue	0 (0)	1 (2)	1.00
- Bone/joint	2 (14)	1 (2)	**0.13**
- Bloodstream infection	5 (36)	9 (20)	0.28
- Surgical site	0 (0)	2 (4)	1.00
- Urinary tract infection	1 (7)	1 (2)	0.41
- Multiple sites infection	6 (43)	10 (22)	**0.16**
Microbiological characteristics			
- Vancomycin MICs value (1.5-2 ug/ml)	6 (43)	11 (24)	**0.19**
- hVISA strain infection**	4 (57)	3 (25)	0.32
- Co-microbe resistant strains infection	8 (57)	22 (48)	0.76

*p-value calculated using a Fisher's exact test

** Only 32 MRSA isolates, which have vancomycin MICs value of 1.5-2 ug/ml, were tested by PAP method.

Table 17: Univariable and multivariable analysis of factors associated with microbiological failure in patients with MRSA infection

Characteristics	Univariate OR	95% CI	*p*-value[a]	Multivariate OR	95% CI	*p*-value[b]
- Age >65 years old	0.60	0.15-2.39	0.47	-	-	ns
- Male sex	3.55	0.96-13.03	0.05	-	-	ns
- Hospitalization within 3 months of MRSA infection	8.80	1.41-54.91	0.02	11.66	1.46-93.09	0.02
- Pneumonia	0.29	0.08-1.01	0.05	-	-	ns
- Bone/Joint infection	7.5	0.62-89.87	0.11	20.30	1.19-345.05	0.03
- Chronic hemodialysis	2.63	0.69-10.00	0.15	-	-	ns
- Multiple sites infection	2.70	0.75-9.60	0.12	5.25	1.10-25.01	0.03
- Vancomycin MIC Creep	2.38	0.67-8.38	0.17	-	-	ns

ns = not significant

[a] Statistically significant in univariate logistic regression analysis, using stepwise backward method (*p*-value <0.2), except age >65 years old and vancomycin MIC creep were included to model for independent variables control.

[b] Statistically significant in multivariate logistic regression analysis, using stepwise backward method (*p*-value <0.05).

4.6 Detection of hVISA strain

A total of 32 MRSA isolates were tested by PAP method, 31% (10/32) of the isolates were hVISA strains. Among the patients with hVISA strains infection (60%) found to be clinical failure. These data indicated that PAP technique is significantly useful for detecting hVISA, which has been associated with vancomycin treatment failure in this study (*p*-value = 0.03) in this study.

Similarly of previous report, treatment failure is common among patients with high bacterial load of hVISA including prosthetic device infection, undrained abscess, endocarditis confirmed by echocardiogram (Charles, 2004). In contrast, another report indicated that failure treatment in case of a small number of hVISA found was not significant leading to limitation of conclusion for clinical failure caused by hVISA (Yasmin, 2007). Therefore, treating of patients with hVISA infection is of great challenge for clinicians, since routine laboratory testing is not yet able to detect hVISA.

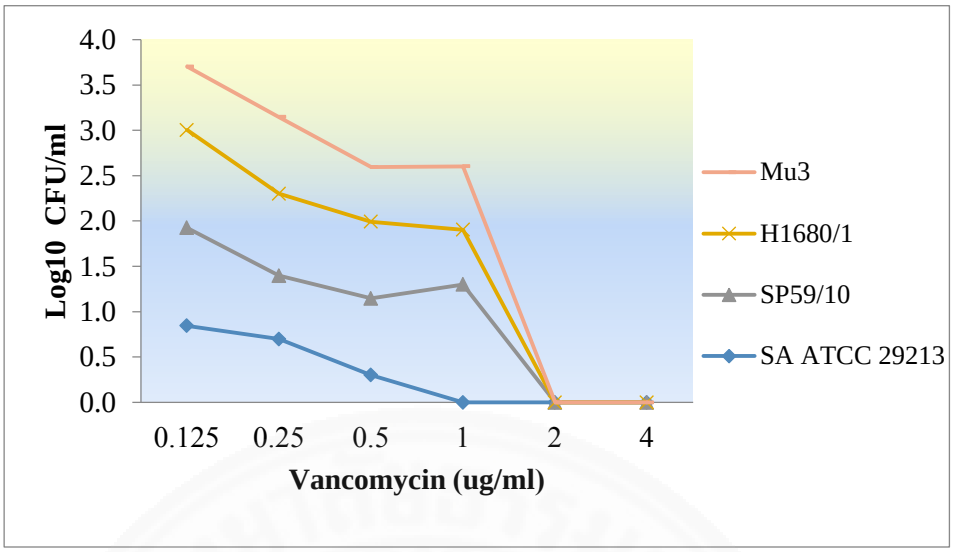


Figure 6 Population Analysis Profiles of hVISA

Mu3 = Postive control of hVISA, H1680/1 and SP59/10 = Tested MRSA MIC vancomycin creep strains and VSSA (SA ATCC 29213) = Negative control.

4.7 Virulence genes of *S. aureus* isolates

The *femA* and *icaA* genes were positive in all isolates similar to previous report indicating the *femA* associated to increased expression of resistance to methicillin (X. Li et al., 2012). An *icaA* gene was involved with clinical persistence (Gordon & Lowy, 2008). In this study, only 17% of all patients were persistent infection. Therefore, there was no significant association between *icaA* gene and infection persistence compared with previous report as mentioned above.

As mentioned earlier, TSST-1 (*tst*) causes toxic shock syndrome (Todd et al., 1978), Exfoliative toxins A and B (*eta* and *etb*) is the cause of staphylococcal scalded skin syndrome (SSSS) (Bukowski et al., 2010). The results of the toxins involved in virulence revealed that none of the tested strains carried *sec*, *sed*, *tst*, *eta* and *etb* genes. All isolates were found *sea* (43%), *seb* (38%) and *see* (43%) genes. The *sec*, *sed*, *tst*, *eta* and *etb* genes were not detected in all strains. Interestingly, the positive toxins were detected but patients chart reviews were not indicated any symptoms involved in food poisoning (Table 19-20).

This result showed that six different virulence genes patterns designated type I, II, III, IV, V and VI were exhibited. The majority of tested MRSA strains were type I and II (Table 18). The most MRSA strains were vancomycin MICs creep belonging to pattern I (48%) and II (39%). Approximately 48% (10/21) of treatment failure were pattern I ($femA^{+}icaA^{+}sea^{+}seb^{-}see^{+}$). The results showed no significance of the clinical outcome associated with each virulence pattern found in the tested strains.

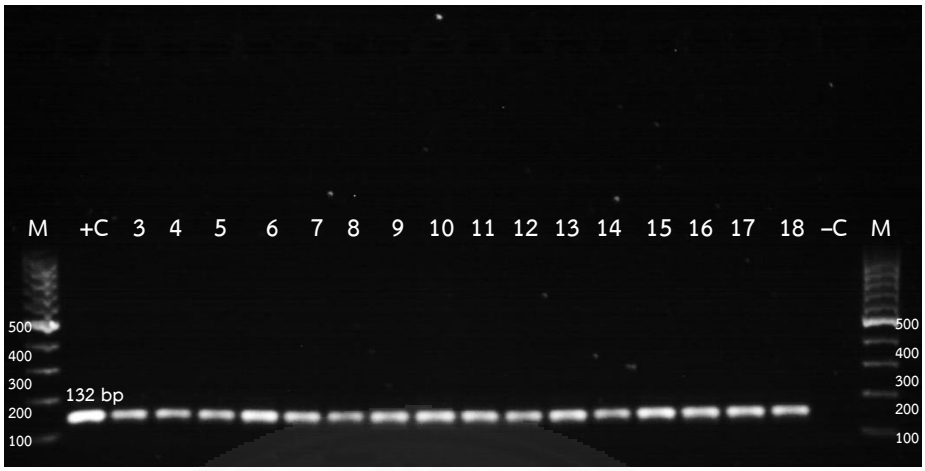


Figure 7 Detection of *femA* gene in MRSA isolates

M= Marker (100 bp DNA Ladder); -C = Negative control; +C= Positive Control (PCR product size=132 bp); Lane 3-18, Representative of MRSA strains

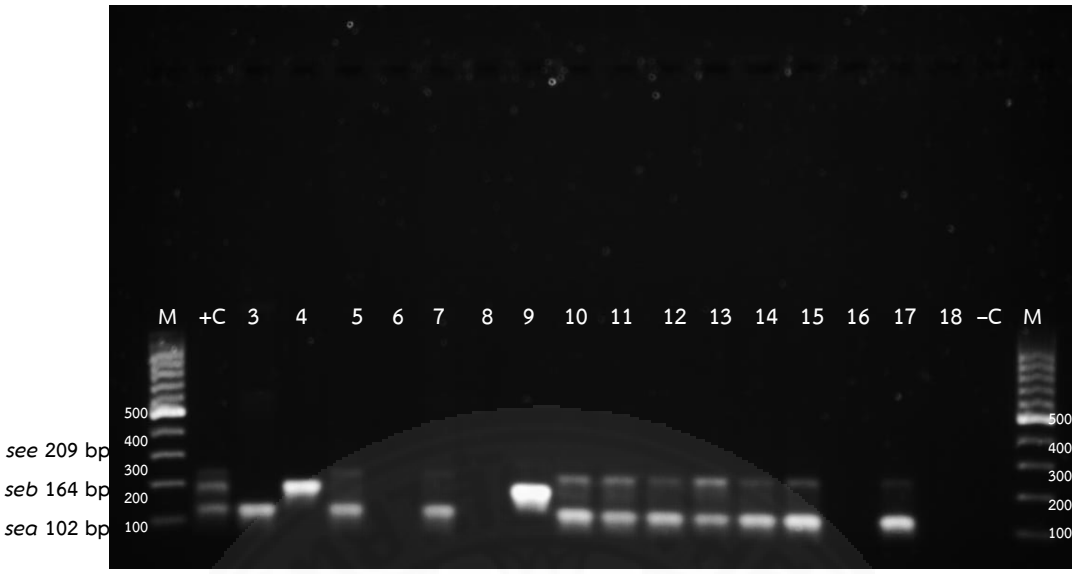


Figure 8 Products of *sea, seb, see* genes found in Representative MRSA strains M= Marker (100 bp DNA Ladder); -C = Negative control; +C= Positive Control (PCR product size; *sea*=102 bp; *seb*=164 bp; *see*=209 bp); Lane 3-18, Representative MRSA strains

Table 18: Virulence genes patterns of tested MRSA isolates

Pattern	*femA*	*icaA*	*sea*	*seb*	*see*	Number of isolates (%)
I	+	+	+	-	+	40 (40)
II	+	+	-	+	-	37 (37)
III	+	+	-	-	-	19 (19)
IV	+	+	+	-	-	2 (2)
V	+	+	-	-	+	2 (2)
VI	+	+	+	+	+	1 (1)
Number of positive isolates (%)	101 (100%)	101 100%)	43 (43%)	38 38%)	43 (43%)	

Table 19: Virulence genes of MRSA isolated from different specimen types

Specimen type	Total number of MRSA isolates	Number of MRSA positive isolates (%)[a]		
		sea	*seb*	*see*
Sputum	66	32 (48)	27 (41)	31 (47)
Blood	18	6 (33)	7 (39)	6 (33)
Pus	15	5 (33)	3 (20)	6 (40)
Urine	2	0 (0)	1 (50)	0 (0)

[a]All isolates were positive to *femA* and *icaA*, negative to *sec*, *sed*, *tst*, *eta* and *etb*.

Table 20: Virulence genes of MRSA isolated from different clinical wards, collected at Thammasat University Hospital, Thailand.

Clinical ward type	Total number of MRSA isolates	Number of MRSA positive isolates (%)[a]		
		sea	*seb*	*see*
Internal medicine	77	32 (41)	30 (39)	32 (41)
Surgery ward	15	5 (33)	7 (47)	5 (33)
Operation theater	6	3 (50)	1 (17)	3 (50)
Pediatric ward	3	3 (100)	0 (0)	3 (100)

[a]All isolates were positive to *femA* and *icaA*, negative to *sec*, *sed*, *tst*, *eta* and *etb*.

Table 21: Virulence genes patterns of MRSA isolated from different clinical wards, collected at Thammasat University Hospital, Thailand.

Clinical ward type	Total number of MRSA isolates	Number of MRSA isolates (%)					
		I	II	III	IV	V	VI
Internal medicine	77	29 (38)	29 (38)	14 (18)	2 (3)	2 (3)	1 (1)
Surgery ward	15	5 (33)	7 (47)	3 (20)	0 (0)	0 (0)	0 (0)
Operation theater	6	3 (50)	1 (17)	2 (33)	0 (0)	0 (0)	0 (0)
Pediatric ward	3	3 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

4.8 SCC*mec* typing

A total of 101 MRSA isolates were tested for SCC*mec* typing. The result revealed MRSA isolates belonged to SCC*mec*-type I, II, and III (Table 22), which corresponds to HA-MRSA. SCC*mec*-type II was highly predominant in this study. The SCC*mec*-type II was also consistently observed in South Korea and Japan (Barada et al., 2007; Kwon et al., 2011). This finding differs from previous studies, SCC*mec*-type III was found in Nakhon Pathom and Khon Kaen, Thailand and other countries in Asia: Vietnam, China, India, Indonesia, Malaysia, Singapore, Saudi Arabia, Sri Lanka, Taiwan and Hong Kong. (Chongtrakool et al., 2006; Lawung, Chuong, Cherdtrakulkiat, Srisarin, & Prachayasittikul, 2014; Lulitanond et al., 2010).

Of interest, SCC*mec*-type III isolates were more resistant to trimethoprim, trimethoprim-Sulfamethoxazole and rifampicin than SCC*mec*-type I and II (Table 23), as previous report (Cavalcante et al., 2013). In contrast, SCC*mec*-type I and II isolates were resistant to clindamycin (100%), while SCC*mec*-type III isolates presented susceptible to the antibiotic (38%). Thus, prevention of the spread of nosocomial SCC*mec* type III to the community is necessary.

SCC*mec*-type III was the most common type in Thailand (Lulitanond et al., 2010) while SCC*mec*-type II was mostly found in this study. It is well known as that SCC*mec*-type III was more resistant than SCC*mec*-type II strain. As SCC*mec* cluster encoded mobile genetic element, SCC*mec*-type III strains are possibly transmitted the resistance gene to SCC*mec*-type II. The SCC*mec* typing is therefore very useful molecular tool for surveillance and control the spread of resistant strains from one to another.

Table 22: Distribution of the detected SCC*mec*-types among different specimen types

SCC*mec*- types	Specimen types				Number of isolates (%)
	Sputum	Blood	Pus	Urine	
I	27 (75)	5 (14)	4 (11)	0(0)	36 (36)
II	28 (68)	9 (22)	3 (7)	1(2)	41 (41)
III	11 (46)	4 (17)	8 (33)	1(4)	24 (24)

Table 23: Antibiotic susceptibility of MRSA isolated from different SCC*mec*-types

	Number of susceptibility isolates (%)		
Antimicrobial agents	**Type I (n=36)**	**Type II (n=41)**	**Type III (n=24)**
Vancomycin	36 (100)	41 (100)	24 (100)
Trimethoprim-Sulfamethoxazole	36 (100)	40 (98)	**16 (67)**
Fusidic acid	36 (100)	41 (100)	21 (88)
Rifampicin	36 (100)	39 (95)	**18 (75)**
Trimethoprim	36 (100)	40 (98)	**11 (46)**
Linezolid	36 (100)	41 (100)	24 (100)
Mupirocin	36 (100)	40 (98)	21 (88)
Teicoplanin	36 (100)	41 (100)	24 (100)
Cefpirome	0 (0)	0 (0)	0 (0)
Clindamycin	0 (0)	0 (0)	9 (38)

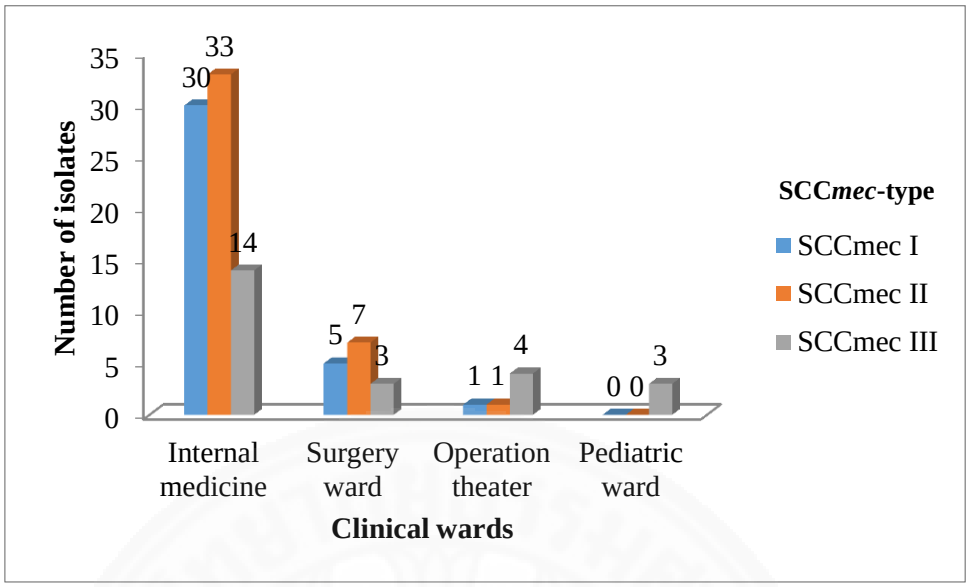


Figure 9 Distribution of the detected SCC*mec*-types among different clinical wards

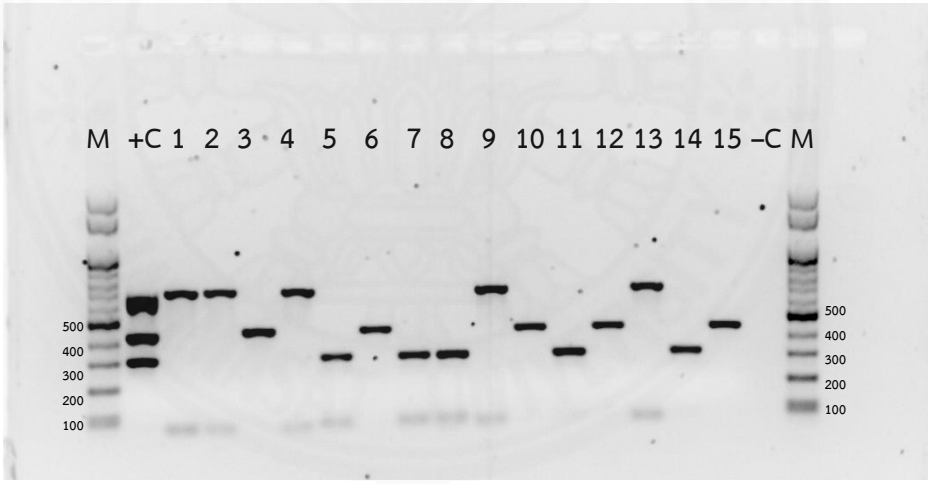


Figure 10 SCC*mec*-types of representative MRSA strains from Thammasat University Hospital

Lane M= Marker (100 bp DNA Ladder); +C= Positive Control (SCC*mec*I=613 bp; SCC*mec* II=398 bp; SCC*mec* III=280 bp); -C = Negative control; Lane 1-15, representative MRSA strains with vancomycin MICs (creep and non-creep)

4.9 Pulse Field Electrophoresis

Seventeen different PFGE types were designated PFGE types “A-Q”. The results indicated that the MRSA isolated from Thammasat University Hospital were high genetic variations. It is suggested that genetic variants occurred in the strains due to mutation or insertion/deletion in the chromosome as previous report (Hallin et al., 2007). The PFGE patterns in this study were different in the number of fragment from previously reports (Wongwanich, Tishyadhigama, Paisomboon, Ohta, & Hayashi, 2000).

The majority of tested MRSA isolates were type A (16%) (Figure 11), followed by PFGE type Q (12%) and PFGE pattern P (10 %). Ten out of 12 isolates belonging to PFGE type Q had vancomycin MICs creep.

Analysis of PFGE pattern P versus clinical outcome revealed that the MRSA belonging to P the association with treatment failure (*p*-value = 0.001). Interestingly, PFGE pattern P was associated with hVISA strains (*p*-value= 0.019). It is therefore indicated that hVISA strains was involved in the failure of treatment among the PFGE pattern P. Since hVISA was not detected by routine laboratory, an inappropriate treatment was therefore underestimate. We proposed that genetic events that caused mutation of MRSA genes involving in genetic changes of cells to survive leading to failure treatment of MRSA infection. These factors including hVISA and the genetic variants could be combination mechanisms involved in failure of treatment as mentioned above. This pattern was mostly found in Internal Medicine Ward and respiratory tract infection. Transmission of vital genetic element from one to another may be higher than other wards since the strains were in the major ward where more patients admitted in Thammasat University Hospital. Thus, the surveillance of potential PFGE patterns P is needed. The PFGE, gold standard molecular tool is useful for epidemiology study which give a crucial information for active surveillance. However, the tool is not practical for routine investigation. Several molecular tools have been studied and proposed to use for epidemiological

study as described in next section (Molecular tools for *S. aureus*). It has been suggested to use at least two different molecular tools to be able to obtain the reliable outcomes for epidemiological interpretation.

Dendrogram showed that the different PFGE patterns consist of different number of fragments. The numbers of fragments within the individual pattern were not exactly the same. In addition, variations are gain or loss of restriction of restriction site, deletion or insertion of DNA fragment (Tenover et al., 1995). The results of dendrogram of PFGE indicated that the isolates were most likely come from the same origin. However, these strains were not comparable with any strains reported in Thailand. We proposed that they are unique strain of Thammasat University Hospital which are needed to be closely surveillance and control. In addition, prevention of transmission should be more concerned before the spread takes place.

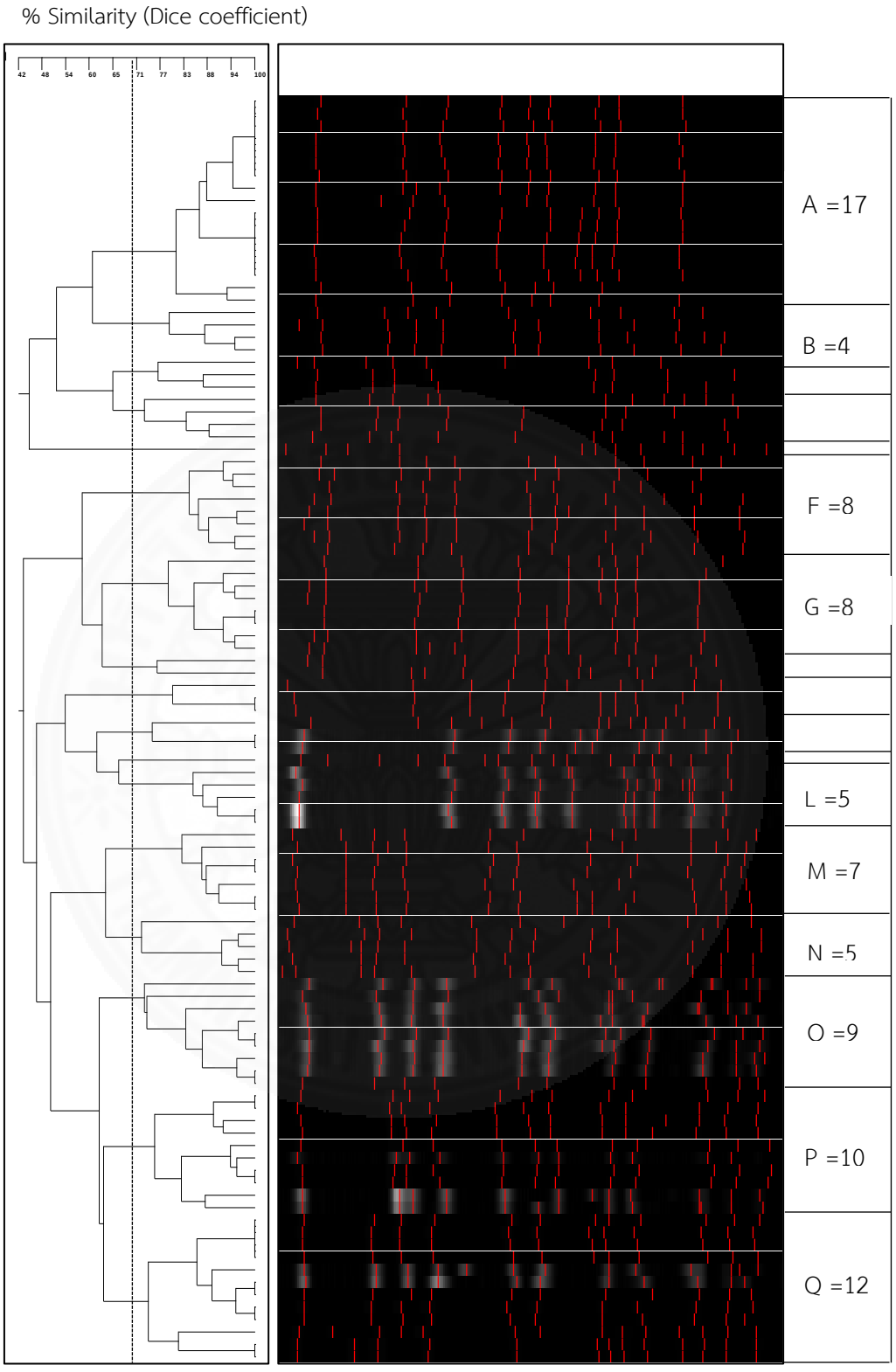


Figure 11 Dendrogram, using GeneDirectory from Syngene with percent similarity calculated by Dice coefficient (2% tolerance position) and represented by UPGMA, clonal group level was set at 71% similarity.

Table 24: Distribution of the PFGE patterns among different specimen types

PFGE patterns	Total number of MRSA isolates	Number of MRSA isolates (%)			
		Sputum	Blood	Pus	Urine
A	16	10 (63)	4 (25)	2 (13)	0 (0)
B	4	3 (75)	1 (25)	0 (0)	0 (0)
C	3	1 (33)	0 (0)	2 (67)	0 (0)
D	4	3 (75)	0 (0)	1 (25)	0 (0)
E	1	0 (0)	0 (0)	1 (100)	0 (0)
F	8	8 (100)	0 (0)	0 (0)	0 (0)
G	8	7 (88)	1 (13)	0 (0)	0 (0)
H	2	0 (0)	1 (50)	1 (50)	0 (0)
I	3	1 (33)	2 (67)	0 (0)	0 (0)
J	3	0 (0)	0 (0)	2 (67)	1 (33)
K	1	0 (0)	0 (0)	1 (100)	0 (0)
L	5	4 (80)	0 (0)	1 (20)	0 (0)
M	7	5 (71)	1 (14)	1 (14)	0 (0)
N	5	5 (100)	0 (0)	0 (0)	0 (0)
O	9	5 (56)	3 (33)	1 (11)	0 (0)
P	10	5 (50)	3 (30)	1 (10)	1 (10)
Q	12	9 (75)	2 (17)	1 (8)	0 (0)

Table 25: PFGE patterns of MRSA isolated from different clinical wards, collected at Thammasat University Hospital, Thailand.

PFGE patterns	Total number of MRSA isolates	Number of MRSA isolates (%)			
		Internal medicine	Surgery ward	Operation theater	Pediatric ward
A	16	12 (75)	3 (19)	1 (6)	0 (0)
B	4	4 (100)	0 (0)	0 (0)	0 (0)
C	3	2 (67)	0 (0)	1 (33)	0 (0)
D	4	2 (50)	0 (0)	1 (25)	1 (25)
E	1	0 (0)	0 (0)	1 (100)	0 (0)
F	8	7 (88)	1 (13)	0 (0)	0 (0)
G	8	8 (100)	0 (0)	0 (0)	0 (0)
H	2	2 (100)	0 (0)	0 (0)	0 (0)
I	3	2 (67)	1 (33)	0 (0)	0 (0)
J	3	1 (33)	2 (67)	0 (0)	0 (0)
K	1	1 (100)	0 (0)	0 (0)	0 (0)
L	5	4 (80)	0 (0)	1 (20)	0 (0)
M	7	7 (100)	0 (0)	0 (0)	0 (0)
N	5	5 (100)	0 (0)	0 (0)	0 (0)
O	9	7 (78)	2 (22)	0 (0)	0 (0)
P	10	6 (60)	4 (40)	0 (0)	0 (0)
Q	12	7 (58)	2 (17)	1 (8)	2 (17)

Table 26: Antibiotic susceptibility of MRSA isolated from different PFGE patterns

Antimicrobial agents	Number of susceptibility isolates (%)																
	A (n=16)	B (n=4)	C (n=3)	D (n=4)	E (n=1)	F (n=8)	G (n=8)	H (n=2)	I (n=3)	J (n=3)	K (n=1)	L (n=5)	M (n=7)	N (n=5)	O (n=9)	P (n=10)	Q (n=12)
Vancomycin	16 (100)	4 (100)	3 (100)	4 (100)	1 (100)	8 (100)	8 (100)	2 (100)	3 (100)	3 (100)	1 (100)	5 (100)	7 (100)	5 (100)	9 (100)	10 (100)	12 (100)
Trimethoprim-Sulfamethoxazole	15 (94)	4 (100)	1 (33)	2 (50)	0 (0)	8 (100)	8 (100)	2 (100)	3 (100)	2 (67)	0 (0)	5 (100)	7 (100)	5 (100)	9 (100)	10 (100)	11 (92)
Fusidic acid	16 (100)	4 (100)	3 (100)	4 (100)	0 (0)	8 (100)	8 (100)	2 (100)	3 (100)	2 (67)	0 (0)	5 (100)	7 (100)	5 (100)	9 (100)	10 (100)	12 (100)
Rifampicin	15 (94)	4 (100)	0 (0)	4 (100)	0 (0)	8 (100)	8 (100)	2 (100)	3 (100)	2 (67)	1 (100)	5 (100)	7 (100)	4 (80)	9 (100)	10 (100)	11 (92)
Trimethoprim	15 (94)	4 (100)	0 (0)	0 (0)	0 (0)	8 (100)	8 (100)	2 (100)	3 (100)	2(67)	0 (0)	5 (100)	7 (100)	5 (100)	9 (100)	10 (100)	9 (75)
Linezolid	16 (100)	4 (100)	3 (100)	4 (100)	1 (100)	8 (100)	8 (100)	2 (100)	3 (100)	3 (100)	1 (100)	5 (100)	7 (100)	5 (100)	9 (100)	10 (100)	12 (100)
Mupirocin	16 (100)	4 (100)	3 (100)	4 (100)	1 (100)	8 (100)	7 (88)	2 (100)	3 (100)	1(33)	0 (0)	5 (100)	7 (100)	5 (100)	9 (100)	10 (100)	12 (100)
Teicoplanin	16 (100)	4 (100)	3 (100)	4 (100)	1 (100)	8 (100)	8 (100)	2 (100)	3 (100)	3 (100)	1 (100)	5 (100)	7 (100)	5 (100)	9 (100)	10 (100)	12 (100)
Cefpirome	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Clindamycin	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (50)	3 (100)	2 (67)	0 (0)	3 (60)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

4.10 Molecular tools for *S. aureus*

Currently, the most frequently used typing methods for *S. aureus* are *spa* sequence typing, Multilocus sequence typing (MLST), SCC*mec* typing, Macrorestriction pattern analysis (PFGE) and Multilocus VNTR analysis (MLVA) (Stefani et al., 2012).

In brief, *spa* typing is a rapid and high throughput, but is misclassification of small number of lineages (Narukawa, Yasuoka, Note, & Funada, 2009). MLST is defines core genetic population, portability and standard nomenclature, but is low throughput and high cost (Struelens, Hawkey, French, Witte, & Tacconelli, 2009). SCC*mec* typing is standard nomenclature, but is low throughput, high cost, no standard protocol and evolving nomenclature (Stefani et al., 2012).

The results of this study conclude that SCC*mec* typing is considered one of the most reliable tools for distinguishing between HA- and CA-MRSA. The majority of HA-MRSA strains carry SCC*mec* types I, II or III, whereas CA-MRSA strains harbor SCC*mec* types IV or V (N. Ahmad et al., 2009; Karahan et al., 2008).

PFGE is high discriminatory index (Petersson, Olsson-Liljequist, Miorner, & Haeggman, 2010; Te Witt, Kanhai, & van Leeuwen, 2009), but is technically difficult, slow, limited portability, multiple nomenclature and misclassification of some lineages.

The epidemiologic characteristics of MRSA are useful for implementation in clinical practice including monitoring and prevention of the multiple drug-resistant of MRSA infection. It is necessary to monitor the spread both within healthcare setting and communities, and also national and international levels.

Table 27: Molecular tools of MRSA collected at Thammasat University Hospital, Thailand.

PFGE patterns	Total number of MRSA isolates	Number of MRSA isolates (%)								
		SCC*mec* typing			Virulence genes patterns					
		Type I	Type II	Type III	Type I	Type II	Type III	Type IV	Type V	Type VI
A	16	0 (0)	16 (100)	0 (0)	0 (0)	15 (94)	1 (6)	0 (0)	0 (0)	0 (0)
B	4	4 (100)	0 (0)	0 (0)	3 (75)	0 (0)	0 (0)	1 (25)	0 ()	0 (0)
C	3	0 (0)	0 (0)	3 (100)	1 (33)	0 (0)	1 (33)	0 (0)	1 (33)	0 (0)
D	4	0 (0)	0 (0)	4 (100)	3 (75)	0 (0)	1 (25)	0 (0)	0 (0)	0 (0)
E	1	0 (0)	0 (0)	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
F	8	7 (88)	1 (13)	0 (0)	6 (75)	0 (0)	0 (0)	0 (0)	1 (13)	1 (13)
G	8	0 (0)	8 (100)	0 (0)	0 (0)	8 (100)	0 (0)	0 (0)	0 (0)	0 (0)
H	2	1 (50)	0 (0)	1 (50)	0 (0)	0 (0)	2 (100)	0 (0)	0 (0)	0 (0)
I	3	0 (0)	0 (0)	3 (100)	0 (0)	0 (0)	3 (100)	0 (0)	0 (0)	0 (0)
J	3	0 (0)	0 (0)	3 (100)	0 (0)	0 (0)	3 (100)	0 (0)	0 (0)	0 (0)
K	1	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)
L	5	0 (0)	0 (0)	5 (100)	0 (0)	0 (0)	5 (100)	0 (0)	0 (0)	0 (0)
M	7	7 (100)	0 (0)	0 (0)	7 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
N	5	0 (0)	5 (100)	0 (0)	0 (0)	5 (100)	0 (0)	0 (0)	0 (0)	0 (0)
O	9	8 (89)	1 (11)	0 (0)	8 (89)	0 (0)	1 (11)	0 (0)	0 (0)	0 (0)
P	10	0 (0)	10 (100)	0 (0)	0 (0)	9 (90)	1 (10)	0 (0)	0 (0)	0 (0)
Q	12	9 (75)	0 (0)	3 (25)	11 (92)	0 (0)	0 (0)	1 (8)	0 (0)	0 (0)

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

MRSA is the causative agent of severe types of infections. Globally, there have been increasing numbers of patients suffered from the MRSA infections. There is a growing concern over the limitation of treatment options for MRSA. While vancomycin is considered as a drug of choice, failure of the vancomycin treatment frequently occurs in about 23% of nosocomial MRSA-infected patients. In this thesis, the important risk factors of failure treatment were identified. These risk factors include chronic hemodialysis, multiple sites infection and co-microbe resistant strains. Furthermore, the treatment failure rate could be significantly increased by infections by hVISA strains. Screening for the hVISA strains may be necessary; although, it currently is not part of the routine microbiological investigations. In the present study, it was found that vancomycin MICs creep was not associated with failure treatment. While linezolid, teicoplanin and vancomycin still remained effective antibiotics for treatment of MRSA infection, extensive use of these antibiotics over a period of time may lead to the generation of selective pressure and emergence of resistance. Therefore, surveillance study for monitoring the antibiotic resistant trend and emerging resistant strains should be continuously conducted.

In addition, this study also revealed the microbiological and molecular characteristics of MRSA isolates collected at Thammasat University Hospital. Antibiogram profiles provided some clue for the epidemiological data for the MRSA strains. Strains with antibiogram pattern 1 were predominant among adult patients while antibiogram pattern 4 strains were more common in children. In this study, it was found that majority MRSA strains at Thammasat University had *SCCmec* type II. PFGE also revealed several types of MRSA, reflecting the variety of this pathogen. It is my great hope that the obtained results of both microbiological and epidemiological characteristics of MRSA isolates will be useful for implementation of monitoring and

prevention program against MRSA and for aiding the development of effective guidelines for the management of MRSA infections.

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APPENDICES

APPENDIX A
REAGENTS AND CHEMICAL SOLUTIONS

0.5M EDTA solution pH 8:

186.1 g EDTA-2H2O (Sigma-Aldrich) added to 800 mL dH2O and mixed thoroughly. pH adjusted to 8 with NaOH pellets (QReC). Volume adjusted to 1 L with dH2O. Autoclaved for 20 minutes at 121°C and store at 4°C.

15% (v/v) Glycerol for bacterial storage:

15 mL Glycerol (Sigma-Aldrich) mixed and dissolved with dH2O. The volume was adjusted to 100mL with dH2O. Dispensed into aliquots of 1 mL in cryotube. Autoclaved for 20 minutes at 121°C and store at 4°C.

0.9% NaCl solution:

9 g NaCl (Biolab) dissolved in 1L dH2O. Autoclaved for 20 minutes at 121°C and store at room temperature.

0.5x TBE (Tris Borate Electrophoresis) buffer:

200 mL 5x TBE mixed with 1,800 mL dH2O.

TE buffer:

5 mL 1M Tris pH 7.6, 1 mL 0.5M EDTA pH 8.0 mixed with 494 mL dH2O. Autoclaved for 20 minutes at 121°C and store at 4°C.

1M Tris-HCl pH8.0:

157.59 g Tris-HCl (Sigma-Aldrich) dissolved in 1 L dH2O. pH adjusted to 8 with NaOH pellets (QReC). Autoclaved for 20 minutes at 121°C and store at 4°C.

APPENDIX B

CULTIVATION MEDIA

MHB (Mueller Hinton broth):

21 g Mueller Hinton broth powder (Difco) dissolved in 1 L dH2O. Dispensed into aliquots and autoclaved for 15 minutes at 121 ºC and store at 4ºC.

MHA (Mueller Hinton agar):

38 g Mueller Hinton agar powder (Oxoid) dissolved in 1 L dH2O. Dispensed into aliquots and autoclaved for 15 minutes at 121 ºC. Once cooled, aliquoted approximately 25 mL into each sterile plate and allowed to set at room temperature. Immediately used or store at 4ºC.

Nutrient broth:

13 g Nutrient-broth powder (Oxoid) dissolved in 1 L dH2O. Dispensed into aliquots and autoclaved for 15 minutes at 121 ºC and store at 4ºC.

NA (Nutrient agar):

23 g Nutrient agar powder (Difco) dissolved in 1 L dH2O. Dispensed into aliquots and autoclaved for 15 minutes at 121 ºC. Once cooled, aliquoted approximately 25 mL into each sterile plate and allowed to set at room temperature. Immediately used or store at 4ºC.

TSB (Tryptic Soy Broth):

30 g TSB powder (Bacto) dissolved in 1 L dH2O. Dispensed into aliquots and autoclaved for 15 minutes at 121 ºC and store at 4ºC.

Brain Heart Infusion broth:

37 g Brain Heart Infusion broth (LABM) dissolved in 1 L dH2O. Dispensed into aliquots and autoclaved for 15 minutes at 121 ºC and store at 4ºC.

BHIA (Brain Heart Infusion Agar):

52 g BHIA powder (Difco) dissolved in 1 L dH2O. Dispensed into aliquots and autoclaved for 15 minutes at 121 ºC. Once cooled, aliquoted approximately 25 mL into each sterile plate and allowed to set at room temperature. Immediately used or store at 4ºC.

BIOGRAPHY

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Publications

Pocaphan, P. & Kondo, S. (2011). Bacterial contamination assay of Thai herbal products. *J Med Assoc Thai, 94*(7), 162-5.

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