Vancomycin Intermediate-Resistant *Staphylococcus aureus*: Characterization of Resistance Development, Detection and Treatment Strategies

Vijaya Chandranna DODDANGOUDAR

Doctor of Philosophy

The Hong Kong Polytechnic University, Hong Kong

2012

The Hong Kong Polytechnic University, Hong Kong

Department of Health Technology and Informatics

Vancomycin Intermediate-Resistant *Staphylococcus aureus*: Characterization of Resistance Development, Detection and Treatment Strategies

By

Vijaya Chandranna DODDANGOUDAR

A Thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy at The Hong Kong Polytechnic University, Hong Kong

May 2012

CERTIFICATE OF ORIGINALITY

I declare that this thesis represents my own work and that, to the best of my knowledge and belief, it reproduces no material previously published or written, nor material that has been accepted for the award of any other degree or diploma, except where due acknowledgement is made.

Signature ----------

Vijaya Chandranna DODDANGOUDAR

ABSTRACT

Since the emergence of vancomycin non-susceptible *Staphylococcus aureus* (hVISA/VISA) efforts have been made to develop a reliable detection method, understand the mechanism of non-susceptibility development and loss, and improve the treatment of VISA infection.

While several methods have been introduced for detection of hVISA/VISA, rapid and correct detection remains difficult due to their various limitations. These limitations could delay appropriate therapy. Therefore, a rapid and reliable resistance detection method is required. The genetic changes associated with development and loss of hVISA/VISA has been tracked in few strains and remains unclear. More work is required to identify important determinants associated with non-susceptibility. Identification of such changes could support development of a molecular detection method. Vancomycin remains the drug of choice for methicillin-resistant *S. aureus* (MRSA) treatment and with the high prevalence of MRSA in Hong Kong, it is not surprising that hVISA/VISA has been reported. VISA has been reported worldwide and determination of the level of hVISA/VISA in Hong Kong is important for formulating infection control guidelines. Additionally, resistance acquisition by MRSA against currently available antibiotics is of concern. Efforts have been made towards the development of new molecules and several new agents are in the pipeline, but such agents are expensive, have possible unknown side effects and may not be available for some time. In

response to these problems, this work aimed to evaluate the spiral gradient endpoint (SGE) as a non-susceptibility detection method, further the understanding of non-susceptibility development and loss by examining genotypic and phenotypic changes, estimate hVISA/VISA prevalence in Hong Kong, and to study the effects of Traditional Chinese Medicine (TCM) herbal extracts alone and in combination with vancomycin against VISA.

SGE was found to have good reproducibility, there being excellent correlation between MICs generated by SGE and agar dilution (r^2 = 0.950). Tracking genotypic and phenotypic changes in both clinical and laboratory-induced VISA strains indicated the importance of mutations in *vraS* and *graR* during development and loss of non-susceptibility, in the development of stable phenotypes, and ability to reach an elevated MIC (20 mg/L) in the absence of *vanA*. This study has also demonstrated the role of stop codons in delaying non-susceptibility development and formation of stable phenotypes. The prevalence rate of hVISA/VISA in Hong Kong hospital was found to be 14.53% (48/330 isolates). Additionally, it was found that strains showing non-susceptible subpopulations rapidly progressed to non-susceptibility in the presence of 2 mg/L vancomycin and that SGE was effective in detecting such strains. Of the three TCM herbs investigated for antimicrobial activity, *Radix scutellariae* was found to be effective against VISA both alone and in combination with vancomycin at 2 g/L and 0.25 g/L respectively.

In summary, SGE offers a reliable alternative for the detection of hVISA/VISA. Mutations in *vraS* and *graR* appear to be important for development of non-susceptibility. In particular the presence of stop codons in two component-regulatory systems appears to be important in non-susceptibility development and loss. The prevalence rate of vancomycin non-susceptibility in local isolates were relatively high and testing revealed additional strains with, resistant sub-populations which could rapidly progress to VISA. Antimicrobial-activity of RS indicated that this herb may have the potential to be used in treatment of MRSA and VISA infections, alone and in combination with vancomycin.

PUBLICATIONS ARISING FROM THIS THESIS

Journal Articles

Doddangoudar VC, O'Donoghue MM, Boost MV, Tsang DNC, Appelbaum PC: Rapid detection of vancomycin-non-susceptible *Staphylococcus aureus* using the spiral gradient endpoint technique. *Journal of Antimicrobial and Chemotherapy* 2010; 65: 2368–2372.

Doddangoudar VC, Boost MV, Tsang DNC, O'Donoghue MM: Tracking changes in the vraSR and graSR two component regulatory systems during the development and loss of vancomycin non-susceptibility in a clinical isolate. *Clinical Microbiology and Infection.* 2011**;**17:1268-1272.

Doddangoudar VC, O'Donoghue MM, Chong E, Tsang DNC, Boost MV: Role of stop codons in development and loss of vancomycin non-susceptibility in MRSA**.** *Journal of Antimicrobial and Chemotherapy.* doi:10.1093/jac/dks171.

Conference Oral Presentations

Doddangoudar VC, Boost MV, O'Donoghue MM, Tsang DNC: Tracking development and loss of vancomycin non-susceptibility in methicillin - resistant *Staphylococcus aureus.* Western Pacific Congress of Chemotherapy and Infectious Diseases, Singapore, 2010.

Doddangoudar VC, Boost MV, O'Donoghue MM, Tsang DNC: Genotypic and phenotypic changes in development and loss of vancomycin non-susceptible *Staphylococcus aureus.* HTI Symposium, Hong Kong, 2011.

Doddangoudar VC, Boost MV, O'Donoghue MM, Tsang DNC: Anti-MRSA and Anti-VISA activity of Cortex Phellodendri and Rhizoma Coptidis**.** Current Trends in Medicinal Plants Research, Pune, 2012.

Conference Poster Presentations

Doddangoudar VC, Boost MV, O'Donoghue MM, Tsang DNC, Appelbaum PC: Evaluation of spiral gradient endpoint technique (SGE) for rapid detection of vancomycin intermediate-resistant *Staphylococcus aureus*. European Congress of Clinical Microbiology and Infectious Diseases, Vienna, 2010.

Doddangoudar VC, Boost MV, O'Donoghue MM, Tsang DNC, Appelbaum PC: Development and stability of non-susceptibility to vancomycin in methicillin - resistant *staphylococcus aureus.* European Congress of Clinical Microbiology and Infectious Diseases, Vienna, 2010.

Doddangoudar VC, O'Donoghue MM, Tsang DNC, Boost MV: Early detection of vancomycin resistant sub-population in clinical MRSA with MIC <2mg/L. European Congress of Clinical Microbiology and Infectious Diseases, London, 2012.

ACKNOWLEDGEMENTS

Completion of this thesis is an important milestone in my career and life and it is my pleasure to acknowledge those who made this possible. First of all I bow to the almighty for blessing my life with this opportunity and wonderful people.

I express my deep sense of gratitude to my supervisor, Dr. Maureen BOOST for her guidance, encouragement and support in this research work. I am grateful to my co-supervisors Dr. Margaret O'DONOGHUE and Dr. Dominic TSANG for their guidance and support.

I sincerely thank Prof. Keiichi HIRAMATSU, Juntendo University (Japan) and Prof. Peter APPELBAUM, Hershey Medical Center (US) for their kind advice.

I thank faculty and staff members of Health Technology and Informatics Department, The Hong Kong Polytechnic University, Hong Kong for their encouragement.

I acknowledge the *Journal of Antimicrobial and Chemotherapy,* and *Clinical Microbiology and Infection* for publishing part of this research.

I thank the Pathology department, Queen Elizabeth Hospital, Hong Kong for their assistance and all others who have directly or indirectly contributed for this research.

I acknowledge The Postgraduate International Studentship, The Hong Kong Polytechnic University, Hong Kong for funding this research.

I appreciate my family and friends for their encouragement and motivation. My parents deserve special mention for aspiring me to dream, showing me the joy of intellectual pursuit, and for their unconditional love and support.

There were times when I wanted to give up my dreams, it is my husband's encouragement and loving support that has revitalized my dreams and words fail to express my appreciation for him.

As a token of appreciation this thesis is dedicated to them who have aspired, supported, and contributed for my dreams.

TABLE OF CONTENTS

TABLE OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AD: Agar Dilution

AST: Antimicrobial susceptibility test

AUC: Area under the Curve

BHA: Brain Heart Agar

BHI: Brain Heart Infusion

BP: Base Pair

BSAC: British Society for Antimicrobial Chemotherapy

CA-MRSA: Community Acquired Methicillin Resistant *Staphylococcus aureus*

ccr: Cassette Chromosome Recombinases

CDC: Centre for Disease Control

CLSI: Clinical and Laboratory Standards Institute

Cmax: Maximum Serum Drug Concentration

CNS: Central Nervous System

CSF: Cerebro-Spinal Fluid

CV: Coefficient of Variation

DNA: Deoxyribose Nucleic Acid

EC: End Point Concentration

EDTA: Ethylene Diamine Tetra Acetic acid

EMA: European Medicines Agency

EP: Confluent Growth End Point

Etest: Epsilometer test

EUCAST: European Committee on Antimicrobial Susceptibility Testing

FDA: Food and Drug Administration

GBH: Glucose BHA

GI: Genomic islands

GIT: Gastro-Intestinal Tract

g/L: Grams/Litre

h: Hour

hVISA: Heterogeneous Vancomycin Intermediate-Resistant *Staphylococcus aureus*

Hla: Alpha-hemolysin

IE: Infectious Endocarditis

ISS: Integration Site Sequence

IV: Intravenous

L: Litre

LPGT: Lysylphosphatidylglycerol Transferase

LTA: Lipoteichoic Acids

MDRO: Multi-Drug Resistant Organism

µl: Microliter

mg/L: Milligrams/Liter

MBC: Minimum Bactericidal Concentration

MBD: Micro Broth Dilution

MET: Macro Etest

MGE: Mobile Genetic Elements

MHA: Mueller-Hinton agar

MIC: Minimal Inhibitory Concentration

MLS_B: Macrolide-Lincosamide-Streptogramin B

Mins: Minutes

MRSA: Methicillin Resistant *Staphylococcus aureus*

NaCl: Sodium Chloride

Orf: Open Reading Frame

PAP: Single-point Population Analysis

PAP-AUC: Population Analysis Profile Area under Curve Ratio

PBP: Penicillin Binding Protein

PCR: Polymerase Chain Reaction

PFGE: Pulsed field gel electrophoresis

PK/PD: Pharmacokinetics/Pharmacodynamics

PMN: Polymorphonuclear

PVL: Panton-Valentine Leukocidin

QD: Quinupristin-dalfopristin

SAB: *Staphylococcus aureus* Bacteremia

SAD: Serial Agar Dilution

SAM: *Staphylococcus aureus* Meningitis

SCC: Staphylococcus Cassette Chromosome

SD: Standard Deviation

SGE: Spiral Gradient Endpoint

SSI: Skin and Soft Tissue Infections

TCM: Traditional Chinese Medicine

TCS: Two-component systems

TEC: Trailing End Point Concentration

TP: Trailing End Point

tRNA: Transfer Ribonucleic Acid

TSS: Toxic Shock Syndrome

TSST-1: Toxic Shock Syndrome Toxin 1

TRIS: Tris(hydroxymethyl)aminomethane

US: United States of America

UDP: Uridine Diphosphate

VISA: Vancomycin Intermediate-Resistant *Staphylococcus aureus*

VRE: Vancomycin Resistant Enterococci

VRSA: Vancomycin Resistant *Staphylococcus aureus*

WTA: Wall Teichoic Acids

PREAMBLE

The research described in this thesis revolves around the health care and management aspects of the common bacteria '*Staphylococcus aureus'*. A normal skin flora but, an opportunistic pathogen by nature, *S. aureus* is known to be the causative agent for both minor and serious conditions in humans. Recommended treatment for *S. aureus* infections includes various antibiotics. However, *S. aureus* has been reported to have acquired resistance to several groups of antibiotics. The acquisition of resistance against methicillin was an important change in *S. aureus*. With this, vancomycin became the drug of choice for treatment of methicillin-resistant *S. aureus* (MRSA) infections. But in the last few years, vancomycin reduced susceptibility has been reported. This resistance development is a cause for concern in both the research and in the health care community with respect to accurate detection, the associated molecular mechanism, clinical prevalence and treatment of vanocmycin intermediate-resistant *S. aureus* (VISA) / heterogeneous VISA (hVISA) infections. The research described here addresses some of these concerns. A brief overview of the structure of the thesis is set down below

Overview of the Thesis

This thesis consists of eight chapters. Chapter 1 consists of the literature review, Chapter 2 outlines the aims and objectives, Chapters 7 integrates the findings and their significance, and Chapter 8 presents the conclusions, while each of the other four Chapters (3 to 6) are targeted towards addressing one of the four objectives of this research. Each of the Chapter's titles and their outline is as follows:

Chapter 1: Literature review - This chapter provides an overview of the rest of the chapters, introduces the background of the research topic, provides a thorough up-to-date literature review and based on this review points out the gaps in knowledge which need to be addressed in improving *S. aureus* related health care and management practices and guidelines.

Chapter 2: Research Objectives - Research gaps identified in Chapter 1 are translated into aims and objectives of the research. Also the significance of the research is highlighted.

Chapter 3: Evaluation of the spiral gradient endpoint technique for rapid detection of vancomycin non-susceptible *S. aureus* - This chapter describes the aim to design a screening tool for rapid and correct detection of vancomycin non-susceptible *S. aureus,* by means of the spiral gradient endpoint (SGE) technique. In this regard, the chapter outlines various vancomycin non-susceptibility screening methods with benefits and associated limitations. SGE method was hypothesized as an option for rapid, reliable and cost effective detection of hVISA/VISA. The experimental design to test the above hypothesis and findings from those experiments are described in detail.

Chapter 4: Tracking the development and reversion of vancomycin non-susceptibility in *S. aureus* with genotypic and phenotypic evidence - The aim of this chapter is to track development and loss of vancomycin non-susceptibility, to understand and explore the molecular mechanism associated with vancomycin non-susceptibility development and loss. The study examines the molecular

changes and correlates these with phenotypic changes and the vancomycin non-susceptibility development and loss pattern.

Chapter 5: Determination of the prevalence of the VISA in clinical isolates from a local hospital - This chapter aims to determine the level of vancomycin non-susceptible *S. aureus* in clinical MRSA isolates in a Hong Kong district hospital, detect isolates likely to rapidly develop non-susceptibility during vancomycin therapy, and to determine the length of time for these isolate to progress into non-susceptibility in the presence of a therapeutic levels of vancomycin. In this regard, this chapter describes the experimental design formulated to accomplish the aim and reported the findings of this study.

Chapter 6: Study of effects of selected TCM herbs on MRSA and hVISA/VISA - The aim of this chapter is to identify possible alternative and/or complimentary therapies for MRSA and vancomycin non-susceptible *S. aureus* among some Traditional Chinese Medicine (TCM) herbs. Emergence of resistance against vancomycin has challenged its clinical value and has limited the treatment options for MRSA infections. In view of restricted treatment options, this study investigated the anti-MRSA and anti-VISA activities of selected herbs alone and in combination with vancomycin. The experimental design was structured to achieve the aim and the findings of this study are described in detail.

Chapter 7: This chapter discusses the major findings from the studies presented in chapters 3-6.

Chapter 8: This chapter summarizes the conclusions, identifies and proposes possibilities for further studies. The findings are generalized for the clinical application where possible and the major findings from Chapter 7 are integrated to provide a framework for rapid detection, and appropriate antimicrobial selection in treatment of MRSA and hVISA/VISA as well as to improve the clinical outcomes and infection control guidelines as listed in chapter 2.

Chapter eight is followed by an appendix section, and the references.

CHAPTER 1: LITERATURE REVIEW

1.1 INTRODUCTION

Antimicrobial resistance acquisition in micro organisms has become a common microbiology research theme in the last few decades, particularly with respect to *S. aureus*. A significant evolution has been the acquisition of methicillin resistance in *S. aureus*, now a major problem worldwide. With the increase in prevalence of MRSA, the glycopeptides, particularly vancomycin became the drug of choice for treatment of MRSA infections. For several years there was no evidence of vancomycin non-susceptibility development in *S. aureus*. However recently, strains with different degrees of vancomycin non-susceptibility have emerged in clinical MRSA strains, generating significant concern in both the clinical and research community. In addition to this, fully vancomycin resistant *S. aureus* (VRSA) with *vanA* gene has emerged. However, to date very few cases of the latter have been reported. Therefore, this study will focus on the more common vancomycin non-susceptibility in *S. aureus* which is associated with cell wall thickening.

1.1.1 Introduction to *S. aureus*

S. aureus is a gram positive coccus, belonging to the family 'Staphylococcaceae', which is in the order Bacillales (Rayn 2004). It has been recognized as a pathogen since 1884 (Ogston 1884). It is frequently part of the human skin flora found in the axillae, the inguinal and perineal areas, and the anterior nares. However, the anterior nares is the most common and natural reservoir for *S. aureus*.

S. aureus is known to cause a wide range of infections from minor skin infections (Sheagren 1985, Roberts and Chambers 2005) to persistent joint infections (Lew and Waldvogel 2004, Davis 2005,) and fatal conditions such as pneumonia, meningitis, endocarditis (Chambers 2005a, Chambers 2005b, Murray 2005) bacteremia and sepsis (Gosbell 2005, Mitchell 2005) and toxic shock syndrome (TSS) (Sheagren 1999).

In the last few decades, treatment of *S. aureus* infection has become increasingly difficult due to its ability to rapidly develop resistance against newly introduced anti-microbial agents. MRSA infection is associated with poorer outcomes than infection with methicillin-sensitive *S. aureus* (MSSA) infection (Cosgrove et. al. 2003). MRSA infection surpassed HIV as a cause of death in US in 2005 with a mortality rate of 6.3% (Camargo and Gilmore 2008). MRSA infection increases the length of hospital stay by 5-8 days compared to MSSA infection, thereby significantly increasing hospital charges (Abramson and Sexton 1999, Engemann et. al. 2003, Cosgrove et. al. 2005).

With the global increase in prevalence of MRSA and reports indicating that Hong Kong has one of the highest prevalence rates of MRSA in the Asia Pacific region (Ip et. al. 2004), vancomycin has been widely used in MRSA treatment leading to the emergence of vancomycin reduced susceptible (VISA) phenotypes in Hong Kong (Wong et. al. 1999). Since the first report of VISA from Japan (Hiramatsu et. al. 1997a), these phenotypes have been recognized worldwide but little information is available about their properties, highlighting the need for understanding the mechanism of this reduced susceptibility, local prevalence levels of these strains,

and the establishment of a standard detection method for effective treatment and infection control.

1.1.2 Taxonomy and Morphology of *S. aureus*

S. aureus is a non-motile, non-spore-forming, facultative anaerobic bacterium, which usually appears in pairs or short chains or grape-like clusters. This is because *S. aureus* divides in two planes, unlike streptococci which divide in one plane forming a chain (Rayn 2004, Bannerman and Peacock 2007).

S. aureus colonies are round, smooth, translucent and large; measuring about 3-4 mm in diameter at 48h. The colonies of most strains are pigmented, ranging from cream-golden yellow to orange and are often associated with hemolysis, when grown on blood agar (Rayn 2004).

S. aureus is resistant to temperatures as high as 50°C, high salt concentrations (up to 10%), and drying. It is catalase positive, being able to convert hydrogen peroxide to water and oxygen due to its ability to produce catalase enzyme. This makes the catalase test a useful tool to differentiate staphylococci from enterococci and streptococci (Rayn 2004, Bannerman and Peacock 2007).

S. aureus is coagulase-positive, being able to clot plasma. Hence, the coagulase test can be used to differentiate *S. aureus* from most other staphylococci. Although the majority of *S. aureus* are coagulase-positive, some atypical strains do not produce coagulase (Rayn 2004).

1.1.3 Growth Characteristics

S. aureus are facultative anaerobes that grow by aerobic respiration or by fermentation that principally yields lactic acid. The nutritional requirements of *S. aureus* are complex and vary both from strain to strain and from the conditions under which it is grown. Environmental factors such as temperature and pH have a enormous impact on growth and enterotoxin production (Bennett et. al. 1986).

In general, *S. aureus* can grow between 7-50^{0}C, with an optimal range of 30-37^{0}C. Enterotoxins are produced between 10-46^{0}C, with most production between 35-45^{0}C. Enterotoxin production is substantially reduced at 20-25^{0}C. It is possible for *S. aureus* to grow without producing enterotoxin and it is generally accepted that enterotoxin production is unlikely to occur at temperatures below 10^{0}C. Optimum enterotoxin production occurs at pH 6-7 and is influenced by atmospheric conditions, oxygen, carbon, nitrogen and salt level (Bennett et. al. 1986).

1.1.4 *S. aureus* Colonization and Infection

As *S. aureus* infections frequently follow colonization, this section will first briefly describe colonization followed by infections.

In healthy subjects, three patterns of carriage have been observed. It has been found that about 20% of humans are long term carriers, 60% are intermittent carriers, and approximately 20% almost never carry *S. aureus* (Kluytmans et. al. 1997)**.** However, recently van Belkum et. al. (2009) classified human nasal *S. aureus* carriers into 2 groups: persistent carriers and others, as both intermittent

carriers and non-carriers share low risk for infection, similar *S. aureus* nasal elimination kinetics and anti-staphylococcal antibody profiles. The carriage rate varies from 11-32% for healthy adults in the general population and is around 25% in hospital personnel (Wenzel and Perl 1995).

Various risk factors have been associated with *S. aureus* carriage and are as follows: age (Lucet et. al. 2003), chronic illness (Jernigan et. al. 2003), companion animals (Andreoletti et. al. 2009), gender (Jariyasethpong et. al. 2010), history of MRSA carriage or infection (Fishbain et. al. 2003), hospitalization especially to intensive care unit (Graffunder et. al. 2002, von Baum et. al. 2002, Jernigan et. al. 2003, Lucet et. al. 2003), nose picking (Wertheim et. al. 2006), occupation (animal husbandry, health care, slaughter house workers, and veterinarians) (Andreoletti et. al. 2009, Van Cleef et. al. 2010, van den Broek et. al. 2008), presence of skin lesions (Lucet et. al. 2003), previous antibiotic treatment (Fishbain et. al. 2003, Harbarth et. al. 2000), previous surgery (Lucet et. al. 2003, Shimada et. al. 1993), stay at nursing homes and elderly homes (von Baum et. al. 2002), travel (Köck et. al. 2010), and use of indwelling devices (Mody et. al. 2007).

A range of infections caused by *S. aureus* is as discussed below.

1.1.4.1 Skin and Soft Tissue Infections (SSI)

Impetigo: Typically, this appears as a small area of erythema. As the disease progresses a bullae (filled with cloudy fluid) appears, that ruptures and heals, leaving a denuded area with a varnish-like coating. Although group-A

Streptococcus was once considered to be the causative agent, *S. aureus* has been recognized as the major pathogen since 1980 and is now accepted as the causative agent of impetigo (Dagan 1991). It occurs in young school going children, and spreads in families through close physical contact. Impetigo is more prevalent in warm and humid climates (Dagan 1991).

In the late 20th century impetigo was rarely observed in the United States. However, in the last decade United States and other parts of the world have experienced a dramatic increase in *S. aureus* SSI in previously healthy individuals due to an increase in community-associated MRSA (CA-MRSA) infections, in persons without traditional risk factors (Crum 2005, Fergie 2001, Gonzalez et. al. 2005, Kaplan et. al. 2005, Davis et. al. 2007). Most of these infections are caused by USA-300 and USA-400 strains (McDougal et. al. 2003), which are resistant to β-lactam antibiotics and erythromycin (Weber 2005).

Folliculitis, furuncle and carbuncle: Folliculitis describes a tender pustule involving a hair follicle, whereas, a furuncle is a small abscess that exudes purulent material from a single opening involving both the skin and the subcutaneous tissues. A carbuncle is a combination of multiple furuncles and has several pustular openings. These are common among patients with impaired neutrophil or immune system function or patients with chronic eczema, impaired circulation, or diabetes mellitus (Sztramko et. al. 2007).

A report has shown that 17% of folliculitis, and furuncles caused by MRSA in HIV positive patients were resistant to ciprofloxacin (92%) and levofloxacin (77%) (Sztramko et. al. 2007).

1.1.4.2 Osteomyelitis and Joint Infections

Bones are usually well protected, but can become infected through the following routes:

- The bloodstream (which may carry the causative agent from the site of infection to the bones)
- Direct infections due to surgical attachments.
- Infections in adjacent bone or soft tissues.

Osteomyelitis simply means an infection of bone or bone marrow. When it is chronic, it can lead to bone sclerosis and deformity (Kumar et. al. 2007). Chronic osteomyelitis may develop due to the presence of intracellular bacteria which are more resistant to antibiotics, resulting in chronic and difficult to treat infections. The infection can spread to joints and can cause arthritis (Kumar et. al. 2007). Because of the nature of blood supply to the large bones, such as tibia, femur and vertebra, these bones are more susceptible to osteomyelitis (Kumar et. al. 2007).

S. aureus is the most common causative agent of bone and joint infection with 80% of osteomyelitis among children and adolescents being caused by this organism (Dohin et. al. 2007, Kumar et. al. 2007). Blood stream sourced osteomyelitis is more common among children, and 50% of vertebral osteomyelitis is caused by *S. aureus* (Kumar et. al. 2007). Korakaki and coworkers (2007) have reported MRSA acute osteomyelitis and septic arthritis cases among neonates in Greece. Most *S. aureus* osteomyelitis in adults is caused by injury exposing the bone to local infection (Kumar et. al. 2007).

Bone infections often require prolonged antibiotic treatment, with courses lasting from several weeks to months. Osteomyelitis also may require surgical debridement and severe cases may lead to loss of a limb (Dohin et. al. 2007, Kumar et. al. 2007).

1.1.4.3 Staphylococcal Pneumonia

Pneumonia is one among the many infections mediated by *S. aureus*, accounting for an estimated 50,000 staphylococcal infections per year in the United States alone (Kuehnert et. al. 2005). As one of the leading causative agents of ventilator-associated pneumonia, *S. aureus* has invaded the intensive care environment (Hidron et. al. 2008). Further, this pathogen is now increasingly recognized as an important cause of community-acquired pneumonia, displaying the ability to infect healthy adults and children (Hidron et. al. 2008).

Recent investigations have highlighted the importance of Panton-Valentine leukocidin (PVL) and alpha-hemolysin (Hla) for the pathogenesis of *S. aureus* pneumonia (Labandeira-Rey et. al. 2007, Wardenburg et. al. 2007).

The increased pathogenesis in *S. aureus* co-infection with influenza is evident in mortality rates of about 50%, highlighting an apparent synergy of these pathogens in the lung environment. Both the dependence of the elderly population on intensive care therapies and the threats of epidemic or pandemic influenza underscore the large population of diverse individuals that are at significant risk for the development of *S. aureus* pneumonia (Hidron et. al. 2008).

Further, complicating the clinical management of staphylococcal pneumonia is that over half of *S. aureus* isolates are currently methicillin-resistant, harboring genes that make these isolates insensitive to most of the antibiotics which were once a potent class of antimicrobial agents (Hidron et. al. 2008). A recent clinical observation suggests that mortality from MRSA pneumonia can exceed 50%, defining the severity of disease caused by this organism (Hidron et. al. 2008).

Reports suggest that there is a considerable economic burden associated with *S. aureus* treatment (Kim et. al. 2001). The estimated medical cost in US to treat a patient suffering with *S. aureus* pneumonia is in excess of USD 35,000-154,605, which can be a significant burden on the nation's economy (Rubin et. al. 1999, Taneja et. al. 2010).

1.1.4.4 Meningitis

Infection of the protective barrier (meninges) of the brain and spinal cord is termed as meningitis; the causative agent may be bacterial, viral, fungal and protozoal.

Although, *S. aureus* meningitis (SAM) is rare, usually seen in patients with connective tissue infections such as bacteremia and osteomyelitis, para-meningeal infections or post-neurosurgical conditions, it accounts for 10.2% of bacterial meningitis and around 51% of SAM occurs following neurosurgery (Pintado et. al. 2002).

In recent years, meningitis caused by MRSA has increased, presenting therapeutic challenges in providing appropriate therapy. It is reported that 79% of SAM is caused by MRSA in adults, that patients with MRSA SAM were older then MSSA

SAM patients and that 75% of MSSA SAM were found to be community-acquired (Chang et. al. 2001). It is also reported that nosocomial infection was more common in post-neurosurgical SAM and patients with MRSA SAM had greater mortality and morbidity rate compared to MSSA SAM (Chang et. al. 2001).

In children, SAM occurs in those with pre-existing conditions of the central nervous system (CNS) such as neurosurgery or trauma. Givner and Kaplan (1993) have reported 80% of SAM pediatric cases had CNS abnormality.

1.1.4.5 Bacteremia

Blood is sterile normally. Presence of bacteria in blood is considered as abnormal and the condition is termed as bacteremia. *S. aureus* is a frequent cause of infection and bacteremia in post-operative patients. *S. aureus* bacteremia (SAB) is usually associated with other serious connective tissue infections.

The port of entry of *S. aureus* resulting in SAB is reported to be post-operative infections in 23.0% of patients, primary surgical wound in 67.1% intravascular catheter infection in 6.4%, pneumonia in 5.5%, other sources in 8.2%, with 2.7% of patients having an unknown source of SAB (Gottlieb et. al. 2000).

It is reported that persistent bacteremia due to MRSA presents a therapeutic challenge, particularly in patients with suspected endocarditis (Khatib et. al. 2006). Even when bacteremia is caused by a *S. aureus,* illness may persist in patients with endocarditis for up to 6 days after initiation of therapy with an antimicrobial agent (Levine et. al. 1991).

1.1.4.6 Endocarditis

Inflammation of the endocardium (valves and septum) is termed as endocarditis. *S. aureus* is a leading cause of infectious endocarditis (IE) worldwide with related high mortality. MRSA accounts for 40% of all IE cases caused by *S. aureus* (Flower et. al. 2005).

The portal of entry for *S. aureus* and causes of IE are drug injection, piercing, and frequent use of echocardiography, organ transplantation, cardiac surgery, bacteremia and dermatitis (Flower et. al. 2005, Giuliana et. al. 2010).

Recently it has been reported that severe dermatitis caused by *S. aureus* is a potential risk for bacteremia and invasive infections such as endocarditis and accounts for 30-35% of native valve endocarditis (Hill et. al. 2007, Mohiyiddeen et. al. 2008).

In cases of post-transplantation endocarditis, *S. aureus* contributes to 80% of the mortality among all liver transplant recipients and 1.7% of mortalities among all the organ transplant recipients (Singh et. al. 2000, Sherman-Weber et. al. 2004). However, even in the absence of identifiable risk factors, the risk of *S. aureus* IE remains high, at up to 16% (Flower et. al. 2003).

1.1.4.7 Toxic Shock Syndrome

Toxic shock syndrome (TSS) is a rare condition with 0.06 cases per 100,000 people *(CDC 1996)*, and is characterized by high fever, rash, hypotension, and multi-organ failure. It is a toxin-mediated acute life-threatening illness, usually precipitated by infection with either *S. aureus* or *Streptococcus pyogenes.*

S. aureus colonization or infection followed by toxin production including toxic shock syndrome toxin 1 (TSST-1) and enterotoxins A, B, C, D, E, G, H and I or exfoliative toxin A or B, and systemic absorption of these toxins produces the systemic manifestations of TSS in people who lack a protective antitoxin antibody (Jarraud et. al. 1999).

TSS was first described in 1978 and since then, it has been associated with high risk groups (Todd et. al. 1978, Matsuda et. al. 2003, Reithinger et. al. 2005). Several studies have correlated TSS with use of high absorbency tampons during menstruation (Berkley et. al. 1987), with use of barrier contraceptives, in postpartum women, patients with surgical wound infections or focal *S. aureus* infection or nasal surgery, in patients with nasopharynax infection (Todd et. al. 1978, Reithinger et. al. 2005, Stevens et. al. 2006) and as a complication of influenza (MacDonald et. al. 1987).

Sinusitis contributes up to 5% of upper respiratory infections, and the sinuses are known to provide a growth environment similar to the vagina in menstruation or other sites of focal infection (Wald 1985). Ferguson and Todd (1990) have described the association of TSS with the sinuses, and other sites of *S. aureus*

infections can result in TSS, including lung, soft tissue and skin, bone and joint infection.

1.1.5 Antimicrobial Resistance

Since the introduction of antibiotics and chemotherapeutic agents, *S. aureus* has been successful in acquisition of antimicrobial resistance. The diversity of antimicrobial resistance acquisition and virulence factors among *S. aureus* has likely occurred through mobile genetic elements (MGE) or by mutation, recombination, and a combination of the above (Gill et. al. 2005). MGE includes bacteriophages, pathogenic genomic islands (GI), plasmids, transposons, staphylococcus cassette chromosome (SCC), and insertion sequences (Gill et. al. 2005). This may have contributed towards change in genetic resistance patterns as well as shift in initial location of infection.

Bacteriophages are considered as transducing phages as they play an important role in horizontal transfer of DNA among strains. Bacteriophages are widely distributed and almost all *S. aureus* carry at least one or more bacteriophages. These are known to encode enterotoxin-A and PVL (Lindsay and Holden 2004).

GI are similar to bacteriophages, but need a helper phage for horizontal transfer. GI encode super antigen genes, including *seb,* and *sec* (TSST-1). GI can also act as a multi-drug resistance transporter, including for the fusidic acid resistance gene (O'Neill and Chopra 2007, Lindsay and Holden 2004).

There are two types of plasmids in *S. aureus*. Small plasmids which code for one or two resistance genes (Khan 2005) and the larger plasmids which carry multiple

resistance genes conferring resistance to several antibiotics including penicillin, trimethoprim, aminoglycosides, heavy metals and detergents (Berg et. al 1998).

Transposons include small resistance genes (e.g. Tn554 carries erm gene which confers resistance to erythromycin, while Tn552 includes *blaz* for penicillinase). These are integrated into SCC or plasmids or the chromosome (Rowland 1989). Some *S. aureus* carry large transposons like the *vanA* cluster genes and exhibit a high level of resistance to vancomycin (Clark et. al. 2005).

1.1.5.1 Resistance to β-lactam Antibiotics

Most cell wall targeting antibiotics function by blocking or disturbing peptidoglycan synthesis or polymerization of peptidoglycan precursors resulting in cell wall degradation and death (Bugg 1999, Pinho et. al. 2001). The two major groups of cell wall active antibiotics are β-lactams and glycopeptides. β-lactams were among the first antibiotics introduced into clinical application. These agents acylate the transpeptidase-active sites of PBPs, thereby producing bactericidal activity (Pinho et. al. 2001). β-lactam resistance in *S. aureu*s has been widely reported, and generally occurs through one of two main mechanisms. The first is by production of penicillinase, mediated by Blaz, which hydrolytically cleaves β-lactams. However, the second mechanism confers broader resistance to both penicillinase sensitive and penicillinase resistant β-lactam, and is defined as methicillin resistance (Hartman and Tomasz 1984, Reynolds and Brown 1985).

MRSA was first described in 1961 (Jevons 1961) but did not become widespread until much later. Since its first report, MRSA generated much interest in both

health care workers and researchers. The interest stems from a number of factors such as magnitude of the infections, antibiotic resistance concern, mechanism of resistance and mode of resistance.

In MRSA, the *mecA* gene, which encodes 78-kDa penicillin-binding protein (PBP2a), results in resistance to semi-synthetic penicillin e.g methicillin, oxacillin, most cephalosporins and all other β-lactam antibiotics. In methicillin-sensitive *S. aureus* (MSSA), the β-lactam antibiotics bind to the native PBPs that are present in the *S. aureus* cell wall, which results in the disruption of the synthesis of the peptidoglycan layer. As a consequence, *S. aureus* will not survive. Acquisition of *mecA* gene by horizontal gene transfer leads to expression of resistance against β-lactam antibiotics by modifying PBP2 (Gill et. al. 2005). However, in the presence of this novel penicillin binding protein PBP2a, peptidoglycan and cell wall synthesis continues, resulting in the growth of MRSA (Berger-Bachi and Rohrer 2002) as shown in Figure 1.1. The *mecA* gene is regulated by the repressor *MecI* gene and the trans-membrane β-lactam-sensing signal-transducer MecR1. *MecI* controls both the transcription of *mecA* and *Mecr1* in the absence of a β-lactam antibiotic. However, in the presence of a β-lactam, MecR1 is auto-catalytically cleaved, and the metalloprotease domain, which is located in the cytoplasmic part of MecR1, becomes active. This metalloprotease cleaves MecI, which, in turn, is bound to the *mecA* operator region, allowing the transcription of *mecA*, and the subsequent production of PBP2a to occur (Berger-Bachi and Rohrer 2002).

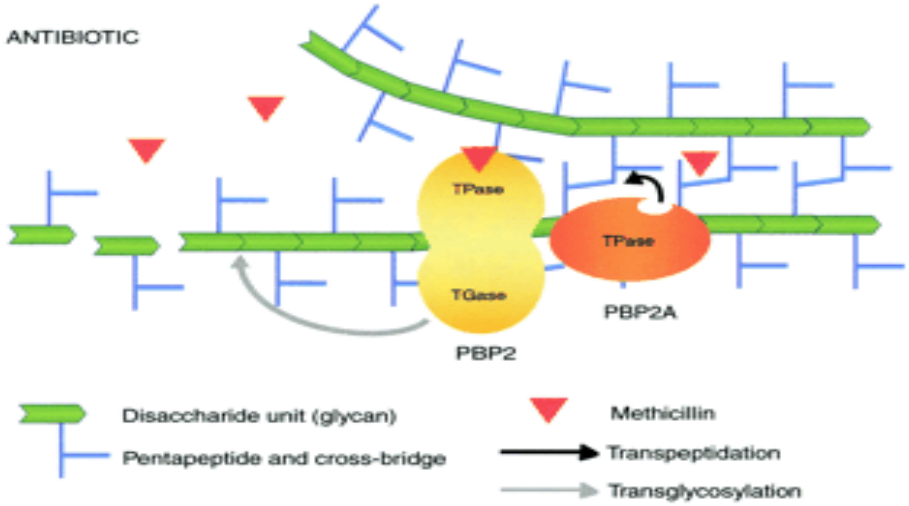


FIGURE 1.1: Model of cell wall synthesis in the presence of Pbp2a
(modified from Pinho et. al. 2001)

The *mecA* gene is 2.1 kb in length, located on a mobile genomic island, called the staphylococcus cassette chromosome *mec* (SCC*mec*) (Ito et. al. 2003).

Mobile resistance element SCC*mec*:

The pathogenicity of *S. aureus* is attributed to genomic islands such as *v*Sa and SCC*mec*, which encode enterotoxins, exotoxins, leukocidins and leukotoxins and accommodate a range of antibiotic resistant genes based on the size of the cassette (Gill et. al. 2005).

The genetic component encoding methicillin resistance and carrying site specific recombinases are termed as cassette chromosome recombinases *(ccr)* and the entire element is defined as SCC*mec*. Several types of SCC*mec* have been identified. These share several common characteristics and carry the following: the *mec* gene complex; *ccr* gene complex; the integration site sequence (ISS), which serves as a target for *ccr*-mediated recombination, and flanking direct repeat sequence (DR).

The SCC*mec* elements are classified based on the combination of *ccr* gene complex (*ccr* gene allotype) and the class of the *mec* gene complex. Currently, eleven types of SCC*mec* (type I to XI) have been recognized as shown in Figure 1.2 and table 1.1 (IWG-SCC 2009, Li et. al. 2011, Shore et. al. 2011).

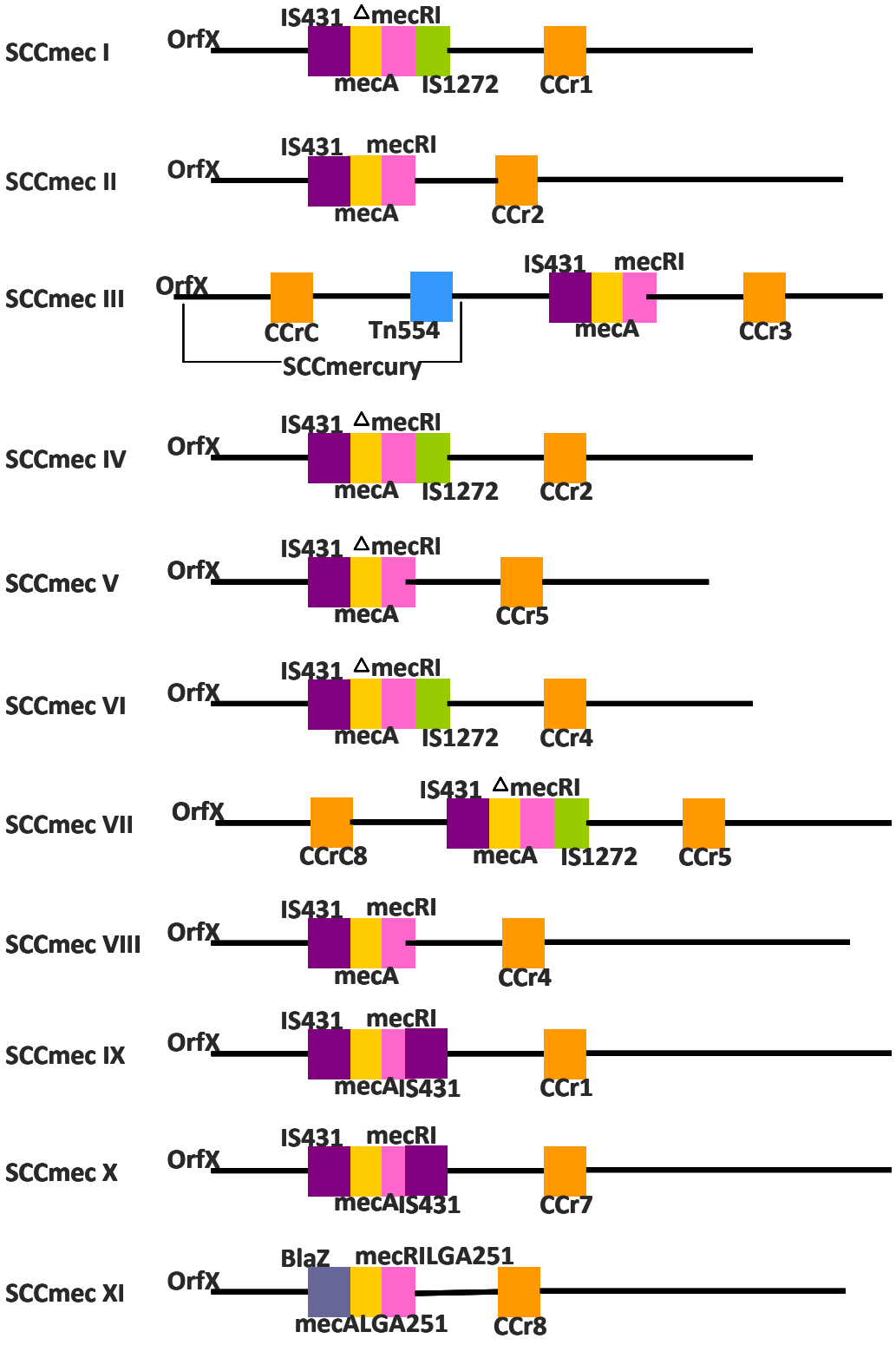


FIGURE 1.2: A schematic drawing of SCC*mec* types I to XI in MRSA

The major elements of the eleven main SCC*mec* types (ccr genes, IS431, IS1272, mecA, mecI/R1, orfX, and Tn554)

TABLE 1.1: SCC*mec* elements identified in MRSA
(Modification of IWG-SCC 2009)

SCC*mec* type	Reported name	Major characteristics of J regions	Reference
I (1B)	I	J1, subtype 1-specific ORFs (*pls*); J3, *dcs*	Ito et. al. 2001, Oliveira et. al. 2001
	I.2	J1, subtype 2-specific ORFs; J3, *dcs* and pUB110	Oliveira et. al. 2001
II (2A)	II	J1, subtype 1-specific ORFs (*kdp*); J2, subtype 1-specific ORFs and Tn*554*; J3, *dcs* and pUB110	
	IIb	J1, subtype 2-specific ORFs; J2, subtype 1-specific ORFs and Tn*554*; J3, *dcs*	Hisata et. al. 2005
	IIB	J1, subtype 3-specific ORFs; J2, subtype 1-specific ORFs; J3, *dcs* and pUB110	Shore et. al. 2005
	IIE	J1, subtype 3-specific ORFs; J2, short J2 region the same as subtype 1 and Tn*554*; J3, *dcs* and pUB110	
	II.4.1.1	J1, subtype 4-specific ORFs; J2, subtype 1-specific ORFs and Tn*554*; J3, *dcs* and pUB110	Kondo et. al. 2007
III (3A)	III	J1, subtype 1-specific ORFs; J2, subtype 1-specific ORFs and ΨTn*554*; J3, subtype 1-specific ORFs and pT181	Ito et. al. 2001, Oliveira et. al. 2001
	IIIA	J1, subtype 1-specific ORFs; J2, subtype 1-specific ORFs and ΨTn*554*; J3, subtype 1-specific ORFs, pT181, and SCC*Hg* carrying *ccrC*	Oliveira et. al. 2001
IV (2B)	IVa	J1, subtype 1-specific ORFs; J3, *dcs*	Ma et. al. 2002
	IVb	J1, subtype 2-specific ORFs; J3, *dcs*	
	IVc	J1, subtype 3-specific ORFs; J3, *dcs* and Tn*4001*	Ma et. al. 2006
	IVd	J1, subtype 3-specific ORFs; J3, *dcs*	IWG-SCC 2009
	IVA	J1, subtype 3-specific ORFs; J3, *dcs* and pUB110	
	IVE	J1, subtype 3-specific ORFs; J3, subtype 2-specific ORFs	Shore et. al. 2005
	IVd	J1, subtype 4-specific ORFs; J3, *dcs*	Ma et. al. 2006
	IVg	J1, subtype 5-specific ORFs; J3, *dcs*	Kwon et. al. 2005
	IVh	J1, subtype 6-specific ORFs; J3, *dcs*	Milheiriço et. al. 2007
	IVi	J1, subtype 7-specific ORFs; J3, *dcs*	Berglund et. al. 2009
	IVj	J1, subtype 8-specific ORFs; J3, *dcs*	
IV (2B&5)	IV variant	J1, subtype 3-specific ORFs; J3, SCC carrying *ccrC*	Heusser et. al. 2007
V (5C2)	V	J1, subtype 1-specific ORFs; J2, subtype 1-specific ORFs; J3, subtype 1-specific ORFs	Ito et. al. 2004
V (5C2&5)	VT, VII	J1, subtype 2-specific ORFs; J2, subtype 2-specific ORFs; J3, SCC carrying *ccrC*	Boyle-Vavra et. al. 2004, Takano et. al. 2008
VI (4B)	VI	J1, subtype 1-specific ORFs; J3, *dcs*	Oliveira et. al. 2006a
VII (5C1)	5C1	J1, subtype 1-specific ORFs; J2, subtype 1-specific ORFs; J3, subtype 1-specific ORFs	Berglund et. al.2008
VIII (4A)	VIII	J1, subtype 1-specific ORFs; J2, subtype 1-specific ORFs; J3, subtype 1-specific ORFs	IWG-SCC 2009
IX	IX	J1, ORF X, type 1 ccr gene complex and class 1C2 mec gene complex	Li et. al. 2011
X	X	J1, ORF, type 7 ccr gene complex (ccrA1+ccrB6) and class 1.2 mec gene complex	Li et. al. 2011
XI	XI	J1, ORF, type 8 ccr gene complex (ccrA1+ccrB3) and class LGA251 mec gene complex	Shore et. al. 2011

The size of these SCC*mec* elements ranges from 20.9 to 66.9 kb. SCC*mec* type I (34.3 kb), IV (20.9–24.3 kb), V (28 kb), VI (20.9 Kb), VII (35.9 kb), VIII (32.17 kb), IX (43.68 kb), X (50.8 kb) and XI (30 kb) cause only β-lactam antibiotic resistance, while SCC*mec* type II (53.0 kb) and III (66.9 kb) are associated with cross resistance to other antibiotics, due to the additional drug resistance genes integrated into SCC*mec*, i.e. integrated plasmids, e.g. pUB110, pI258 and pT181, and two transposons, e.g. Tn*554* and ΨTn*554*. Integrated plasmid pUB110 harbors the *ant*(*4′*) gene, encoding resistance to several aminoglycosides, including kanamycin, tobramycin and bleomycin. Resistance to penicillins and heavy metals, such as mercury, is encoded by pI258, whilst tetracycline resistance is encoded by pT181. Transposon Tn*554* harbors the *ermA* gene, which confers resistance against macrolides, lincosamides and streptogramins (MLS), while ΨTn*554* encodes for resistance to cadmium (Ito et. al. 2001, Leclercq 2002, Ito et. al. 2003, Oliverira et. al. 2006a, Oliverira et. al. 2006b, Takano et. al. 2008). In addition to the resistance genes carried on SCC*mec*, *S. aureus* can also harbor resistance genes on other sites of the genome, such as Tn*554*, as well as on plasmids (Lindsay et. al. 2006) emphasizing the diversity and plasticity in their structural organization and genetic content.

It has been shown that SCC*mec* type III is a composite element that consists of two SCC elements, i.e. SCC*mec* type III and SCC*mercury*, harboring *ccrC*, pI258 and Tn554 (Chongtrakool et. al. 2006).

Furthermore, SCC*mec* carries several insertion sequences, such as IS431 and IS1272, as well as the genes responsible for the regulation of the transcription of

mecA, i.e. Δ*mecR1* (SCC*mec* type I, IV, V, VI and VII), or *mecR1* and *mecI* (SCC*mec* type II and III) (Ito et. al. 2001, Daum et. al. 2002, Ito et. al. 2003, Ito et. al. 2004, Oliverira et. al. 2006b, Takano et. al. 2008).

Both IS431 and IS1272 can abbreviate *mecI* and *mecR1* and this can result in a de-repression of the *mecA* gene (Katayama et. al. 2001). These genes are situated on *mec* complexes, and five major classes of *mec* complexes (A to E) have been distinguished; of which A to C are the most common in the SCC*mec* elements (Ito et. al. 2001, Ito et. al. 2003, Katayama et. al. 2001).

The *ccr* genes, which are of the invertase / resolvase class, are located on all SCC*mec* elements. Their function is the integration of SCC*mec* into and excision of SCC*mec* from the *S. aureus* genome at the specific site, the SCC*mec* attachment site (*attBscc*) at the 3′ end of an open reading frame (orf) the function of which is not known (*orfX*) (Ito et. al 1999). The *ccr* genes are designated *ccrA1* and *ccrB1* (SCC*mec* type I), *ccrA2* and *ccrB2* (SCC*mec* type II and IV), *ccrA3* and *ccrB3* (SCC*mec* type III), *ccrA4* and *ccrB4* (SCC*mec* type VI) and *ccrC* (SCC*mec* type V and VII). The SCC*mec* type is determined by the *mec* complex and the *ccr* genes (Figure 1.3).

The regions that are not part of the *mec* complexes and *ccr* genes are called J (junkyard) regions (Daum et. al. 2002, Hiramatsu et. al. 2001, Ito et. al. 2004, Oliveira et. al. 2006a Oliveira et. al. 2006b, Takano 2008).

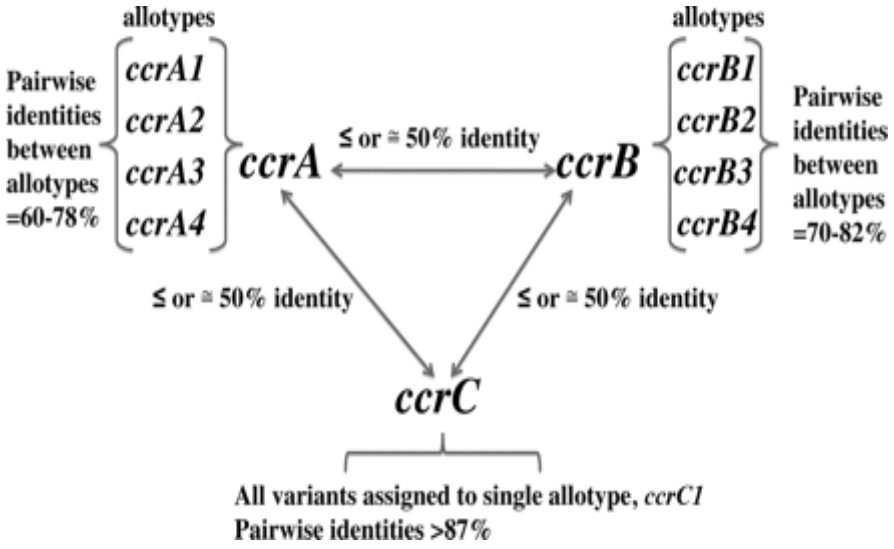


FIGURE 1.3: Model of the naming conventions for *CCR* genes in *S. aureus*
(IWG-SCC 2009)

Each SCC*mec* element is divided into three J regions. The J1 region is located between the genome right junction and the *ccr* genes, while the region from the *ccr* genes to the *mec* complex is called the J2 region. The J3 region spans from the *mec* complex to *orfX* (Fig 1.2) (Shore et. al. 2005, Chongtrakool et. al. 2006). A SCC*mec* element has the following composition: J3-*mec*-J2-*ccr*-J1. Several variants of the SCC*mec* type I to IV, which differ in the J regions, have been described in *S. aureus* (Oliveira et. al. 2001, Ma et. al. 2002, Oliveira and de Lencaster 2002, Ito et. al. 2003, Hisata et. al. 2005, Kwon et. al. 2005, Shore et. al. 2005, Milheirico et. al. 2007a, Milheirico et. al. 2007b). The relatively large number of SCC*mec* type IV variants compared to variants of the other SCC*mec* types could be due to the genetic plasticity of the MRSA lineages (Jansen et. al. 2006). It is also evident that *ccrAB* gene is an independent mobile SSC element that mediates interspecies transfer of antimicrobial and virulence genes (Katayama et. al. 2000, Katayama et. al. 2003).

1.1.6 Types of MRSA and Impact

The most common type of MRSA is healthcare associated MRSA (HA-MRSA) which has typically been linked to persons with health care associated risk factors such as hospitalization or nursing home care, chronic dialysis, antibiotic treatment, or exposure to invasive devices or procedures (Lowy 1998). HA-MRSA is a highly resistant and important nosocomial pathogen in both acute care and chronic care settings. It is also known to cause infections associated with increased morbidity, mortality, and cost (Abramson and Sexton 1999, Engemann et. al. 2003, Cosgrove et. al. 2005).

Community associated MRSA (CA-MRSA) was first reported in 1993 in a healthy adult in Australia (Udo et. al. 1993) and later in USA in children in 1998 (Adcock et. al. 1998), while recently these phenotypes have been reported world wide (Vandenesch et. al. 2003). Genetic and epidemiologic evidence shows that CA-MRSA is caused by strains of *S. aureus* different from those associated with HA-MRSA. CA-MRSA is defined as an infection without established risk factors for MRSA acquisition (Vandenesch et. al. 2003). CA-MRSA are genetically different from HA-MRSA strains, and contain the SCC*mec* IV and V, a smaller versions of the genetic package in comparison to the SCC*mec* I, II, and III found in HA-MRSA (Baba et. al. 2002). The smaller size of the SCC*mec* IV and V confer less resistance than the larger SCC*mec* cassettes (Baba et. al. 2002), and explains why CA-MRSA is susceptible to more classes of antibiotics than HA-MRSA. The CA-MRSA is always resistant to the beta-lactams and usually to erythromycin, but remains susceptible to several other antimicrobial agents (Baba et. al. 2002, Naimi et. al. 2003). Resistance to most classes of antibiotics has been reported in HA-MRSA (Campanile et. al. 2009) except tigecycline. Most CA-MRSA strains carry the PVL gene that allows the production of a necrotizing cytotoxin, which may be responsible for the invasiveness and virulence of the organism (Lina et. al. 1999, Gillet et. al. 2002). The differences between HA-MRSA and CA-MRSA are as summarized in Table 1.2.

TABLE 1.2: Differences between HA-MRSA and CA-MRSA

Characteristics	HA-MRSA	CA-MRSA	Reference
Definition & source	Hospital associated	Community associated	Vandenesch et. al. 2003, CDC.gov
Colonization	In high risk groups	Younger and healthy population	Adcock et. al. 1998, Udo et. al. 1993
SCCmec type	I, II, III	IV, V	Baba et. al. 2002
PVL	Most are - ve	Most are +ve	Lina et. al. 1999, Gillet et. al. 2002
Resistance	β-lactams & up to 8 other antibiotics	β-lactams, Ciprofloxacin, Erythromycin & Tetracycline	Baba et. al. 2002, Naimi et. al. 2003
Treatment options	Vancomycin, Linezolid, Daptomycin, Co-trimoxazole	Several options	Baba et. al. 2002, Naimi et. al. 2003

However, it is suggested that the distinction between HA-MRSA and CA-MRSA has diminished, as CA-MRSA have caused infections among hospitalized patients (David and Daum 2010)

The most frequent infections caused by CA-MRSA are skin and soft tissue infections that are typically present as boils, abscesses, or cellulitis and a more serious necrotizing fasciitis. Early lesions often appear as spider bites. Although less common, CA-MRSA can cause fatal conditions such as bacteremia, endocarditis, and necrotizing pneumonia (Couppie´et. al. 1994, Miller et. al. 2005).

Although CA-MRSA was initially found in a small percentage of healthy adults and children, it has now emerged as epidemic strains and outbreaks among sportsmen, prisoners, military recruits, and drug users have been reported (MMWR 2003, Zinderman et. al. 2004, Kazakova et. al. 2005). Transmission mainly occurs by direct contact from person to person but may also occur through contaminated surfaces or fomites. Little is known about risk factors for transmission in the community, except that common factors observed from outbreak investigations were crowding, frequent skin to skin contact, compromised skin, contaminated surfaces and shared fomites, injection drug use, HIV infection, lack of cleanliness and prior exposure to hospitals (Charlebois et. al. 2002). Several reports have indicated the expansion of the CA-MRSA reservoir i.e. infiltration into hospital settings, as well as the possibilities of predominance of CA-MRSA over HA-MRSA strains as a cause of Hospital-associated infection (Seybold et. al. 2006, Popovich et. al. 2008, D'Agata et. al. 2009).

The pathogenicity of MSSA/MRSA is associated with surface components (e.g., capsular polysaccharide and protein A), includes adhesive matrix molecules (e.g., clumping factor and fibronectin binding protein medicates binding to host cells), and extra-cellular proteins (e.g., coagulase, hemolysins, enterotoxins, TSST-1, exfoliatins, (see Figure 1.4) while the high level of virulence of CA-MRSA is mainly attributed to production of PVL (Archer 1998). PVL has been largely linked to furuncles, cutaneous abscesses, and severe necrotic skin infections (Cribier 1992, Couppie´et. al. 1994, Miller et. al. 2005). However, recently novel Atl and AtlE adhesions have been suggested to be associated with internalisation and pathogensis of *S. aureus* (Hirschhausen et. al. 2010).

PVL, g-hemolysin and other leukocidins belong to the family of synergohymenotropic toxins (Supersac et. al. 1993). These toxins damage host defense cells and erythrocytes by the synergistic action of secretory proteins, (Prevost et. al. 1995). g-hemolysin is produced by most *S. aureus* clinical strains; its activity results from three proteins (HlgA and HlgC, belonging to class S, and HlgB, belonging to class the F) forming 2 pairs: HlgA1HlgB and HlgC1HlgB (Prevost et. al. 1995).

All PVL producing CA-MRSA strains produce LukS-PV and LukF-PV, as well as the three proteins forming g-hemolysin. Hence these strains are able to produce following biologically active pairs: HlgA1HlgB, HlgC1HlgB, LukS-PV1HlgB, HlgA1LukF-PV, HlgC1LukF-PV, and LukS-PV1LukF-PV (Prevost et. al. 1995).

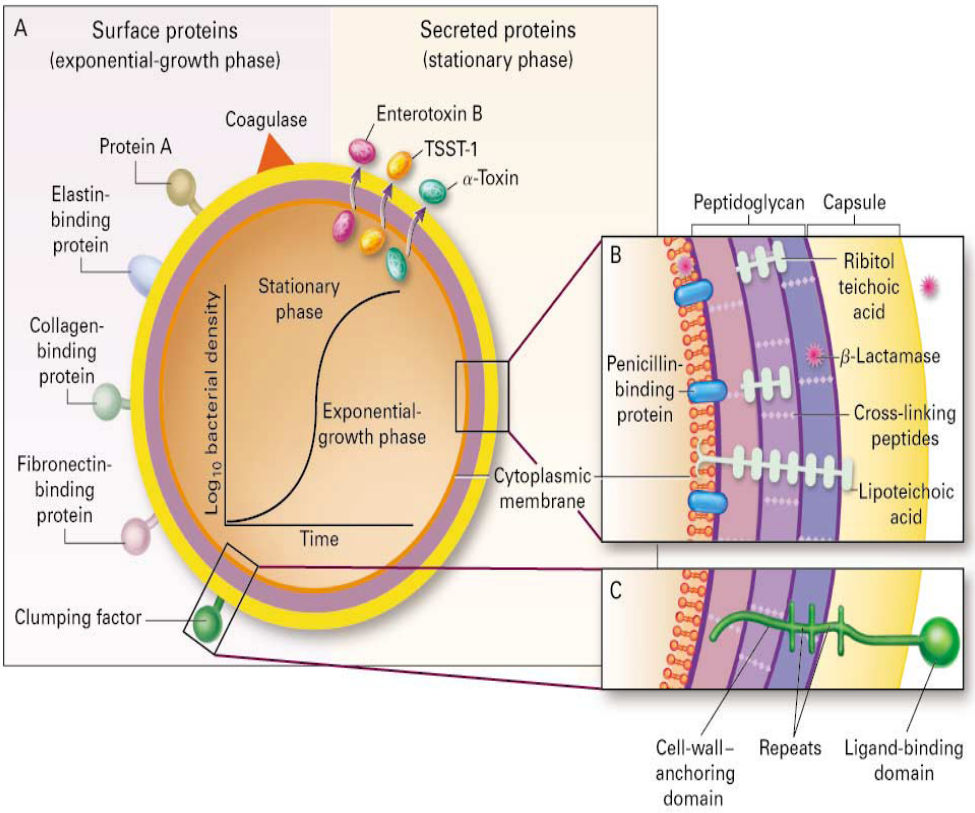


FIGURE 1.4: Diagram illustrating surface protein structure and sereatory proteins of *S. aureus*
(Lowy 1998)

Panel A shows the surface and secreted proteins, *panel B* show cross sections of the cell envelope and *panel C* shows cross section of TSST-1 denotes toxic shock syndrome toxin 1.

The 2 pairs composing g-hemolysin have leukotoxic properties and are also able to lyse human erythrocytes. Purified PVL is able to lyse human polymorphonuclear (PMN) cells and macrophages by pore induction (Finck-Barbancon et. al. 1993, Prevost et. al. 1995, Loffler 2010) resulting in severe inflammatory lesions, leading to capillary dilation, chemotaxis, PMN infiltration, PMN karyorrhexis, lung and skin necrosis (Ward and Turner 1980, Prevost et. al. 1995, Gillet et. al. 2002, Bocchini et. al. 2008, Dipe et. al. 2010, Lin et. al. 2011).

1.1.6.1 Impact of MRSA

The clinical symptoms of multi-drug resistant organisms (MDRO) are similar to infections caused by susceptible isolate. However, the treatment options for MRDO are very limited. MRSA has been observed to behave differently when compared to other MDRO. Reports suggest that patients with MRSA colonization frequently develop symptomatic infections (CDC 2006). Furthermore, higher fatality rates and persistence infections have been reported in certain MRSA infections (CDC 2006). Reports also indicate a strong association between MRSA infection and increase in mortality rate, length of hospital stay and health care costs (Abramson and Sexton 1999, Engemann et. al. 2003, Cosgrove et. al. 2005). Some hospitals have even observed an increased overall incidence of *S. aureus* infections after introduction of a patient infected with MRSA to the hospital (Abramson and Sexton 1999, Engemann et. al. 2003, Cosgrove et. al. 2005).

1.1.6.2 Therapeutic Options for MRSA

A number of antimicrobial agents retain *in-vitro* anti-MRSA activity. Daptomycin, linezolid and a number of other new agents have been developed. In addition, there are some agents in clinical trial that are yet to be released into the market.

Historically, MRSA has been successfully treated in outpatients with oral sulfonamides, clindamycin, rifampin, doxycycline, tetracycline or a combination of these agents (Bishop and Howden 2007). With the increasing drug resistance of MRSA to these traditional antimicrobials, there has been a search for effective antibiotics. These include combination of old agents such as rifampin and fusidic acid. A recent study reported that vancomycin, linezolid, and quinupristin-dalfopristin were the most effective antibiotics against multiple strains of MRSA (Draghi 2007). The parenteral administration of vancomycin and quinupristin-dalfopristin has limited their use in the outpatient setting; however, the availability of an oral formulation of linezolid has lead to its increasing utilization.

Linezolid: Linezolid is a synthetic antimicrobial agent belonging to the class of oxazolidinones. Although, it has been reported to have an *in-vitro* bacteriostatic effect against *S. aureus* and a mild-to-moderate anti-MRSA effect, a number of serious MRSA cases have been cured including bacteremia and endocarditis *(Howden et. al. 2004, Huang et. al. 2008).* It is also suggested that the effectiveness of linezolid is similar to other comparators agents in treatment of gram positive bacteramia and pneumonia (Beibei et. al. 2010). Recent reports indicate resistance development against this agent (Kola et. al. 2007) due to mutation of rRNA (Gould et. al. 2011b, Meka and Gold 2004) and other resistant

determinants including presence of Cfr gene (Morales et. al. 2010). Bishop and co-workers (2006) have reported a high rate of toxicity, in complicated diseased patients and have suggested cautious use in prolonged therapy. The common side effects are diarrhea, nausea, and headache. Less common side effects include hypertension, lactic acidosis, and elevated liver enzymes. The most toxic effects include irreversible peripheral neuropathy, optic neuropathy, and reversible myelosuppression (Huang et. al. 2008).

Quinupristin-dalfopristin (QD): This combination was introduced to improve water-solubility of streptogramins. It is active against MRSA (Jevitt 2003), but has poor bio-availability, and a considerable toxic profile including hepatotoxicity, hyperbilirubinemia and thrombophlebitis, as well as needing to be administered through a central vein (Aksoy and Unal 2008). QD is known to be ineffective in strains exhibiting MLS_B gene, but it is reported that a combination of QD with rifampin is effective against MRSA with an MLS_B resistant gene (Brown and Freeman 2004).

Rifampin and fusidic acid: Some studies have reported that rifampin and fusidic acid both possess good in-*vitro* anti-MRSA activity and the combination has been particularly useful for oral treatment of multi-drug resistant MRSA (Turnidge et. al. 1996, Zinn et. al. 2004). Reports also indicate rapid resistance development with monotherapy with either of these drugs (Howden 2005) and have suggested that these drugs should be always used in combination with other effective anti-MRSA drugs. It is also reported that vancomycin does not provide adequate protection against rifampin resistance development when used in vancomycin-rifampin

combination (Ju et. al. 2006). However, a report indicates that rifampin and vancomycin to be effective in treatment of HA-MRSA pneumonia (Jung et. al. 2010). The combination of rifampin and fusidic acid is a mainstay in many parts of the world for treatment of complicated MRSA infection. However, this combination is not approved in the US.

Daptomycin: Daptomycin is an expensive cyclic lipopeptide antibiotic with *in-vitro* activity against MRSA and is recommended for treatment of severe MRSA infections. Motrin et. al. (2007) have reported a more rapid and enhanced bactericidal effect against MSSA and MRSA infections in mice. It is suggested to be effective and safe in treatment of gram positive infection in cancer patients (Chaftari et. al. 2012). Reduced daptomycin susceptibility and treatment failure has been documented during the course of therapy and it appears to be related to high-bacterial load infections (Werner et. al. 2001, Boyle-Vavra et. al. 2011, van Hal et. al. 2011b) and daptomycin resistance has been suggested to result in cross-resistance to vancomycin (Camargo et. al. 2008, Boyle-Vavra et. al. 2011). Werner et. al. (2001) reported daptomycin resistance in MRSA isolates from hospitals in France and Spain and some reports indicate a daptomycin-rifampin combination to be effective for treatment of MRSA with reduced susceptibility to daptomycin (Ahmad and Rojtman 2010). In order to reduce the risk of resistance development during daptomycin therapy higher dose (>6 mg/kg/day) has been suggested (Liu et. al. 2011) and a report suggests that high dose of daptomycin may improve the clinical outcome than the concentional dose of 4-6 mg/kg/day (Bassetti et. al. 2010). But, there is limited evidence about safety assoaciated with use of higher dose (Moise et. al. 2009, Bassetti et. al. 2010, Liu et. al. 2011).

Tigecycline: Tigecycline is the first antibiotic of the class of the glycylcyclines *(Noskin 2005)*, effective against MRSA and other multi-resistant organisms (Draghi et. al 2008). It is similar to the tetracycline group of antibiotics, with structural modifications that allow for broad-spectrum activity and defense against antimicrobial efflux pumps (Olson et. al. 2006). The broad spectrum activity and enhanced protection against several mechanisms of resistance is attributed to stronger binding to 30S ribosomal subunit and inhibiting protein translation (Olson et. al. 2006). It was reported to have an *in-vivo* effect against MSSA and MRSA strains in an intraperitoneal systemic murine infection model (Petersen et. al. 2002). A report suggests that tigecycline is effective in treatment of MRSA pneumonia (Saner et. al. 2006). Tigecycline is reported to have excellent effect against MRSA and better eradication rates for MRSA than for MSSA (Cai et. al. 2011).

5th generation cephelosporins

Fifth generation cephalosporins were developed to specifically target multi-drug resistant bacteria, particularly ceftobiprole and ceftaroline (Kollef 2009). These group of agents exhibit anti-MRSA activity by binding to PBP2a and forming a stable inhibitory acyl-enzyme complex (Hebeisen et. al. 2001).

Ceftobiprole: Ceftobiprole is a broad-spectrum, parenteral cephalosporin, confers anti-MRSA effect by inhibiting the cell-wall synthesis of PBP and PBP2a (Entenza et. al. 2002, Kollef 2009). Although, ceftobiprole was approved for use in Canada, the US FDA and European EMA (EMA 2010) have declined to approve ceftobiprole use due to data integrity concerns with the supporting studies. In

response to failuer to gain approval from US FDA and EMA (EMA 2010), Health Canada has issuded the notice for discontinuation of sale of ceftobiprole (Health Canada 2010).

Ceftaroline: Ceftaroline is a novel broad spectrum cephalosporin and is currently under investigation for CA-pneumonia and SSI caused by MRSA. Several reports have indicated that it is effective against MRSA and have suggested ceftaroline as a promising agent for monotherpay of SSI caused by MRSA (Jones et. al. 2010, Jones et. al. 2011).

Glycopeptides

Newer agents with proven efficacy against MRSA infections (eg, linezolid, quinupristin-dalfopristin, daptomycin, and tigecycline) are available but are not routinely prescribed because of higher drug acquisition costs and/or lack of clinical experience compared with glycopeptides particularly teicoplanin and vancomycin.

Teicoplanin: Teicoplanin is a potent anti-MRSA agent and acts by hindering peptidoglycan synthesis and thereby inhibiting the cell wall synthesis. However, reports indicate resistance against teicoplanin and treatment failure with teicoplanin therapy *(Mainardi 1995, Winebren 2002, Cepede et. al 2003)*.

Vancomycin: Vancomycin is a tricyclic glycopeptide antibiotic, with molecular weight of 1486. Introduced in 1958, vancomycin has been drug of choice for treatment of multi-drug resistant MRSA (Garau et. al. 2009, Gould et. al. 2009, Gould et. al. 2011) Vancomycin is an inhibitor of cell wall synthesis, by binding to C-terminal D-alanyl-D-alanine residues of peptidoglycan forming a stable complex, thus inhibiting late-stage peptidoglycan synthesis by inhibiting transglycosylation and preventing the cell wall synthesis as shown in Figure 1.5 (Pootoolal et. al. 2002, Courvalin 2006). Any process that interferes with binding of vancomycin to the target site will decrease the efficacy of vancomycin (Allen et. al. 1997).

For many years vancomycin has been the frontline drug in treatment of MRSA as there was no indication of resistance and the treatment was far more cost-effective when compared to other competitive agents.

However, an initial report of vancomycin non-susceptibility in MRSA (VISA) from Japan (Hiramatsu et. al. 1997a, Hiramatsu 1997b) and later evidence of such phenotypes around the world has generated concern in the healthcare community. Teicoplanin reduced susceptibility in MRSA was reported prior to the VISA report (Brunet et. al. 1990) and VISA strains exhibit reduced susceptibility to teicoplanin, indicating cross resistance among this class of antibiotics (Liu and Chambers 2003). Later vancomycin resistant *S. aureus* with *vanA* gene was reported in 2002 (Sievert et. al. 2002).

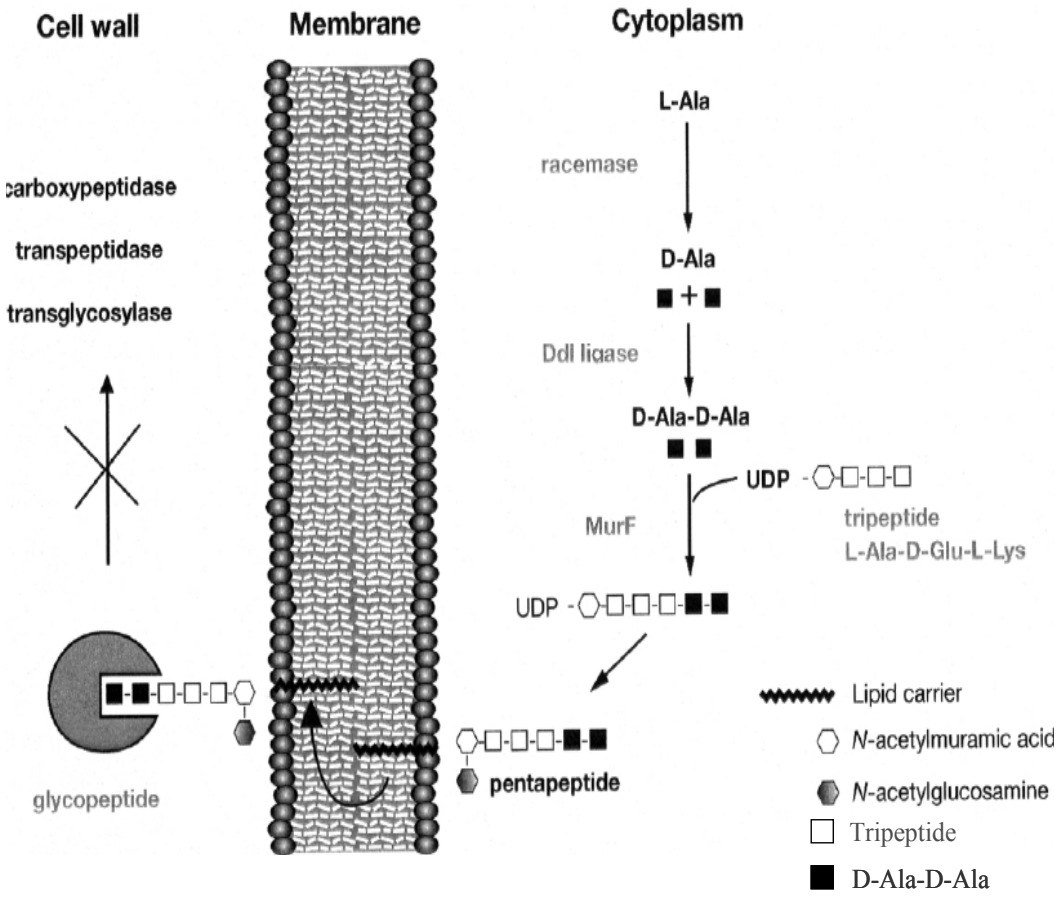


FIGURE 1.5: Mechanism of action of vancomycin
(Courvalin 2006)

VISA development has been associated with physicochemical properties of vancomycin which are known to determine the pharmacokinetic and pharmacodynamic parameters and the clinical outcome. In this context, the next sub-section will briefly cover the pharmacokinetic and pharmacodynamic parameters of vancomycin for clearer understanding of risk factors associated with vancomycin non-susceptibility.

1.1.7 Vancomycin-Pharmacokinetics/Pharmacodynamics (PK/PD)

Vancomycin has no appreciable absorption upon oral administration and needs to be administered intravenously while it has good solubility in water and is compatible with dextran and sodium chloride solutions (Matzke et. al. 1984). Vancomycin is mainly cleared via the renal route and about 80-90% of the drug is cleared unchanged in urine within 24h of administration in patients with normal renal function (Raybak et. al. 2009).

Various models have been used to understand PK parameters for vancomycin as the concentration-time profile of vancomycin is complex and has been characterized by one-, two-, and three-compartment PK models. The distribution phase ranges from 30 to 60 minutes, the elimination half-life ranges from 6 to 12 hours and the volume of distribution is 0.4–1 L/kg in patients with normal renal function (Blouin et. al. 1982, Rotschafer et. al. 1982, Matzke et. al. 1984, Golper et. al.1988, Rodvold et. al.1988).

Reports suggest vancomycin protein binding of 50-55% (Albrecht et. al. 1991) and indicate that the tissue penetration of vancomycin can be affected by disease and

inflammation. Vancomycin skin penetration is considerably lower in diabetic patients ranging between 0.01-0.45mg/L in comparison to non-diabetic patients (0.46-0.94mg/L) (Skhirtladze et. al. 2006). The cerebral spinal fluid (CSF) vancomycin concentration is found to be between 0-4mg/L in non-inflamed meninges and the vancomycin concentration in CSF in inflamed meninges has been reported to be 6.4-11.1mg/L (Ackerman et. al. 1988).

1.1.7.1 PK/PD Parameters

A variety of PK/PD parameters have been proposed for vancomycin, which includes time *(t)* of the vancomcyin concentration above MIC (t > MIC), ratio of area under the curve (AUC) of serum drug concentration and the MIC (AUC/MIC) and the ratio of the maximum serum drug concentration (Cmax) and the MIC (Cmax/MIC) as shown in Figure 1.6 (Craig 1998, Craig 2003, Drusano 2004, Rybak 2006a, Rybak 2006b).

Reports on animal models, in-vitro studies and limited human studies suggest AUC/MIC as the preferred parameter. Investigators have demonstrated AUC/MIC as a suitable PK/PD parameter for measuring the vancomycin effectiveness in treating *S. aureus* (MSSA, MRSA, and VISA) infections in mouse models (Craig 2003, Rybak 2006b) and that the free vancomycin AUC_{0-24hr}/MIC (*f* AUC/MIC) is an important parameter for predicting vancomycin activity against VISA, hVISA, and MSSA in the mouse model (Moise-Broder et. al. 2004). The same report also indicated that the *f*AUC/MIC varied based on the vancomycin MIC and bacterial density at the site of infection (Moise-Broder et. al. 2004).

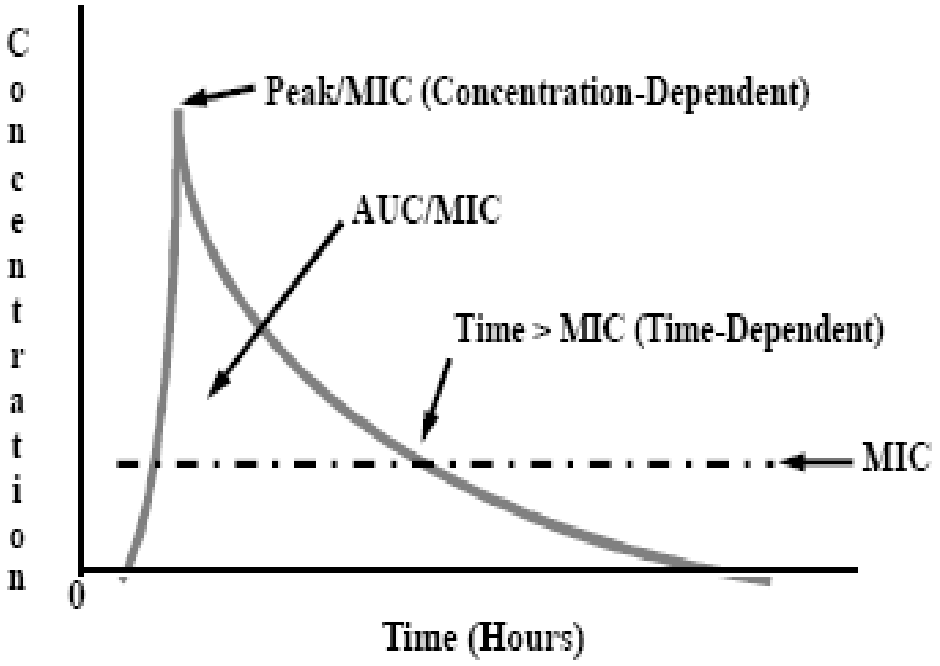


FIGURE 1.6: Pharmacokinetic and pharmacodynamic parameters for antibiotic efficacy
(Quintiliani 2004)

t > MIC: β- lactams, erythromcyin, and linezolid.

AUC/MIC: tetracyclines, glycopeptides and dalfopristin-quinupristin.

Cmax/MIC: fluoroquinolones, daptomycin and the ketolides.

Another study evaluated AUC/MIC to predict clinical success in treating ventilator-associated *S. aureus* pneumonia and found that a PK/PD index (AUC/MIC) of 345 is required for a successful clinical outcome in treating isolates with vancomycin MIC of 1 mg/L and actual body weight (ABW) of 80 kg. Based on these results AUC/MIC of 400 is advocated for positive clinical outcome with vancomycin therapy of *S. aureus* (Moise-Broder et. al. 2004, Moise-Broder et. al. 2007, Kim et. al. 2009).

1.1.7.2 Effective vancomycin trough concentration

Early reports suggested a trough concentration of 5-10mg/L of vancomycin was adequate for clinical success (Geraci 1977). However, a trough concentration of 5-10mg/L may result in a negative clinical outcome in strains with higher MICs within the susceptible range. Therefore, a higher trough serum concentration of vancomycin is suggested to achieve PK/PD index of 400 for effective treatment of strains with higher MIC values (Kim et. al. 2009, Rybak et. al. 2009b*).* A initial vancomycin dosage of 15 mg/kg/12h in patients with normal renal function is suggested to attain and maintain a vancomycin trough concentration at 15–20 mg/L (American Thoracic Society 2005, Rybak et. al. 2009a, Gould 2011, Lin et. al. 2011). However, there is limited data on the safety and the possibility of producing trough concentrations of 15–20 mg/L in patients with a normal body weight and renal function with vancomycin dose of 15 mg/kg/12h (Rybak et. al. 2009b).

1.1.7.3 Vancomycin Trough Concentration and Clinical Outcome

Vancomycin is a bactericidal antibiotic. However, the rate of bactericidal activity is affected by both MIC and the bacterial load (Craig 2006). In recent years there has been a significant increase in reports of clinical failure of vancomycin therapy which is attributed to hVISA and the emergence of hVISA/VISA has been associated with low vancomycin trough concentration. Moreover, reports indicate that a vancomycin trough concentration of <10 mg/L is associated with poor clinical response and emergence of hVISA/VISA (Howden et. al. 2004, Sakoulas et. al. 2006). Reports also indicate that the possibility of achieving the PK/PD index of 350-400 with a trough serum concentration of 10-15 mg/L is only 40-60% when the MIC is 1 mg/L, and cannot be achieved when the MIC is 2 mg/L (Moise-Broder et. al. 2004, Soriano et. al. 2008).

1.1.8 Problems with Vancomycin

Therapeutic drug monitoring (TDM)

TDM mainly of the trough plasma drug concentration is necessary in patients on haemodialysis, receiving high dose of vancomycin therapy, on prolonged courses of vancomycin therapy, receiving multiple antibiotics particularly aminoglycosides, and with impaired renal function (Devabhakthuni 2011). However, vancomycin has been reported to have varied levels of non-protein bound drug within and among patients (Berthion et. al. 2009) further increasing the difficulty of TDM optimization (Gould et. al. 2012)

Administration

Vancomycin must be administered by IV route, since it is a large hydrophilic molecule and its ability to cross the GIT mucosa is poor (Moellering 1984).

Adverse effects

Vancomycin has been reported to have a narrow therapeutic window and high dose is associated with adverse effects (Lodise 2009, Gould et. al. 2011). The common adverse effects with vancomycin are thrombophlebitis and pain. Earlier reports on nephtotoxicity and ototoxicity have been atributed to the impurities in vancomycin formulations (Farber and Moellering 1983, Moellering 2006). However, a recent report suggests a higher trough concentrations, particularly >20 mg/L increases the risk of nephrotoxicity (Lodise 2009). Another common adverse effect is "red man syndrome" characterized by erythematous rash or flushing affecting the face, neck, and torso and sometimes accompanied by pruritus and, in severe cases, hypotension or shock is attributed to histamine release (Davis et. al. 1986, Renz et. al. 1998). This syndrome usually appears soon after the completion of a rapid infusion (Davis et. al. 1986, Renz et. al.1998). However, it is suggested that preadministration of H1 or H2 anti-histamines can reduce the histamine-related side effects of rapid vancomycin infusion (Renz et. al. 1998).

With this understanding of PK/PD parameters and problems associated with vancomycin the next sub-section will describe the definition and mechanism of vancomycin non-susceptibility.

1.1.9 Mechanism of Resistance to Vancomycin

There is a significant controversy over the current and future role of vancomycin in MRSA treatment. To resolve this controversy a clear understanding of the mechanism and definition of vancomycin non-susceptibility is important.

1.1.9.1 Definition for Vancomycin Non-susceptibility

In order to improve the correlation of *in-vitro* susceptibility with clinical outcome of vancomycin therapy, the Clinical and Laboratory Standard Institute (CLSI) lowered the breakpoints of the vancomycin MIC in 2006. Therefore, the current CLSI resistance breakpoints definitions for vancomycin against *S. aureus* are as follows: Strains with MIC <2mg/L are deemed as vancomycin susceptible *S. aureus* (VSSA), strains with MIC >4-8mg/L are defined as VISA and those phenotypes exhibiting MIC >16mg/L with *vanA* gene are defined as VRSA (CLSI 2010). In addition, hVISA; phenotypes with MIC <4mg/L with mixed (heterogeneous) population of susceptible and resistant phenotype (typically 1 organism per 10^5 to 10^6 colony forming units) have been reported (Howden et. al. 2010). Similarly, the European Committee of Antimicrobial Susceptibility Testing (EUCAST) recently changed the definition of vancomycin non-susceptibility to term all strains with MIC >2mg/L as vancomycin resistant *S. aureus* (VRSA) in recognition of the problems of treatment failure with strains with elevated MICs (EUCAST 2009).

1.1.9.2 Vancomycin Resistant Enterococci (VRE)

Vancomycin non-susceptibility was first noted in enterococci and *S. aureus* has acquired the *vanA* gene from enterococci. Hence, this section will first summarize the mechanism of vancomycin resistance in enterococci, followed by vancomycin non-susceptibility in *S. aureus*.

VRE was first reported in 1986 in France, later in UK (Uttely et. al. 1988) and eventually worldwide (Cetinkaya et. al. 2000). It took nearly three decades for development of resistance after vancomycin was clinically introduced. Vancomycin resistance in enterococci may have occurred mainly due to oral administration of vancomycin for treating diarrhea in hospitals.

Vancomycin resistance in human species is conferred by one of two similar operons, *vanA* or *vanB (Arthur et. al. 1996).* The *vanA* and *vanB* operons are complex resistance determinants, which suggest that they evolved in other species and were acquired by enterococci. Typical enterococcal genes have 35% to 40% guanine-cytosine (G-C) content (Murray 1990). However, the *van* operon of resistant enterococci has roughly 50% G-C content (Evers et. al. 1993). This evidence supports the hypothesis that the *van* gene has been acquired. High levels of glycopeptides in the gastrointestinal tract are achievable by oral administration. Failure to absorb the agent results in fecal vancomycin concentrations favoring vancomycin-resistant streptomycetes and tolerant enterococcus colonization suggesting that oral administration of glycopeptides to humans was a major factor in the emergence of VRE (Rice 2001).

Avoparcin a glycopeptide antibiotic and structurally similar to vancomycin (Ellestad et. al. 1982) was widely used as a growth-promoting additive in animal feeds (Elwinger et. al. 1998). Development and spread of VRE in Europe has also been attributed to wide use of avoparcin in animal farming (Donnelly et. al 1996, Barger et. al. 1997). VRE was more prevalent in the 1980s (Cetinkaya 2000). However, due to the early development of easily recognizable genotypic marker for VRE (Clark et. al. 1993), implementation of infection control procedures and the ban of avoparcin as an animal food additive by European Union, these phenotypes are now less prevalent (Boyce 1995, van den Bogaard et. al. 2000).

1.1.9.3 Vancomycin Resistant S. aureus (VRSA)

Since the first report of VRE, concern existed in the health-care community in regard to potential for VRSA development, by acquisition of *vanA* gene from VRE (Leclercq et. al. 1988). As mentioned earlier VRE were prevalent in 1980s and during this period, vancomycin was widely used in treatment of MRSA, adding sufficient pressure on MRSA to acquire *vanA* gene. Although shown to be possible in *in-vitro*. MRSA was not reported to have acquired *vanA* gene until 2002 when the first VRSA was reported in US (Sievert et. al. 2002). However, these strains appear to be rare and to date, only small numbers of VRSA cases have been reported; including nine cases form US, one from India and one from Iran. Due to the rapid and correct identification of VRSA by genotypic method and prompt infection control and these strains did not spread causing outbreaks (Howden et. al. 2010). However, more recently VRSA outbreaks have been reported in India (Thati et. al. 2011, Banerjee and Anupurba 2012). Acquisition of *vanA* gene can

result from one step or two step genetic events. In first step the plasmid transfer occurs by conjugation from the VRE to the *S. aureus*. In step two, the *vanA* transpose from the doner plasmid to the recipient plasmid or chromosome. The horizontal transfer of the *vanA* gene from VRE to MRSA, results in production of a new strain with high vancomycin resistance (as shown in Figure 1.7a). The *vanA* gene exhibits its resistance by replacing the carboxyl terminal D-alanyl-D-alanine with a structurally distinct D-alanyl-D-lactate in the peptidoglycan cell wall precursor (Figure 1.7b), resulting in a 1,000-fold lower affinity for vancomycin (González-Zorn and Courvalin 2003).

1.1.9.4 Vancomycin Non-susceptible S. aureus (hVISA/VISA)

Before clinical VRSA strains were reported, a different type of vancomycin non-susceptible strains lacking *van-A* and having 4mg/L (Mu3) and MICs of 8mg/L (Mu50) had been isolated in 1997 in Japan (Hiramatsu et. al. 1997a, Hiramatsu et. al. 1997b) and were later reported from other parts of the world (Howden 2005). Mu3 (hVISA) was first isolated in 1996 from the sputum of a 64year old patient with MRSA pneumonia, who failed to respond to vancomycin therapy and this isolate had an MIC of 4 mg/L with a more resistant sub-population (Hiramatsu et. al. 1997a). However, with revised CLSI breakpoints for VISA, Mu3 would now fall into the class of VISA. Later, Mu50 was isolated from the cardiac surgery wound of 4 month old patient. This isolate had an MIC of 8 mg/L (Hiramatsu et. al. 1997b).

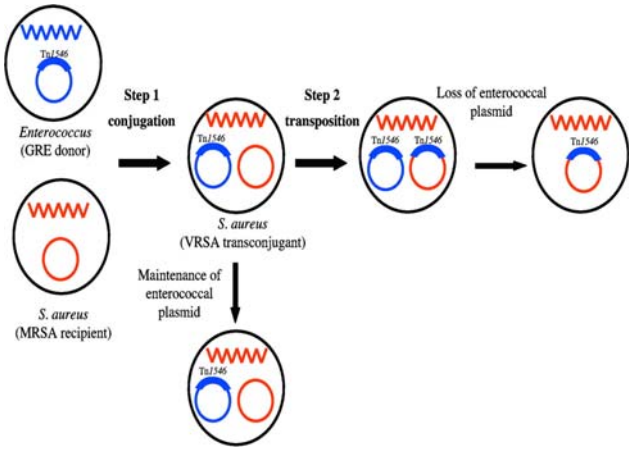


FIGURE 1.7a: Schematic representation of *vanA* transfer from Enterococcus to *S. aureus*
(Perichon and Courvalin 2009)

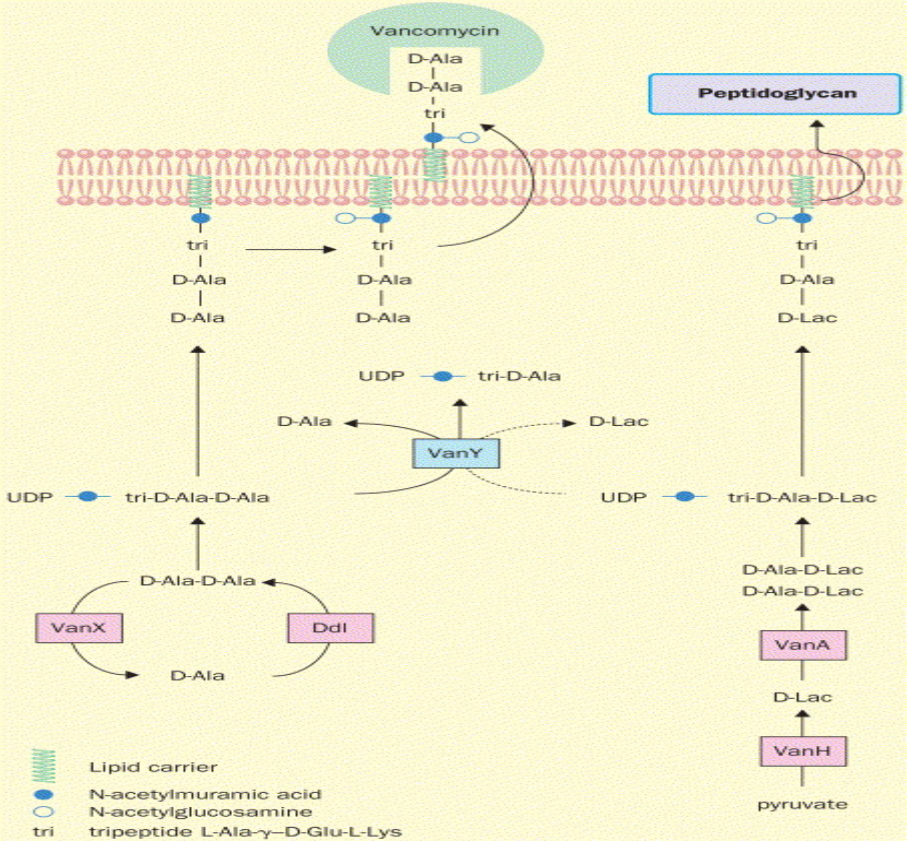


FIGURE 1.7b: Vancomycin resistance due to *vanA* operon
(González-Zorn and Courvalin 2003)

VRSA strains are resistant to vancomycin due to the acquisition of the *vanA* operon from VRE that allows synthesis of a cell wall precursor that ends in D-Ala-D-Lac dipeptide rather than D-Ala-D-Ala

After the first report on hVISA and VISA, retrospective analysis performed on stored MRSA clinical isolates indicated the presence of unrecognized hVISA/VISA isolates stretching back to 1987 in US, and Europe (Cercenado 2000, Rybak et. al. 2005, Robert et. al. 2006). However, the impact of prolonged storage on results of retrospective analysis of stored strains is of concern, due to the possibilities of loss of reduced susceptibility on prolonged storage (Robert et. al. 2006). These findings are evidence for the clinical failure of vancomycin and advocate the need of a new drugs, standardized detection method and categorization of vancomycin non-susceptible strains to overcome the negative clinical outcomes in treatment of MRSA infection.

1.1.9.5 Risk factors for Vancomycin Non-susceptible S. aureus (hVISA/VISA)

The major risk factor for hVISA/VISA infection is prior infection with MRSA, previous and prolonged exposure to vancomycin (Moise et. al. 2008). The physico-chemical properties of vancomycin result in poor tissue penetration, low bactericidal activity and increased risk of toxicity, including nephrotoxicity and red man syndrome with increase in dose, resulting in poor elimination of MRSA (Charles et. al. 2004, Rybak et. al. 2009b). hVISA/VISA infection mostly occurs in patients with serious underlying disease and high bacterial load at the infection site, such as in endocarditis, bone infection, and deep infection of prosthetic joints and valves, possibly due to low blood supply and poor drug penetration, resulting in poor elimination of MRSA (Charles et. al. 2004). A report indicates that 1/4th of

endocarditis MRSA strains are hVISA and patients with infective endocarditis are more likely to harbour hVISA (Bae et. al. 2009). It is also suggested that a sub-inhibitory concentration of vancomycin therapy in early treatment of MRSA infection may mediate emergence of hVISA/VISA (Charles et. al. 2004).

1.1.9.6 Impact of Vancomycin Non-susceptible S. aureus

Reports have indicated that patients with serious MRSA infection have persistence of infection and poor clinical outcomes even while on appropriate doses of vancomycin (Charles et. al. 2004, Howden et. al. 2004, Howden et. al. 2006). Retrospective analysis have associated VISA strains with vancomycin clinical failure and death in patients with MRSA infections (Musta et. al. 2009, Khatib et. al. 2011), however resistant sub-populations in susceptible strains is suggested to reduce only the effectiveness of vancomycin without affecting the mortality and increase the persistence of MRSA (Maor et. al. 2009, van Hal et. al. 2011a). Strangely, low rate of mortality has even been described in patients infected with MRSA strains with vancomycin MIC >1.5mg/L than in patients infected with strains with MIC < 1.0 mg/L in UK (Price et. al. 2009).

Using an *in-vitro* model, effects on hVISA of MIC of 2mg/L demonstrated that it required 10mg/L vancomycin for complete suppression of growth (Hiramatsu et. al. 2001a Hiramatsu 2001b). Animal and *in-vitro* models on hVISA/VISA have demonstrated the reduced efficacy of vancomycin (Backo et. al. 1999, Leonard and Rybak 2009). Furthermore, Rose et. al. (2009) have demonstrated poor vancomycin activity against clinical hVISA even at appropriate dose (*f*AUC/MIC=105–317) in an *in-vitro* PK/PD model and their study also revealed

that vancomycin had limited activity against hVISA even at simulated doses of 5.0 g every 12 h (*f*AUC/MIC=799).

Vancomycin has been shown to be inferior to beta-lactams in treatment of *S. aureus* (VSSA) infection (Chang et. al. 2003). Vancomycin clinical failure is not uncommon, even in isolates with MIC < 2 mg/L (Moise and Schentag 2000, Howden et. al. 2004, Hidayat et. al. 2006, Soriano et. al. 2008). Reduced vancomycin efficacy against MRSA strains with MIC of 1-2 mg/L has been reported (Sakoulas et. al. 2004, Moise-Border et. al. 2007). Soriano et. al. (2008) have suggested increase in mortality rate in patients with MRSA bacteremia when the antibiotic therapy was inappropriate and when vancomycin was used as frontline drug in treatment of infection caused by MRSA strains exhibiting vancomycin of MIC >1 mg/L, indicating that a subtle change in MIC may explain negative clinical outcome with vancomycin therapy. It is therefore suggested that patients with MRSA infection having vancomycin MIC >1 mg/L, vancomycin should be replaced with alternatives including surgical interventions and use of new and effective drugs (Rubinstein et. al. 2011). However, this could lead to increased use of expensive alternative drugs associated with increase in treatment cost and unknown potential toxicity, as well as increasing the likelihood of resistance development in these agents.

1.1.9.7 Problems Associated with hVISA/VISA

At present, the proportion of MRSA with reduced susceptibility to vancomycin (VISA/hVISA) is unknown to many healthcare centers due to lack of a simple standardized method for detection of hVISA/VISA and poor understanding of the non-susceptibility mechanism.

Detection methods

Antimicrobial susceptibility tests (AST) were developed soon after commercial availability of antibiotics (Poupard et. al. 1994). AST are performed on clinical strains to determine the effectiveness of an antimicrobial agent, to design the dosage regimen and to monitor the trends in resistance patterns. Usually, AST are not performed on normal microbial flora from non-sterile sites. However, species generally regarded as normal flora but considered to be opportunistic organisms or which were previously susceptible to antibiotics are now resistant, e.g. *S. aureus*. Such situations may demand AST. Regular performance of AST has numerous advantages; as it can avoid the risk of negative clinical outcome due to use of inappropriate agents, allow change from a broad spectrum to a narrow spectrum agent reducing the risk of inducing further resistance, enable use of cost-effective antimicrobials, generate information on resistance rates and patterns, and therefore helps in formulating guidelines on empirical therapy. Antibiotic-resistance surveillance is important to prevent and control the development and spread of resistant phenotypes.

Various organizations (CLSI, EUCAST and other local bodies) have listed the guidelines to perform AST by using an appropriate agent to test various groups of organisms. The primary objective of these guidelines is to create awareness on prudent use of antibiotics as well as to ensure use of less toxic, cost-effective and clinically appropriate antimicrobial agents, to minimize the selection of multi-resistant nosocomial strains by imprudent use of antibiotics and to maximize positive clinical outcomes with antimicrobial therapy.

The antimicrobial susceptibility of an organism can be assessed by numerous methods and the factors considered in selecting an AST include: ease of performance, cost, nature of organism under examination and degree of precision required. There are two common approaches to AST: diffusion method and dilution methods (Andrews 2001). Adoption of dilution methods have led to development of gradient and automated methods. However, in recent years there has been an increase in use of molecular methods for direct detection of the resistance determinants.

Despite considerable efforts, molecular mechanisms of hVISA/VISA have not been clearly understood and no genotypic assay is available for rapid detection of these phenotypes. With various methods available for detection and confirmation of hVISA/VISA, accurate detection of these phenotypes still appears to be a difficult process. Possible methods that can be used for defining vancomycin MIC and / or screening hVISA/VISA are:

Disk Diffusion method

This is a qualitative method and is the most commonly used technique in clinical laboratories. In this method 0.5 McFarland suspension of the test organism is swabbed onto the agar plate and the reservoir of the antibiotic (paper discs) to be tested is placed on the plate and incubated. After incubation the diameter of zone of inhibition is measured and is compared with published breakpoints. There are two types of disk diffusion test: the comparative method and the standardized method.

Comparative methods, such as the Stoke's method (Stokes 1993), in which the zone of inhibition obtained with the test organism is compared directly with a fully sensitive control strain on the same agar plate. However, this method is no longer advocated by any guideline as the limitations of this method have reduced the value and application of the Stoke's method as an AST.

The most commonly used standardized method is the CLSI method (CLSI 2010), which is a modification of the Kirby-Bauer approach (Bauer 1966). Other standardized methods are the result of International collaborative study supported by the World Health Organization (WHO) (Ericsson 1971), by The European Committee on Antimicrobial Susceptibility Testing (EUCAST) and by British Society for Antimicrobial Chemotherapy (BSAC) (Andrews 2004) to control variation by standardizing all the details of the tests, such as media-Mueller Hinton agar and density of bacterial suspension-0.5McFarland with the aim to achieve reproducibility between different clinical laboratories. This method however,

suffers from the inherent limitation, i.e. considerable difference in the batches of agar medium affecting the performance of the test itself.

The disk diffusion method is simple to perform and reproducible if performed carefully (Amsterdam 1996). It is cost-effective and requires very few or no special equipment. It allows flexibility for the choice of antimicrobials for testing and provides results that can be easily interpreted by physicians. However, the test should be applied only to bacterial species that have been completely evaluated and standardized (Turnidge and Jorgensen 1999). Problems with diffusion of the drug and apparent sensitivity display in testing of some of β-lactams can reduce the predictability of susceptibility of disk diffusion. Moreover, disk diffusion test is not suitable for slow growing, anaerobic or fastidious bacteria (Woods and Washington 1995). The disk diffusion method is not suitable for intermediate resistance such as VISA and was removed from the CLSI guidelines for identification of hVISA/VISA. It provides only qualitative results of resistance and is not suitable for cases when a more accurate assessment of susceptibility is needed.

Dilution Method

The increase in antimicrobial resistance and the need to tailor the treatment regimen to individual cases requires the MIC (Minimum inhibitory concentration) the lowest concentration of the drug which inhibits growth of an organism (Turnidge and Paterson 2007). The most common approach of AST to determine MIC is use of a dilution method. Usually, serial two-fold dilutions, representing the *in-vivo* attainable concentrations are used. The concentration range varies with

the drug, and the organism under test. The tested concentration range must include concentrations that allow categorization of organisms into susceptible and resistant. The antibiotic stock solutions are prepared and stored as specified by CLSI guidelines for dilution test (CLSI 2010). CLSI recommends use of Mueller-Hinton agar (MHA) / broth for AST of aerobic organisms, whereas the BSAC recommends Iso-sensitest agar / broth. The interpretive criteria of susceptibility for each organism and antibiotic are provided by CLSI (CLSI 2010). Currently there are two approaches of dilution method i.e. agar dilution method and broth dilution method.

a. Agar dilution test

This method uses agar plates, prepared using a specific volume of sterilized and cooled (around 50-60^0 C) agar supplemented with a specific volumes of antibiotic to create different concentrations, subsequently poured into petri dishes and allowed to solidify. Then a standard concentration of bacteria (around 10^5CFU/inoculam) is deposited to the surface of the plate using a multipoint inoculator and the plate incubated. Following incubation, the MIC is determined as the lowest concentration of antibiotic that inhibits the growth of the test organism. A complete description of the method has been published in several standardized guidelines (EUCAST 2009, CLSI 2012).

Up to 34 isolates can be tested on one set of plates for their AST and MIC, but this method cannot be used to determine MBC (Minimum bactericidal concentration). Bacteria multiply *in-vivo* on solid surfaces where they adhere and the agar provides

solid surface and hence the bacterial growth on agar is more closely related to *in-vivo* conditions.

b. Broth dilution method

Broth dilution tests are performed in test tubes (macro) or in micro-titer plate (micro) with serial two-fold dilution of antibiotic using 10^5 CFU/ml of bacterial suspension and MIC is determined as described in agar dilution method (CLSI 2012).

The major advantages with broth dilution test is susceptibility testing can be performed on a single isolate for drugs not routinely tested, and this method can be modified making it suitable for automation. The use of micro dilution method allows for use of small volumes and automated equipment can be used for preparation of micro-titer plates for tailored panel of antibiotics with flexibility for changes to reduce the cost and preparation time.

In comparison to disk diffusion method dilution methods are laborious, time-consuming and more expensive. The accuracy of MIC obtained by dilution method is within one log dilution difference for most combination of antibiotics and organisms, as long as the test organism is susceptible (Amsterdam 1996). This doesn't provide the exact MIC of the test strain. Moreover, this method is not suitable for checking acquired mechanisms of resistance, especially if the test organism is producing inactivating enzymes. Another common limitation of broth dilution is the MICs obtained are three to five dilutions higher than on agar therefore, the organism appears to be more resistant in broth (Acar and Goldstein

1996). For this reason when testing a new method using agar media, agar dilution test should be used as the reference method.

Dilution methods are used when a more accurate estimation of susceptibly is needed or when disk diffusion method is not appropriate (Andrews 2001). However, the conventional dilution method has the limitation of large increment of higher concentration, and a high standard allowable rate of error (Wexler et. al. 1990), thus makes this methods unsuitable for detection of hVISA/VISA.

Dilution method is a CLSI approved method for defining vancomycin MIC and / or screening hVISA/VISA. Prakash et. al. (2008) reported reasonable correlation between CLSI agar dilution and MBD. However, Swenson et. al. (2009) reported an inconsistency with these methods, with 20.8% of isolates having an MIC of 0.5mg/L by MBD, while agar dilution indicated only 1.0% of strains had an MIC of 0.5mg/L. This study also investigated a number of commercial methods and reference methods and has found that commercial methods such as Microscan and Phoenix tend to categorize VISA as VSSA and VSSA as VISA (Swenson et. al. 2009).

Breakpoint method

This method is similar to the agar dilution method, but the antibiotic concentrations are selected to categorize the strain as sensitive, intermediate or resistant. Generally, two appropriate drug concentrations are used, when growth occurs at both concentrations, it is interpreted as resistant, whilst growth on only the lower

concentration indicates intermediate resistance. When there is no growth at both concentrations, it is categorized as susceptible.

The breakpoint method gained wide acceptance in United Kingdom as an alternative to disc diffusion (Faiers et. al. 1992) due to its advantages such as ease of data handling, clear-cut endpoints and reduced interpretive difficulties. Moreover, the method is suitable for laboratories with high workloads, since large number of test strains can be tested using multi-point inoculators. However, the limitations include lack of an exact MIC, inability to discriminate the finer degrees of susceptibility and low reproducibility for organisms with MICs close to a breakpoint. This has led to discontinuation of its use.

Automated methods

The development of robotics, electronics and microprocessors in recent years has lead to the development of automated methods with clinical applications. Automated AST methods are simple and rapid as the incubation time is shorter.

Automated methods range in choice from simple to highly complex, with instrument based broth micro-dilution method being the least automated. The automated methods differ in the optical method used for examining the growth endpoint. Turbidimetric detection is commonly employed for detection of resistance, the growth of bacteria in the antibiotic supplemented broth being detected by use of a photometer. Some use fluorimetric detection by incorporating fluorescent indicators or detecting the hydrolysis of fluorogenic substrates in the liquid medium (Nolte et. al. 1998). Most of these depend on microprocessing, to

generate and store the data. Some instruments with expert software are able to provide automated reviews of the test results for errors and resistance interpretation (Ferraro and Jorgensen 2004).

A wide range of commercial systems are available including Vitek (BioMerieux), AutoSCAN WalkAway W/A (Baxter Diagnostics), Sensititer (Radiometer), AutoSceptor (Becton Dickinson) ALADIN (analytab Products), ATB Expression (BioMerieux) and Cobas Micro (Roche). Only Baxter AutoSCAN and Vitek system are capable of generating rapid AST results and are approved by Food and Drug Authority (FDA) (Ferraro and Jorgensen 2004, Ferraro and Jorgensen 2009).

The major advantage of automated AST is the reproducibility of results since the procedures are highly standardized and many of antimicrobial agents and test organism combinations have been approved by regulatory authorities (Berke and Teeino 1996). Another advantage of automated methods is the link between the computer and the laboratory information system and thus the expert system is able to detect rare resistance patterns and avoid possible human error on reporting. The automated methods and the laboratory information system can generate rapid AST results, allowing timely changes to appropriate antibiotics (Trenholme et. al. 1989) and thereby, reducing the hospitalization costs.

Although, automated methods appear to be more efficient for performing AST and for data management and data storage, the initial investment for purchasing the system and maintenance costs are relatively high (Ferraro and Jorgensen 2004). Moreover, use of such systems requires purchasing of the antimicrobial panels from the manufacturer and flexibility to change test panels is limited. It may be

necessary to use a combination of panels to have a complete set of antimicrobials for AST, further increasing the cost of each run.

The major disadvantage of such AST systems testing is lack of ability to test some clinically significant bacteria needing an alternative MIC method for susceptibility testing. Moreover, these systems lack reproducibility and accuracy in identification of hVISA/VISA (Jones 2006, Swenson et. al. 2009).

Single-point population analysis method (PAP)

Brain Heart Agar (BHA) plates supplemented with 4 mg/L vancomycin has been described for screening VISA (Hiramatsu et. al. 1997a). However, CDC recommends use of BHA supplemented with 6mg/L of vancomycin for detection of VISA. The growth of two or more colonies after 48hrs of incubation is considered to be positive (Walsh and Howe 2002, Yusof et. al. 2008).

Studies indicate that PAP method to be a poor predictor for isolates having MIC less than 6 mg/L (Walsh et. al. 2001, Wooton 2001). A number of variations of this method have been reported, including use of different media, with different vancomycin concentrations, and use of a higher inoculum density with no significant improvement in screening of hVISA/VISA (Hubert et. al. 1999, Reverdy et. al. 2001, Trakulsomboon et. al. 2001).

Jung et. al. (2002) modified the technique using BHA supplemented with 4 mg/L vancomycin plates with addition of 4% NaCl but found that the modified method was not superior to the original method (Jung et. al. 2002). BHA supplemented with 3 mg/L resulted in unacceptable levels of false positive isolates (Kosowska-

Shick et. al. 2008), although a similar agar additionally supplemented with a non-inhibitory dye was reported to have 65% specificity (Burnham et. al. 2010). Five mg/L Teicoplanin-supplemented MHA has been reported to have better specificity, but requires 48h incubation (Walsh and Howe 2002).

Supplementation of BHA with 5% blood or 20% serum was found to enhance growth of hVISA, suggesting that supplementation could improve the detection of hVISA (Howden et. al. 2004). However, more detailed analysis of these media did not confirm the initial findings (Horne et. al. 2009).

Population analysis profile area under cure ratio (PAP-AUC)

PAP-AUC is also termed as modified PAP. PAP-AUC is the gold standard for detection of hVISA, performed by preparing 10^{-3} and 10^{-6} bacterial suspensions in saline and then depositing these on BHA plates containing 0.5, 1, 2, 2.5 and 4mg/L of vancomycin using spiral plater. The number of colonies are counted after 48h of incubation at 37^{0}C. The viable count is plotted against vancomycin concentration and used for calculation of AUC. The ratio is obtained by dividing the AUC of test strain by AUC of mu3 and isolates with AUC ratio of $\geq$ 0.9 are defined as hVISA (Wootton et. al. 2001). Although PAP-AUC is the gold standard for detection of hVISA, it is labor-intensive, time consuming and needs 48h of incubation, thus unsuitable for routine screening in clinical laboratories.

Gradient Methods

Antimicrobial gradient diffusion methods work on the principle of formation of an antibiotic concentration gradient on an agar plate. The common approaches of gradient creation are: Etest and Spiral Gradient Endpoint (SGE) method:

Epsilometer test (Etest)

Etest (AB Biodisk) is the most common agar gradient diffusion method for direct quantification of antimicrobial susceptibility of micro-organisms. E-test strips are inert thin plastic carriers of the predefined concentration of dried antibiotic on one side and a continuous concentration scale on the other. MIC determination by E-test is performed by swabbing the agar plate with a pre-adjusted concentration of bacterial suspension in the same manner as for disc diffusion. One to three strips of the antimicrobial agents under the test are placed on the inoculated agar surface and incubated overnight, the effect of gradient concentration of antibiotic gives rise to an elliptical inhibitory zone. The interphase of the ellipse margin indicates the MIC of the test drug for the organism (AB Biodisk).

Etest is a simple and robust method for determining the MIC and is versatile in identification of high and low level resistance and subtle changes in susceptibility. The major advantage is its ability to detect the resistant subpopulations (gradient Etest) and to test anaerobic bacteria or fastidious organisms. This is because the Etest strips can be placed on enriched media or in special incubation conditions without affecting the accuracy of the result. Etest is an FDA approved method for MIC determination and reports have demonstrated that MICs obtained by Etest

have excellent agreement with the reference methods against the commonly isolated bacteria (Baker et. al. 1991, Brown and Brown 1991, Ngui-Yen et. al. 1992).

Etest strips are quite expensive and their use for routine testing in the clinical laboratory can become a significant burden on the healthcare systems and may limit the number of agents tested. Etest is also known to produce minor discrepancies, when compared with reference methods (Macias et. al. 1994, Skulnick et. al. 1995, Leonard and Rybak 2009). Differences in results reported in previous studies indicate that variations in results can occur if conditions are not carefully controlled by the user, such as the thickness and the wetness of the plates which may affect the results. The quality of the result entirely depends on the quality of the test strips. There can be storage problem associated with Etest strips, which should be kept at -20^0C and extreme care must be taken to avoid any moisture (AB Biodisk). Another major limitation is that Etest is unreliable for accurate detection of vancomycin non-susceptibility, as this method provides MIC values one dilution higher than dilution methods (Walsh and Howe 2002, Prakesh et. al. 2008, Leonard and Rybak 2009). Other modified versions of Etest for testing vancomycin non-susceptibility are discussed below.

Macro Etest (MET)

Macro Etest was developed to improve the detection of resistance, mainly for glycopeptide intermediate resistance. Vancomycin and teicoplainin Etest strips are placed on a Brain heart infusion agar plate that has been swabbed with a bacterial suspension at 2.0 McFarland concentrations and incubated at 35^0C. The plates are

read after 48h (Wootton 2007). Although, MET produces fewer false-positive results in comparison to other methods, the method is expensive and the need for 48h incubation may delay the appropriate therapy to the patient (Wootton 2007, Rybak et. al. 2008). Whilst, MET improves the detection of hVISA the MICs generated by this method are not true vancomycin MICs. Thus, this method is not suitable for generating epidemiological data about changes in MIC levels (Leonard and Rybak 2009).

GRD Etest

GRD Etest is a modified Etest to improve the detection of hVISA while overcoming the disadvantages associated with PAP-AUC. In this method the GRD strip (Vancomycin and Teicoplanin) is placed on Mueller-Hinton agar supplemented with 5.0% blood and swabbed with 0.5 McFarland bacterial suspensions. The plate is incubated at 35^0C and the results read at 24h and 48h. At 24 h. GRD Etest has a sensitivity of around 70-77% and at 48h this improves to 94% which is almost equivalent to PAP-AUC method and is superior to MET and Etest (Yusof et. al. 2008, Leonard et. al. 2009). However, a report indicates that GRD has low positive predictive value for hVISA (16.2%) and confirmatory testing with PAP-AUC is necessary, suggesting poor specificity of GRD in hVISA detection (Richter et. al. 2011). In addition, the need for 48h incubation for optimum sensitivity may delay a change to appropriate treatment for the patient indicating a need for rapid and reliable method for detection of resistant sub-population. In addition, this method is too expensive to allow for routine screening for epidemiological purposes (Howden et. al. 2010).

Spiral Gradient Endpoint (SGE) method

This method also works on the gradient principal. A small volume of a drug solution of known concentration is deposited onto an agar plate in a precise spiral pattern creating a radial concentration gradient of the antimicrobial agent as shown in Figure 1.8a, with higher concentration near the center of the plate and the lower concentration at the periphery of the plate, thus creating the gradient. A bacterial suspension of 0.5 McFarland concentrations is then swabbed across the gradient at a 90^0 angle to the spiral using a template as guide. Following incubation, the growth endpoint measurement of the test bacteria provides the MIC (Schalkowsky 1985). The concentration of the antimicrobial at the growth endpoint can be calculated using the SGE software developed by the manufacturer of the spiral plater after the measurement of the radial distance of the growth transition endpoint to the commencement of the antibiotic deposition using SGE template (Figure 1.8b).

SGE is not a diffusion based AST as it does not rely on the drug diffusion to create the concentration gradient, but rather depends upon the precise deposition of the known concentration of antimicrobial stock solution. However, drug diffusion time will alter the deposited gradient and the MIC. The relevant diffusion rate has been initially investigated during the development of SGE by using spectrophotometer reading of agar plugs collected at different position of the gradient (Schalkowsky 1985).

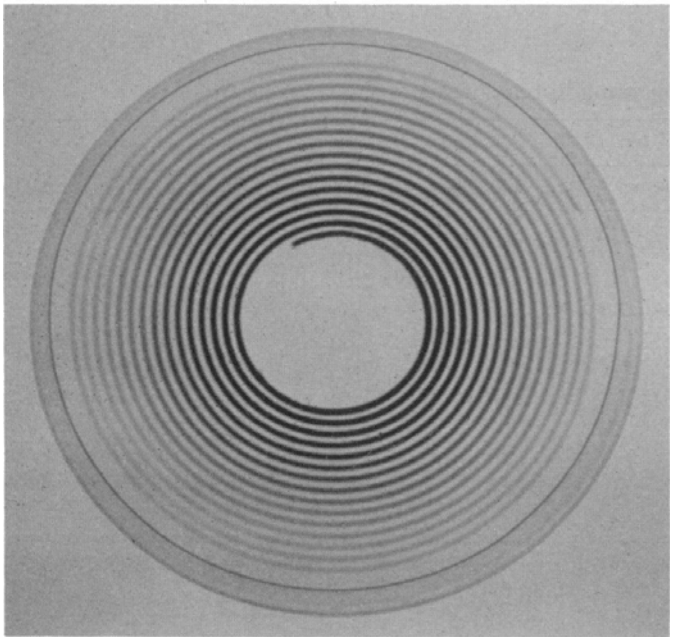


FIGURE 1.8a: Radial concentration gradient of the antibiotic on the agar plate created by the spiral plater
(Auto plate 4000-Spiral Biotech)

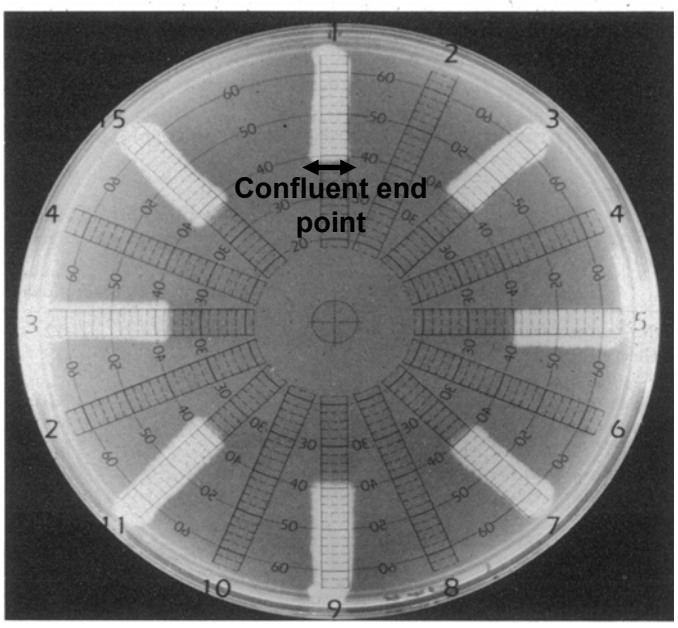


FIGURE 1.8b: SGE template
(Auto Plate 4000-Spiral Biotech)

The important determinants of diffusion rate are drug molecular weight, diffusion time and incubation time (1h). Software is required to calculate the required stock concentrations and to calculate the endpoint MIC of the test strain and the drug. Several studies have demonstrated that there is a good agreement between the MIC obtained by SGE and serial agar dilution (SAD) (Hill and Schalkousky 1990, Paton et. al. 1990, James et. al. 1991).

Although reports indicate that SGE is reproducible and accurate in determining MIC for aerobic and anaerobic organisms (Hill and Schalkousky 1990, James 1990, Paton et. al. 1990, James et. al. 1991, Wexler et. al. 1991), the method was neglected due to complex calculations involved in MIC determination and the availability of automated methods (Pong et. al. 2010). Recently, SGE has been shown to be suitable for fastidious organisms due to the introduction of a software for calculation (Pong et. al. 2010), but there appears to be no report on its use as a detection tool for hVISA/VISA.

The major advantages of SGE as an AST are decreased labor and materials, more accurate dilution compared with SAD and ease of visualization of the growth endpoint in comparison to dilution method. It is cost-effective as the consumables are low in comparison to other AST such as dilution method and Etest. Although there is an initial large investment for the spiral plater purchase, it could be soon recovered as the set up could be used as an AST tool for other antimicrobial agents. One or more drugs and strains can be tested economically with reduced turn around time (Pong et. al. 2010).

Whilst, others have suggested combination of vancomycin and rifampin (Pallares et. al. 1998) or vancomycin and gentamicin (Cottagnowd 2003) such synergistic combinations have been suggested for treatment of hVISA/VISA infections e.g: linezolid and co-trimaoxazole or linezolid and rifampin (Howden et. al. 2010) and SGE could be readily adapted for testing the effectiveness of combination therapy for treatment of MRSA/hVISA/VISA and other resistant organisms.

With the inconsistencies associated with available methods CLSI recommends a combination of disc diffusion and MBD for MIC or disc diffusion and PAP using 6mg/L plate or MBD for MIC and PAP using 6mg/L plate (CLSI 2010). Requirement of performing multiple methods appear to be time consuming and labor intensive. Thus, to assess the prevalence and clinical relevance of vancomycin non-susceptible *S. aureus,* a suitable detection method that can overcome the above limitations is needed.

There are large number of reports on investigation of various screening methods for hVISA/VISA. The findings of these studies have been summarized in Table 1.3

TABLE 1.3: Laboratory detection of hVISA and accuracy of methods compared to those of PAP-AUC method

Method	Sensitivity	Specificity	Time	Remarks	Reference
Vancomycin broth MIC	11%	100%	24 h	Sub-optimal in detecting hVISA	Walsh et. al. 2001
BHA + Vancomycin 6 mg/L	4.5-11.5%	68-97.4%	48 h	Least effective in detecting hVISA	Voss et. al. 2007, Wootton et. al. 2007
MHA + teicoplanin 5 mg/L	65-92%	35-85%	48 h	Better at predicting VSSA but less accurate at hVISA.	Voss et. al. 2007, Wootton et. al. 2007
MHA + vancomycin 5 mg/L	1-20%	59-99%	48 h	Poor sensitivity due to the high number of false negatives	Walsh et. al. 2001, Voss et. al. 2007
Simplified PAP	71%	88%	48 h	False-negative due to lack of detection of hVISA	Walsh et. al. 2001
Macromethod Etest (MET)	69.3-98.5%	87.2-97%	48 h	MET has the advantage of producing fewer false-positive	Walsh et. al. 2001, Voss et. al. 2007, Wootton et. al. 2007
Etest GRD	70-77%	98-100%	24 h	Useful test for clinical detection of hVISA	Yusof et. al. 2008, Leonard et. al. 2009
	93-94%	82-95%	48 h		

1.1.9.8 The Staphylococcal Cell Wall

A clear understanding of cell wall structure is important for better understanding of the mechanism of glycopeptide reduced susceptibility as well as to aid in the development of new cell wall active anti-MRSA agents.

The outermost surface of the Gram positive cell wall is a polysaccharide. The cell wall is made up of highly cross-linked peptidoglycan units, wall teichoic acids (WTA), lipoteichoic acids (LTA) and proteins. The peptidoglycan is composed of amino sugars (*N*-acetlyglucosamine and *N*-acetylmuramic acid). The carboxyl group of each amino sugar is attached to stem pentapeptides (L-Ala-D-iso-Gln-L-Lys-D-Ala-D-Ala). The stem pentapeptides are inter-connected by inter-peptide bridges; these bridges connect the lysine component of one stem peptide to the D-alanine of another stem peptide (Tomasz 2006).

Teichoic acid chains are attached to the 6-hydroxyl group of *N*-acetylmuramic acid of the glycan chains (Tomasz 2006). Therefore muramic acid is the stress-bearing component of the cell wall (Diep et. al. 2008).

The functions of WTA and LTA are still unclear. Reports indicate that teichoic acids are important in protecting the cell wall from antibiotic stress as alanylation of teichoic acid results in increased positive charge which is involved in repelling positively charged antibiotics and host defense molecules (Collins et. al. 2002, Peschel et. al. 1999, Peschel et. al. 2000). WTA is important in attachment to host cells and is also known to contribute towards lysozyme resistance by preventing

lysozyme binding to peptidoglycan (Bera et. al. 2007). The structure of gram positive cell wall is shown in Figure 1.9.

A large number of genes and enzymes appear to be involved in the biosynthesis of peptidoglycan cell wall. The *femA, femB, femC* and *femX (fem genes)* are important genes involved in synthesis of the pentaglycine bridge that attaches to the lysine residue of the stem peptide (Rohrer et. al. 1999, Rohrer and Berger-Bachi 2003). *glnR* and *glnA* genes are regulated by the *fem* group of genes (Ornelas-Soares et. al. 1993, Gustafson et. al. 1994). These genes are also known to regulate the glutamine (amino donor) availability for synthesis of peptidoglycan (Ornelas-Soares et. al. 1993, Gustafson et. al. 1994). The PBP's are important in cell wall synthesis as these are involved in transglycosidase function to bridge N-acetylglucosamine to N-acetlymuramic acid and transpeptidase function to bridge penultimate D-ala to a glycine acceptor in the nascent cell wall (Moreillon et. al. 2005, Pinho and Errington 2005).

The *mur* group of genes *(murB, murC, murD, murE, murF,* and *murZ)* are known to encode a number of mur enzymes (muramyl peptide ligases) which are involved in the primary stage of peptidoglycan synthesis mainly UDP-N-acetlyglucosaminenopyruvate transferase, glutamate racemase and UDP-N-acetlylglucosamine carboxylvinylntransferase (Kuroda et. al. 2003, Sobral et. al. 2006).

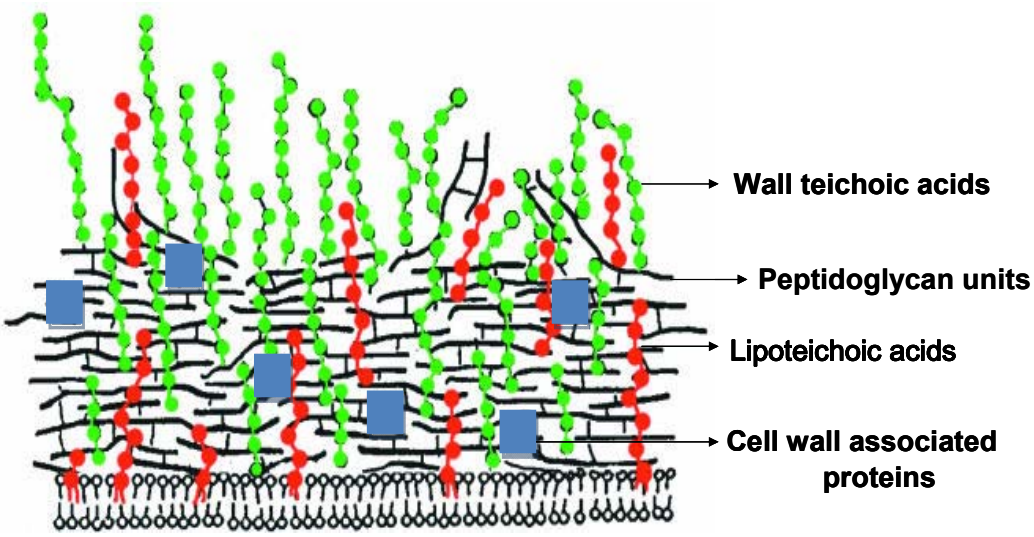


FIGURE 1.9: Simple structure of Staphylococcal cell wall
(Modified from Neuhaus and Biddiley 2003)

MurZ is involved in production of an enzyme, enolpyruvyl transferase that supports UDP-N-acetylglucosamine condensation with phosphoenolpyruvate (PEP) (El Zoeiby et. al. 2003). *murF* gene is known to be involved in attachment of dipeptides, first the *dala* gene produces the dipeptide and *murF* bridges the dipeptide to the UDP-n-acetylmuramic acid (MurNAc-)-tripeptide, to complete the construction of the peptidoglycan precursor, the UDP-linked MurNAc-pentapetide (Sobral et. al. 2006). Phosphoglucosamine mutase gene known as *glmM* is involved in an early step of UDP-N-acetylglucosamine synthesis, which has a role in peptidoglycan and WTA synthesis (Fischer 1990).

Several Two-component systems (TCS) including vancomycin resistance associated SR (vraSR), glycopeptide resistance associated SR (graSR) and walKR have been recognized to be involved in regulating cell wall biosynthesis (Kuroda et. al. 2003, Gardete et. al 2006, Dubrac et. al 2008, Cui et. al 2009) and in the environmental sensing systems that integrate a broad range of input stimuli to effector proteins, often involved in transcription factors (Galperin 2006, Gao and Stock 2009). Several studies have indicated that a TCS is composed of a membrane sensor histidine kinase and a cognate response regulator. The environmental signals captured by the sensing kinase results in histidine autophosphosphorylation, followed by phosphotransfer from histidine kinase to a conserved aspartate in the receiver domain of the response regulator resulting in alteration in regulatory genes, altered enzymatic activity and increased cell wall synthesis (Galperin 2006, Gao and Stock 2009).

Mechanism of vancomycin non-susceptibility:

Development of hVISA/VISA has been attributed to alterations in cell wall structure that inhibit vancomycin access to its site of action (Sieradzki and Tomasz 1999b). Common biochemical and phenotypic changes contribute to vancomycin reduced susceptibility. When examined in detail, the thickened cell wall is a consistent feature, as shown in Figure 1.10. Using electron microscopy, it has been shown that VSSA has an average cell wall thickness of about 20nm. In the case of hVISA/VISA, the cell wall thickness is found to be around 40nm (Cui et. al. 2003). Cell wall thickening is due to activated cell wall synthesis, increased production of peptidoglycan and increased levels of enzymes (Glutamine synthetase and L-glutamine-D-fructose-6-phosphate aminotransferase), which are involved in peptidoglycan biosynthesis (Hanaki et. al. 1998, Sieradzki et. al. 1999a, Sieradzki and Tomasz 1999b, Cui et. al. 2000). In addition, VISA isolates have an increased proportion of glutamine non-amidated muropeptide components in their peptidoglycan (Hanaki et. al. 1998).

The increased production of glutamine non-amidated muropeptides is due to increased functioning of the GlmS pathway (involved in conversion of Fru-6-P into GlcN-6-P) (Hanaki et. al. 1998), contributing towards increased production of peptidoglycan with reduced cross-linkage between the peptidoglycan units (Hanaki et. al. 1998, Sieradzki et. al. 1999a, Sieradzki and Tomasz 1999b, Cui et. al. 2000). All the above changes result in accumulation of free peptidoglycan D-ala-D-ala termini which act as false targets to trap vancomycin.

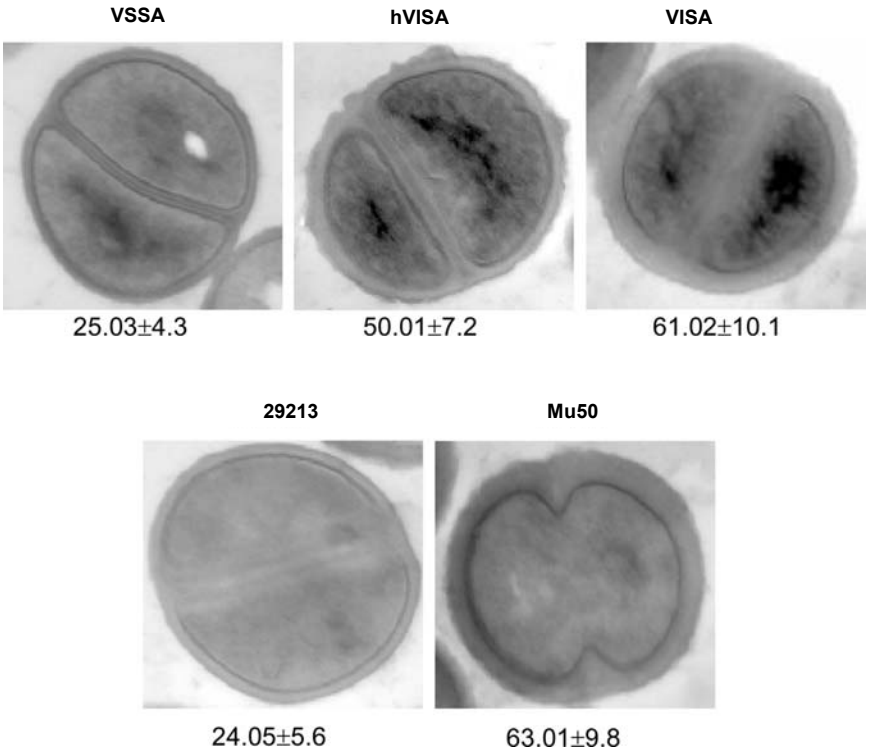


FIGURE 1.10: Micrographs of VSSA, hVISA and VISA
Values given under each image are mean ± SD of the cell wall thickness in nanometers. (Modified from Sola et. al. 2011)

In agreement with these findings, Mu50 and Mu3 were found to bind 2.4 and 1.1 times greater amounts of vancomycin than VSSA, the vancomycin binding assay of peptidoglycan determined by incubating a peptidoglycan suspension with vancomycin for 15mins at 37^0C (Hanaki et. al. 1998). This accumulation of vancomycin in the cell wall suggests that non-amidated murein monomers have greater affinity for vancomycin than amidated murein units (Hanaki et. al. 1998). This reduces the amount of vancomycin reaching the target located in the cytoplasmic membrane, thus providing protection to the C-terminal of the D-ala-D-ala residue of the peptidoglycan precursor, further facilitating cell wall synthesis and survival of the organism (Hanaki et. al. 1998, Cui et. al. 2006).

Reduced autolytic activity is another common change observed in hVISA/VISA. Some reports suggest a possible role of wall teichoic acid in suppressing peptidoglycan degradation (Sieradzki and Tomasz 2003). While, others suggest that altered activity of peptidoglycan hydrolase is responsible for reduced autolytic activity (Koehl et. al. 2004). A study reported a decrease in acetate catabolism (around 71%) indicating that vancomycin non-susceptibility results in impaired acetate catabolism (Nelson et. al. 2007). It has been suggested that altered acetate catabolism could lead to altered growth characteristics, change in cell death rate and antibiotic tolerance (Nelson et. al. 2007). There has been a large number of reports of development of VISA strains and there characteristics have been investigated. The results of these studies are summarized in Table 1.4.

TABLE 1.4: Phenotypic characteristics of clinical and laboratory-induced hVISA and VISA strains

(Modified from Howden et. al. 2010)

Isolate studied	Phenotype (vancomycin MIC mg/L)	Isolate description	Phenotypic feature(s) of hVISA or VISA isolate compared to VSSA	Reference
Mu3	hVISA (2-4)	First reported hVISA and VISA strain	Increased production of PBP2 and PBP2'; activated cell wall synthesis; increased glutamate-containing muropeptides; correlation between cell wall thickness and vancomycin MIC; reduced whole-cell autolytic activity; vancomycin clogging of cell wall	Hanaki et. al. 1998,Cui et. al. 2000, Cui et. al. 2003, Utaida et. al. 2003, Cui et. al. 2006
Mu50	VISA (8)	First reported VISA strain		
Mu50Ω	VSSA/hVISA	Mu50Ω isolated 18 months later from same patient as Mu50		
BR1	VISA (8)	Clinical isolates from Brazil	Cell wall thickening	Oliveira et. al. 2001, Cui et. al. 2003
MI	VISA (6)	Michigan VISA isolate, July 1997, CAPD-associated peritonitis	Cell wall thickening; increased extracellular matrix; increased glutamate-containing muropeptides; reduced Triton X-induced autolysis	Smith et. al. 1999, Boyle-Vavra et. al. 2001, Boyle-Vavra et. al. 2003,Cui et. al. 2003
NJ	VISA (5)	New Jersey VISA isolate, August 1997, bacteremia		
AMC11094	VISA (8)	South Korea, clinical isolate	Cell wall thickening	Kim et. al. 2000, Cui et. al. 2003
99/3759-V	VISA (8)	Scotland, UK	Cell wall thickening	Cui et. al. 2003
99/3700-W				
28160	VISA (8)	South Africa	Cell wall thickening	Ferraz et. al. 2000, Cui et. al. 2003
LIM-2	VISA (8)	France; isolated from patient who failed teicoplanin therapy	Cell wall thickening	Ploy et. al.1998, Cui et. al. 2003
98141	VISA (8)	France, clinical isolate	Cell wall thickening	Cui et. al. 2003
Hershey MC 10	VISA (daptomycin resistant)	clinical isolate	Reduced muropeptide cross-linking; reduced O-acetylation of muramic acid	Julian et. al. 2007

Continued on following page

Table 1.4 Continued

Isolate studied	Phenotype (vancomycin MIC mg/L)	Isolate description	Phenotypic feature(s) of hVISA or VISA isolate compared to VSSA	Reference
JH9 JH14	VISA (8)	Baltimore patient with endocarditis who failed vancomycin therapy	Cell wall thickening; reduced cross-linkage; reduced PBP4; decreased cell wall turnover and autolysis; changes in wall teichoic acids	Sieradzki et. al. 2003, Sieradzki and Tomasz 2003, Pereira et. al. 2007
MRGR3 14-4	VSSA (1), TSSA (0.5) VISA(4; teicoplanin, 16)	Teicoplanin-resistant subclones emerged in rat model of foreign-body infection without antibiotic exposure.	Unstable resistance in rat model without antibiotic exposure; increased fibronectin-mediated adherence; reduced autolytic activity; reduced extracellular hydrolase activity;	Renzoni et. al. 2004, Renzoni et. al. 2006
NM18	Teicoplanin MIC, 16	In vitro-derived strains, teicoplanin selected	Slower growth; thickened cell wall; reduced fitness in resistant strain	McCallum et. al. 2006
IL-A IL-F	hVISA VISA (8)	VISA emerged from hVISA during 13 days of persistent bacteremia	Cell wall thickening; no increase in glutamate-containing muropeptides; reduced lysostaphin susceptibility; reduced Triton X-induced autolysis	Boyle-Vavra et. al. 2001, Boyle-Vavra et. al. 2003, Cui et. al. 2003
PC-1 PC-3	hVISA (2) hVISA (8)	Paired isolates from patient with vancomycin treatment failure	Cell wall thickening; reduced resistance after 15 days of serial passage; no increase in glutamate-containing muropeptides; reduced Triton X-induced autolysis	Rotun et. al. 1991, Sieradzki et. al.1999b, Boyle-Vavra et. al. 2001, Boyle-Vavra et. al. 2003, Cui et. al. 2003
SA137/93A	VISA (8)	Clinical isolate	Increased cell wall thickness; reduced beta-lactam resistance	Reipert et. al. 2003
VM3	hVISA / VISA	Induced from MRSA COL by vancomycin selective pressure	Gradual alterations in cell wall with increasing resistance; vancomycin trapped in cell wall; reduced growth rate; inactivated PBP4; reduced methicillin MIC, inactivation of *mecA;* delayed access of vancomycin to active site in division septum	Sieradzki and Tomasz 1997, Sieradzki et. al. 1999a, Sieradzki and Tomasz 1999b, Sieradzki and Tomasz 2003, Pereira et. al. 2007

It has been proposed that change in cell wall biosynthetic pathway in hVISA/VISA have evolved in strains with changes in DNA sequences and these mutations support the above biochemical and morphological changes in hVISA/VISA.

Schaaff et. al. (2002) have demonstrated that knockout of *mutS* gene increased the rate of non-susceptibility development and level of vancomycin resistance. Additionally, it was proposed that mutations in *mutS* gene had contributed towards emergence of Mu50 (Avison et. al. 2002); however, sequence analysis of *mutS* in Mu50 and laboratory developed VISA did not confirm changes in *mutS* (O'Neill et. al. 2002, Muthaiyan et. al. 2004).

Kuroda et. al. (2003) have demonstrated the regulatory role of *vraSR* in early and late steps of peptidoglycan synthesis and cDNA differential hybridization has indicated that over-expression of *vraR* results in increase in vancomycin resistance. Complete genomic analysis of hVISA has indicated that mutations in *vraR, yycF* and *agr* increases tolerance to vancomycin (Mwangi et. al. 2007). More recently mutation in w*alKR* was shown to be an important mechanism for vancomycin non-susceptibility and daptomycin cross-resistance (Howden et. al. 2011). Additionally, transcription profiling has indicated that mutations in *walKR* controls autolysis and metabolic activities within the cell (Howden et. al. 2011).

Reports have suggested a role for *graRS* two-compartment regulatory system (TCS) and mutation in *graR* N197S in Mu50. Introduction of N197S into Mu3 resulted in further increase in vancomycin tolerance and formation of VISA. However, when N197S was introduced into *graR* of N315, the isolate did not develop vancomycin tolerance, thereby suggesting that an additional genomic change in N315 was

required for development of VISA. Micro array studies has shown that *graR* N197S up-regulated at least 14 genes involved in cell wall synthesis (Cui et. al. 2005, Neoh et. al. 2008).

A comparative genomic study investigated iso-genic isolates and found 6 mutations. One of the mutations was in *graS* (T136I). Its importance was confirmed by introduction of *graS* (T136I) into VSSA. However, the cloned strain did not build up the cell wall to form a full VISA phenotype, indicating that one or more other mutations are required for development of VISA (Howden et. al. 2008).

Recently, Mu50Ω isolated from a patient 18 months after initial Mu50 infection (VSSA) and its comparative genomic analysis revealed loss of mutations in *vraS* and *graR.* Restoration of *vraS* (I5N) and *GraR* (N197S) of Mu50 into Mu50Ω resulted in development of VISA, clearly indicating that point mutations in these two regulatory systems are essential for development of VISA (Cui et. al. 2009) (see Figure 1.11).

Kato et. al. (2010) have performed complete sequencing of *yvqF, vraS and vraR* genes in iso-genic teicoplanin non-susceptible strains and have found a possible mutation in *yvqF* in 21% of samples and in *vraS* in 16.0% of samples. This study also indicated that use of β-lactam antibiotics to treat MRSA infections might be one of the risk factors for development of hVISA/VISA.

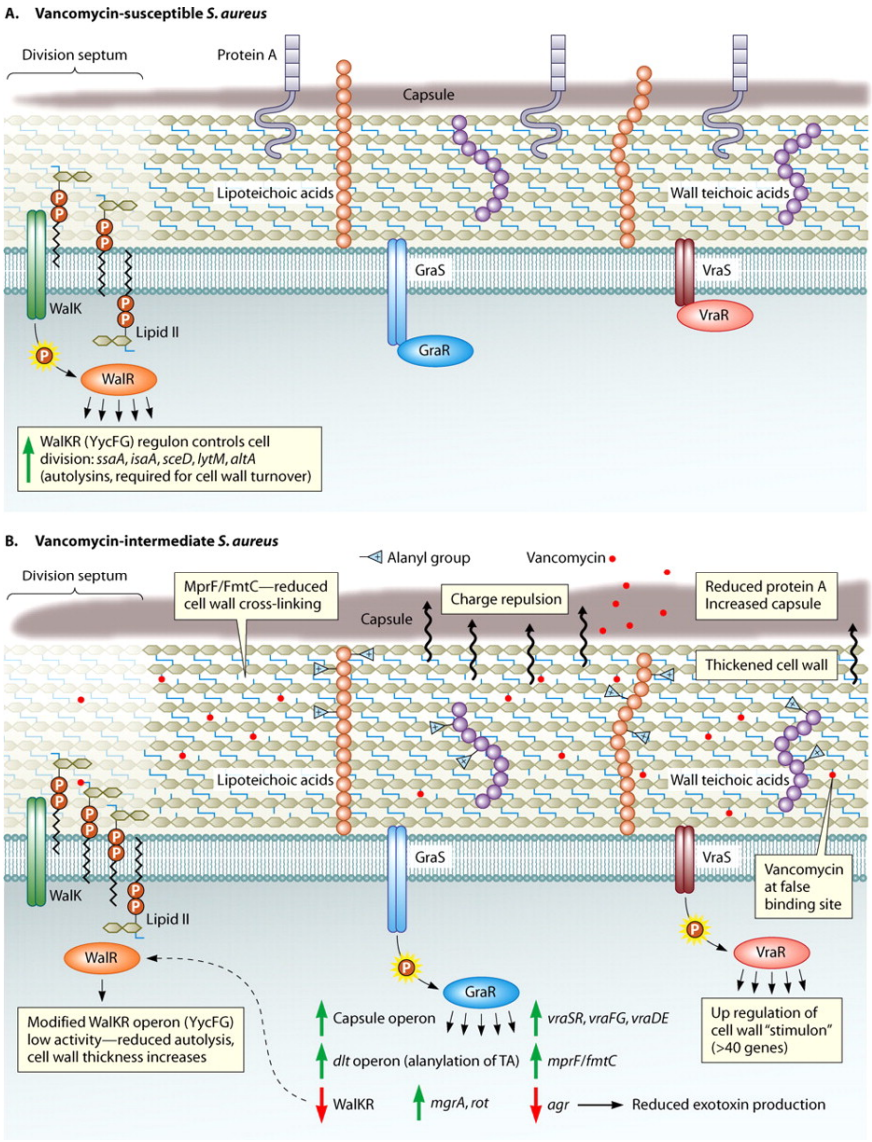


FIGURE 1.11: Mechanism of cell wall thickening
(Howden et. al. 2010)

Cui et. al. (2010) reported the role of a mutation of the *rpoB* gene at amino acid position A621E in development of hetero-resistance to daptomycin and vancomycin. A recent study reported that 71.0% of VISA clinical isolates carried mutations in RpoB in rifampin resistance determining region (RRDR) (Watanabe et. al. 2011). Moreover, mutations in RRDR region spanning amino acids 463-550 conferred resistance against both rifampin and vancomycin, whereas mutations identified in rifampin susceptible VISA strains were found to carry mutations outside the RRDR region of the RpoB (Watanabe et. al. 2011).

A recent study has demonstrated that point mutation in multiple peptide resistance factors, *mpr*F, a gene which encodes for the enzyme lysylphosphatidylglycerol transferase (LPGT) is associated with daptomycin and vancomycin non-suceptibility. This enzyme is known to alter the cell membrane charge by transfer of L-lysine from lysyl-tRNA to phosphatidylglycerol (Boyle-Vavra et. al. 2011). This study also indicated that there was no increase in cell wall thickness in daptomycin non-suceptible strains despite reduced susceptibility to both daptomycin and vancomycin (Boyle-Vavra et. al. 2011). A more recent report suggests that L826F *mprF* mutation accompanied with up-regulation of vraSR two component regulatory system (TCS) is involved in modulation of cell wall bio-sysnthesis (Mehta et. al. 2012). Another recent report indicates that mutation in *mprF* and *dlt* accompanied with mutation in *graR* and *vraS* results in altered surface-positive charge, this in turn contributes to reduced susceptibility to cationic antimicrobial peptides, including mammalian platelets, neutrophils and polymyxin B (Yang et. al. 2012). More importantly, up-regulation of *mprF* and *dlt* via *graRS*-

vraFG pathway was selective to a sub-lethal dose of platelets and polymyxin B, but not by other cationic agents such as vancomycin, daptomycin and gentamicin (Yang et. al. 2012) suggesting involvement of multiple and interconnected pathways in development of resistance against cationic agents.

Recently, Shoji et. al. (2011) demonstrated that mutations in walk and ClpP are responsible for reduced vancomycin susceptibility with mutation prevalence of 61.5% and 7.7% in walK and ClpP respectively.

Fifteen years after the first description of this mode of vancomycin reduced susceptibility development, the molecular mechanism still remains unclear and due to the lack of a universal genetic marker, no molecular method has been developed for determining hVISA/VISA, which would help in rapid detection of hVISA/VISA.

Reports suggest that two component-regulatory systems (TCS) are widely employed by bacteria to monitor cell wall stress. TCS utilize phospho-transfer cascades to alter the gene expression leading to reduced susceptibility against cell wall active agents by sensing cell wall damage and activating the transcription of enzymes and transporters associated with cell wall synthesis (West and Stock 2001) (see Figure 1.12).

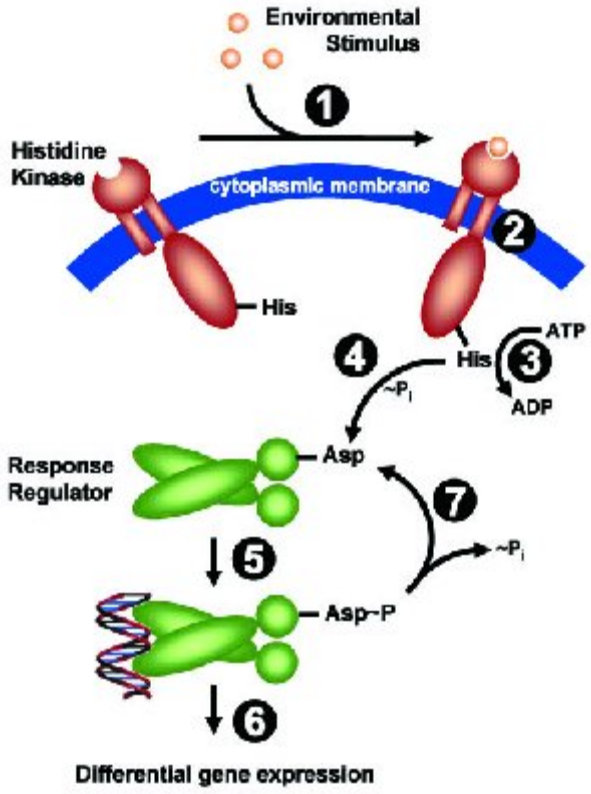


FIGURE 1.12: Schematic representation of two component regulatory systems (tcs) in response to stress
(Parkinson 1993)

Vancomycin being a cell wall active agent, the role of TCS towards reduced vancomycin susceptibility has been postulated and previous studies have identified TCS *vraSR* and *graSR* as glycopeptides resistance-associated regulatory genes (Kuroda et. al. 2003, Cui et. al. 2005) and mutation in these genes is known to modulate cell wall bio-synthesis (Howden et. al. 2008, Neoh et. al. 2008). The results of the studies to date are summarized in Table1.5

Cell wall thickening appears to be a consistent mechanism for vancomycin resistance, associated with the peptidoglycan clogging mechanism that prevents passage of vancomycin through the multilayer peptidoglycan (Cui et. al. 2000, Cui et. al. 2003, Cui et. al. 2006a, Cui et. al. 2006b, Hiramatsu et. al. 2001a, Hiramatsu 2001b). RpoB gene and TCS *vraSR* are known to play important roles in cell wall synthesis (Kuroda et. al. 2003, Cui et. al. 2010) and mutation outside the rifampin resistance determining region (RRDR) at 621 amino acid in RpoB gene is known to cause repression of genes associated with metabolic pathways of purine, pyrimidine, arginine, galactose, urea cycle and enhancement of biosynthetic pathway of vitamin B2, K1, and K2 and cell wall metabolism. It has been suggested that these changes result in up to 63% increase in cell wall thickness in comparison to the susceptible parent strains and decreased cell surface negative charge which reduces binding of vancomycin and daptomycin to the cell, correlating to the reduced susceptibility and contributing towards development of hetero-resistance to vancomycin and daptomycin (Cui et. al. 2010).

TABLE 1.5: Genes associated with hVISA/VISA

Gene, Mutation	Impact of mutation	Reference
graR, nucleotide substitution	Led to a change N197S in GraR, cloning of N197S in GraR of mu3 converted hVISA strain to VISA	Neoh et. al. 2008
graRS, DNA deletion	Deletion of *graR* led to increased susceptibility to vancomycin	Meehl et. al 2007
vraFG, DNA deletion	Deletion of *vraG* led to increased susceptibility to vancomycin	
graS, nucleotide substitution	Led to change T136I of in GraS, mutations detected in VISA strain JKD6008, Cloning of T136I of in GraS in VSSA strain JKD6009 resulted in an increase in the MET MIC from 2 to 6 mg/L	Howden et. al 2008
graRS, DNA deletion	Loss of GraRS function in VSSA JKD6009 and resulted in loss of resistance with decrease in the MET MIC from 2 to 1 mg/L	
vraS, premature stop codon	Loss of VraS function in Mu50 omega; replacement of disrupted with intact *vraS* from Mu50 resulted in an increase in MIC from 0.5 to 3.5 mg/L	Cui et. al. 2009
Intact *graR* with no change	Replacement of *vraS and graR* gene with of *vraS and graR* gene from Mu50 resulted in an increase in the vancomycin MIC from 0.5 to 6.0 mg/L	
walKR, IS*256* upstream insertion	Insertion of IS*256* led to up-regulation of *walKR and* increase in vancomycin resistance	Jansen et. al. 2007
mgrA and sarA, DNA deletion	SarA and MgrA are negative regulators of murein hydrolases or autolysins; these enzymes are required for cell wall turnover. Loss of SarA and MgrA function increased Triton X-100-induced autolysis and increased sensitivity to killing by vancomycin and oxacillin	Trotonda et. al. 2009
spoVG, deleted	Decreases resistance to oxacillin and teicoplanin, with less impact on vancomycin resistance loss of capsule production	Schulthess et. al. 2009
vraS, nucleotide substitution	Led to change a I5N in VraS, this mutation observed in Mu3 and Mu50 and not in VSSA isolates	Ohta et. al. 2004
mprF, Tn917 insertion	Insertion of Tn*917* led to MprF loss and decreased vancomycin resistance; (MprF is involved in synthesis of lysyl-phosphatidylglycerol)	Ruzin et. al. 2003
mprF, Tn551 insertion	Insertion of Tn*551* led to MprF loss and decreased vancomycin resistance in VISA strains but slightly increased vancomycin resistance in VSSA strains	Nishi et. al 2004

Continued in the following page

Table 1.5 Continued

Gene, Mutation	Impact of mutation	Reference
trfA/trfB, insertion inactivation	Led to an increase in susceptibility to teicoplanin, oxacillin, and vancomycin; function of TrfAB is still unknown	Renzoni et. al. 2009
ccpA, insertion inactivation	Reduces growth, carbon metabolism, RNAIII expression, and capsule synthesis; loss of CcpA also reduced resistance to teicoplanin; the effect of this mutation on vancomycin resistance is not reported	Seidl et. al. 2006
agr, insertion inactivation	Loss of Agr led to increased probability of hVISA when population was exposed to 1 mg/L vancomycin	Sakoulas et. al. 2002, Sakoulas et. al. 2005
rsbU, DNA deletion	RsbU is an anti-sigma factor; selection for teicoplanin resistance in an *rsbU* mutant resulted in GISA	Bischoff and Berger-Bachi 2001
pbp4, DNA deletion	Led to increased vancomycin resistance in VSSA, while overexpression of Pbp4 reduced vancomycin resistance in VISA (Pbp4 may be involved in transpeptidation, i.e., formation of peptidoglycan cross-linking)	Finan et. al. 2001
SA1702, nucleotide substitution	Led to a change H164R in SA1702 (SA1702 a protein of unknown role) and upstream of *vraS ,* strain isolated when MIC increased from 1 to 4 mg/L	Mwangi et. al. 2007
SA1249, frameshift	Loss-of-function in SA1249 (Role of SA1249 is unknown, its location suggests that it maybe part of the *murG* with a role in peptidoglycan synthesis) strain isolated when MIC increased from 4 to 6 mg/L	
agrC, frameshift	Loss of function in *agrC*; this gene is part of the *agr*quorum-sensing locus; this is one of six mutations affecting protein coding Sequences and the vancomycin MIC increased from 6 to 8 mg/L	
yycH, premature stop codon	loss of function in *yycH (* YycH is a hypothetical protein within an operon containing TCS WalKR that controls cell wall synthesis by expression of genes involved in autolysis) this is one of six mutations affecting protein coding sequences, the vancomycin MIC increased from 6 to 8 mg/L	
isdE, nucleotide substitution	Led to change IsdE, A84V; (IsdE involved in heme transport); this is one of six mutations affecting protein-coding sequences, the vancomycin MIC increased from 6 to 8 mg/L	
prsA, frameshift	loss of function in PrsA; (PrsA is a putative membrane-linked ribose-phosphate pyrophosphokinase that guides secreted proteins) this is one of six mutations affecting protein-coding sequences, the vancomycin MIC increased from 6 to 8 mg/L	
SA2094, nucleotide substitution	Led to a change A94T in SA2094,; (The role of SA2094 is predicted to be membrane associated) this is one of six mutations affecting protein-coding sequences, the vancomycin MIC increased from 6 to 8 mg/L	

Activation of *vraS* then phosphorylates *vraR,* converting it into its active form, this then up-regulates at least 50 genes encoding enzymes involved in the peptidoglycan synthesis pathway such as PBP1A, PBP1B, PBP2, *SgtB, FmtA, tacA/tacB, spsA, ctpA, prop, opuD, drp35, PTS, SA1255 and MurZ*. This overall change could occur thorough point mutations in *vraS* supporting development of hVISA (Kuroda et. al. 2003, Gardete et. al. 2006) and a mutation in *graR* may be essential for increment in the level of vancomycin resistance, formation of VISA, to up regulate other genes involved in cell wall thickening, glycopeptide resistance development and cross resistance against other cell wall acting antibiotics (Neoh et. al. 2008).

Although, Cui et. al. (2010) have strongly advocated the importance of changes in RpoB gene, the combined effect of mutated *vraS* and *graR* contributing to vancomycin resistance development (Cui et. al. 2009), studies on several resistant isolates are required to confirm this hypothesis and to find a universal genetic marker in all hVISA/VISA phenotypes. This could lead to development of a molecular detection method, which can help to establish proper treatment protocols for using cell wall active antibiotics and to reduce vancomycin resistance development.

hVISA / VISA prevalence

Since 1997, there has been a growing concern over vancomycin clinical failure and many countries have reported incidence of hVISA (Howden et. al. 2010) in patients receiving vancomycin therapy for prolonged periods. Reports suggest that there is considerable variability in the prevalence of vancomycin non-susceptible

phenotypes in clinical isolates (Howden et. al. 2010). The prevalence rates in isolates reported ranges from less than 1.0% to as high as 65.0% (Ariza et. al. 1999, Bierbaum et. al. 1999). However, more recent reports indicate prevalence level from 1.3% to 50% (Horne et. al. 2009, Adam et. al 2010, Howden et. al. 2010) as shown in Table 1.6.

It has been suggested that the problem of vancomycin non-susceptibility is increasing and that there has been a slow increase in the base levels of vancomycin resistance. This gradual increasing in vancomycin MICs over time is termed as "vancomycin creep" (Wang et. al. 2006, Rybak et. al. 2008). However, the evidence from the prevalence studies suggests that this phenomenon is not universal and regional difference in prevalence of these phenotypes or lack of standardized definition and detection method for accurate detection may have resulted in variations. Therefore, a standardized screening method is necessary as a part of the routine screening of clinical isolates at hospitals to determine presence of non-susceptibility, to allow comparison of rates both geographically and over time. This can allow better use of antimicrobial therapy, guide dosage regimens as well as helping to formulate infection control guidelines.

Although, the community level of MRSA colonization in Hong Kong is low (O'Donoghue and Boost 2004), high rates of MRSA infection in hospitals and colonization in elderly homes have been reported (Ip et. al. 2004, Ho et. al. 2007).

TABLE 1.6: The prevalence of hVISA in different countries

Country	Year	Sample size	Test methods	Prevalence of hVISA / VISA	Reference
Australia	2001-2002	#53	PAP-AUC	9.4%	Charles et. al. 2004
	2009	#117	MBD, MET, PAP-AUC, BHST4, BHBV2	50.0%	Horne et. al. 2009
Belgium	1999	*2145	MBD, PAP-AUC, BHAV6	0.18%	Denis et. al. 2002
Canada	1995-2006	#475	MET, GRD, PAP-AUC	1.3%	Adam et. al 2010
China	2005-2007	*1012	MET, PAP-AUC	13%-16%	Sun et. al. 2009
France	2003	*1070	BHAT6, GRD	0.7 %	Cartolano et. al. 2004
	2006	*2300	BHAT4, GRD, PAP-AUC	11.0% (7 strains MSSA)	Garnier et. al. 2006
Germany	1999	*101	BHAV4, MBD	2.9%	Bierbaum et. al. 1999
Hong Kong	1999	*112, #52	BHAV4, MBD, Etest,	5.8%	Wong et. al. 1999
Ireland	1999-2003	#3189	BHAT4, MET, PAP-AUC	2.6%	Fitzgibbon et. al. 2007
Israel	2003-2004	*264	Etest, MET	6.0%	Maor et. al. 2007
Italy	1999	#179	BHAV4, AD	1.1%	Marchese et. al. 2000
Japan	1997	#1149	BHAV4	1.3-20%	Hiramatsu et. al. 1997b
Korea	2001-2006	*4483	BHAV4, PAP-AUC	0.09%	Kim et. al. 2002
Netherlands	2003	*107, #250	MET, PAP-AUC	6.0%	Van Griethuysen et. al. 2003
Spain	1990-1997	#19	MHV4	65.0%	Ariza et. al. 1999
Thailand	2002	#533	PAP-AUC	0.8%	Lulitanond et. al. 2009
	2007	#361		3.0%	
Turkey	1998-2001	#256	BHAV4, MET	17.97%	Sancak et. al. 2005
USA	1986-1993	#225	MET, PAP-AUC	2.2%	Rybak et. al. 2008
	1994-2002	#356		7.6%	
	2003-2007	#917		8.3 %	

*_S. aureus,_ #MRSA, MBD=Micro broth dilution, MET=Macro Etest, PAP-AUC=Population analysis profile-area under the curve, BHAT4=BHA+teicoplanin 4 mg／L, BHST4=BHAT4+ 20% horse serum, BHBV2=BHA+vancomycin 2 mg/L+5% horse blood, BHAT6=BHA+ teicoplanin 6 mg／L, BHAV6=BHA+ vancomycin 6 mg／L, BHAV4=BHA+ vancomycin 4 mg／L, MBD=Micro broth dilution method, MHAV4= MHA+ vancomycin 4 mg／L

Vancomycin remains drug of choice for MRSA treatment in Hong Kong and consequently vancomycin non-susceptible phenotypes have been reported locally (Wong et. al 1999). Screening of blood cultures showed that 5.8% of isolates were hVISA/VISA suggesting vancomycin non-susceptible strains to be prevalent at a low level in Hong Kong (Wong et. al. 1999). However, the report was based on the screening of only MRSA strains obtained from blood culture and thus would not provide the accurate prevalence level of hVISA/VISA among *S. aureus* strains and the reliability of AST method used to detect the non-susceptibility is under question as the study screened the potential vancomycin resistance by performing the aztreonam (30mg) disk diffusion test using Mueller-Hinton agar supplemented with 4 mg/mL vancomycin and 4% NaCl and incubation condition 37°C for 48 h and isolates exhibiting heteroressitance in zone of inhibition was further confirmed by Etest and MBD. Also the study was performed about eleven years ago and current situation is unknown. Thus, there is need to investigate the prevalence of hVISA/VISA strains in Hong Kong.

Treatment

If there is failure of vancomycin in treatment of MRSA infection, there are very limited therapeutic options available (Mergenhagen and Pasko 2007). Currently, there is no formal recommendation for treatment of hVISA/VISA.

Surgery

As many hVISA/VISA cases are associated with high bacterial load and deep infections, surgical debridement can be an important adjunct to antimicrobial therapy. Howden et. al. (2004) have indicated that 60% of patients in an Australian

hospital were successfully treated by a combination of surgical debridement and antimicrobial therapy.

Linezolid

Reports have indicated that a number of serious cases of hVISA/ VISA infection were successfully treated by linezolid therapy (Howden et. al. 2004, Huang et. al. 2008). However, the key issue with linezolid is toxicity associated with extended therapy and needs to monitor the effects, though the incidence of adverse effect is similar to those of other competitor drugs (Jauregui et. al. 2005, Jaksic et. al. 2006).

Quinupristin-dalfopristin (QD)

Testing in i*n-vitro* models of infection has indicated that QD is effective against MRSA and has good *in-vitro* activity against hVISA/VISA strains. (Jevitt et. al. 2003). However, resistance to macrolide-lincosamide-streptogramin B (MLS_B) is common in strains harboring the *erm* gene which causes target site alteration of the ribosome (Arthur et. al. 1987). Erm resistance has replaced macrolides only in the US as strains with *erm* gene and MLS_B are common in the US (Arthur et. al. 1987, Howden et. al. 2010).

Rifampin and fusidic acid

Although, this combination is not available in US as fusidic acid is not approved in the US, but in Australia and other parts of the world this oral combination therapy is the mainstay of therapy in treatment of complication MRSA infections and this combination has been shown to be effective in the treatment of hVISA/VISA in patients who had failed to respond to vancomycin therapy (Howden et. al. 2004).

However due to the development of resistance if either of these drug is used in monotherapy it is suggested that they should be always used in combination *(Howden et. al. 2004).*

Daptomycin

Reports have indicated cross resistance between vancomycin and daptomycin, i.e vancomycin exposure can induce low-level daptomycin resistance (Sakoulas et. al. 2006, Rose et. al. 2008). However, this association has been found to be strain specific and unstable (Sakoulas et. al. 2006). An i*n-vitro* study suggested that daptomycin may a have low rate of activity against hVISA/VISA which may be due to its larger molecular weight resulting in poor permeability across the thick cell wall in vancomycin non-susceptible phenotypes (Leonardo et. al. 2009).

Tigecycline

Tigecycline has been found to have very good activity when tested against a small number of VISA isolates. However, more extensive investigation for anti-hVISA/VISA activity is needed (Huang et. al. 2008).

Ceftaroline

Ceftaroline has been reported to be effective against hIVSA/VISA/VRSA/daptomycin non-susceptible strains (Sarvolatz et. al 2010, Zhanell et. al. 2011). A study has demonstrated that MSSA, MRSA, hVISA/VISA did not develop resistance to this 5^{th} generation β-lactam and there was a lack of cross-resistance with other antibiotic classes following 50 passages in ceftaroline

(Clark et. al. 2011). These reports indicate that ceftaroline is a promising agent for the treatment of multi-drug resistant MRSA strains.

Old agents

A report indicates that the safety and efficacy of co-trimoxazole is comparable to that of vancomycin and may be considered as an alternative in treatment of MRSA infections (Goldberg et. al. 2010) and more recently about 70.0% of VISA strains have been reported to be susceptible to co-trimoxazole (Saravolatz et. al. 2012a) but further investigations are required to determine the role of co-trimoxazole in the treatment of hVISA/VISA. More recently, nybomycin is shown to be effective against *S. aureus* strains including hVISA/VISA (Hiramatsu et. al. 2012). Further investigations are required to determine the role of fosfomycin against hVISA/VISA as fosfomycin has been reported to improve the effectivenss of linezolid, minocycline, vancomycin and teicoplanin in treatment of catheter-related or prosthetic joint MRSA infections (Tang et. al. 2012). The same study has also indicated that these combinations are better than rifampin combination regimens.

New agents

Several new glycopeptides (lipoglycopeptides): dalbavancin, and oritavancin are in final stages of US FDA approval and telavancin has been approved for the treatment of complicated skin infections in adult patients (Sader et. al. 2005, Streit et. al. 2005, Corey et. al. 2009, Butler and Copper 2011). Telavancin has been reported to be more effective than vancomycin (Logman et. al. 2010, Rubinstein et. al. 2011) with cure rate of 82.0-91.0% against MRSA (Stryjewski et. al. 2008,

Rubinstein et. al. 2011) and oritavancin has been shown to be effective in treatment of complicated SSI caused by MRSA (Dunbar et. al. 2011). Additionaly, delafloxacin (fluoroquinolone), and EDP-420 (ketolide) are in final stage of US FDA approval. However, their superiority to vancomycin against MDR strains has not been determined nor their likelihood to induce resistance. Several other agents: AFN-1252 (FabI inhibitor new class), BC-3781 (pleuromutilin), finafloxacin (fluoroquinolone), GSK1322322 (Peptide deformylase inhibitor new class), LTX-109 (synthetic antimicrobial peptidomimetics), nemonoxacin (C8-methoxy non-fluorinated quinolone), omadacycline (novel tetracycline; aminomethylcycline) PMX-30063 (defensin-mimetics new class), solithromycin (fluoroketolide), TD-1792 (glycopeptide-cephalosporin hybrid), TP-434 (fluorocycline), torezolid, radezolid (oxazolidinone), and XF-73 (dicationic porphyrin new class) are in the development stage (Farrell et. al. 2010, Li et. al. 2010, Butler and Copper 2011, Coates et. al. 2011, Noel et. al. 2012, Saravolatz et. al. 2012b, clinicaltrials.gov). But new agents are expensive, have possible unknown side effects and may not be available for some time. The future role of potentially active and available antibiotics in treatment of hVISA/VISA infection is unknown as the antimicrobial drug development and approval is much slower in comparison to emergence and spread of resistant strains. In the last few decades, a large number of antimicrobials have been identified but very few agents of new class of agents have been introduced into the market (Zucca and Savoia 2010). Despite significant effort the result of antimicrobials clinical trials is frequently disappointing (Zucca and Savoia 2010). Several agents have been aborted at a late stage of clinical trials e.g cethromycin (ketolide), iraprim (dihydrofolate reductase selective inhibitor), and PTK0796 (tetracycline) (clinicaltrials.gov). Considering the aforementioned results

and the time required for a new antimicrobial to be available in the market, it is imperative to investigate other sources for new antimicrobials and also to extend the usefulness of current antimicrobial agents by combining them with other agents including plant extracts (Zucca and Savoia 2010).

Historically, natural products have been the source of antimicrobials and the search for new agents needs to be intensified in both natural and chemical potential sources to develop agents with novel mechanism of action. In this respect a range of natural products have been investigated for anti-microbial activity and *Acacia aromo* extract has been reported to have the anti-staphylocooi and anti-MRSA properties (Mattana et. al. 2010). Antimicrobial activities of some medicinal bark have indicated that extracts of *Naucleopsis glabra* exhibited strong activity against gram-positive bacteria (Kloucek et. al. 2007). Kuzma et. al. (2007) have reported the anti-MRSA, anti-hVISA and anti-biofilm property of Abietane diterpenoids from *Salvia sclarea.* Anti-MRSA activity of ellagic acid and syringic acid of *Quercus infectoria* has been reported and the mechanism of action of these compounds is based on their ability to produce cytoplasmic membrane damage (Chusri and Voravuthikunchai 2011).

Anti-microbial activities of dietary supplements including green tea, turmeric, and pomegranate have also been reported. Several studies have demonstrated the antimicrobial effects of tea. Anti-MRSA activity and synergistic effects of a combination of epigallocatechin gallate a major active ingredient from green tea and ampicillin / sulbactam have been demonstrated (Hu et. al. 2001). It is also reported that epigallocatechin gallate acts on peptidoglycan units of the cell wall

(Zhao et. al. 2001) and antimicrobial activity of epicatechin gallate has been shown to be associated with binding to the cytoplasmic membrane, thereby reducing the membrane fluidity and perturbating cell wall synthesis (Bernal et. al. 2011).

Combination therapy has been suggested to improve the clinical outcome, potency, spectrum of action, safety and reduce the possibility of resistance development, and recently several combinations of herbs with available antibiotics have been investigated. A study indicated synergistic effects of phenolic acids in combination with oxacillin including caffeic acid, ellagic acid, salicylic acid, and P-anisic acid, flavonides in combination with oxacillin including catechin, chrysin, epicatechin, hesperidin, hesperitin, rutin, and quercetin and alkolides in combination with oxacillin including berberine, piperine and aesculetin (Basri et. al. 2008).

Several Traditional Chinese Medicine (TCM) herbs have been demonstrated to be effective in treatment of various diseases, and they are very likely to possess phytochemicals of potential therapeutic value against infections. Their use in treatment of infections has been established for many years and several have been shown to have *in-vitro* antimicrobial activity (Jiang 2005). The therapeutic benefits of three TCM herbs, *Cortex Phellodendron, Rhizoma Coptidis* and *Radix scutellariae* used in treatment of infections have been reported. These include anti-cancer, anti-inflammatory, anti-microbial, resistance modulatory effects (Li et al. 2000, Liu 2001, Tian et. al. 2001, Li et al. 2006, Xu et al. 2010). These are commonly used in combination in TCM practice to treat various infections. However, these herbs have not been investigated for anti-MRSA, anti-VISA and / or resistance modulatory effects.

1.2 RESEARCH GAP

Until the first report of VISA in Japan, MRSA strains did not require vancomycin susceptibility test since MRSA were consistently susceptible to the empirical therapy. However, with the evidence of vancomycin resistance worldwide emperical therapy can no longer be used with out performing susceptibility test. CLSI recommended methods are standard types of AST - quantitative dilution and qualitative diffusion tests. Both depend on growth of test strains with overnight incubation. Moreover, neither of the recommended method is able to detect heterogenecity within the bacterial population and produces only an approximate value for MIC (Tenover et. al. 1998, Tenover et. al. 2001, Walsh et. al. 2001). Several reports have indicated that hVISA/VISA can emerge during the course of vancomycin therapy of MRSA infection (Soriano et. al. 2008, Howden et. al. 2010). Therefore there is need of a reliable and cost-effective hVISA/VISA detection technique to detect and track non-susceptibility before and during the course of treatment to achieve patient favorable outcome, and to determine the prevalence rate of non-susceptibility.

The evidence suggests that point mutations are important for vancomycin resistance development (Cui et. al. 2009, Kato et. al. 2010). However, the genetic determinants of hVISA/VISA have not been conclusively determined. Evidence also suggests that emergence of resistance against vancomycin has restricted the treatment options and according to the Infectious Diseases Society of America (IDSA 2010) at least ten antibiotics of novel class active against MDRO are required to reach the market within the next 10 years to avoid the running out of

antibiotics. Therefore, further work is required to identify and develop new antibiotics.

1.2.1 Detection of Vancomycin Non-Susceptible *S. aureus*

It is suggested that hVISA is precursor for VISA and standard methods cannot be used to detect hVISA as they grow slowly, may require supplemental nutrients or simply that there is need of special conditions for accurate detection. In addition, standard detection methods are labor intensive, lack ability to detect heterogenecity, suffer from inaccuracy due to larger increments and allowable error (Wexler et. al. 1990, Jorgensen et. al. 1994) and may need 48h of incubation (Fitzgibbon et. al. 2007) which may delay accurate therapy for the patient. As the treatment options for MRSA infection with reduced susceptibility to vancomycin are limited, it is essential to determine if a patient receiving vancomycin therapy is harbouring hVISA/VISA strains and thus may require a change in therapy for positive clinical outcome. Rapid and reliable non-susceptibility detection method allow physicians to prescribe more appropriate therapy thereby significantly lowers mortality rate and shorten hospital stay (Doern et. al. 1994, Barangfranger and Short 2001). Therefore, a more precise, cost-effective and reliable method of susceptibility testing is required. Accurate MIC determination is also important in controlling the dissemination and selection of non-susceptible strains. To attain this, a rapid, simple, and inexpensive hVISA/VISA detection method is necessary for epidemiological surveillance.

1.2.2 Mechanism of Vancomycin Non-Susceptibility in *S. aureus*

Several genetic targets have been investigated to understand the mechanism and these genetic targets have been associated with the changes mainly phenotypic, biosynthesis and metabolic pathway observed in hVISA/VISA strains. Several genes have been implicated with non-susceptibility development, but most work has been done on TCS which are known to regulate various genes associated with various pathways based on environmental stimuli. However, most studies have investigated a single set of iso-genic upon development and loss of non-susceptibility i.e. Mu3, Mu50, and Mu50Ω (Cui et. al. 2009) mainly of Japan origin and further work is needed to investigate the genetic changes in local isolates. More work is required on development and loss of non-susceptibility to identify important determinants associated with non-susceptibility and the time required for these mutations to occur, and to identify a consistent marker that confers resistance in all vancomycin non-susceptible phenotypes which may allow for development of a rapid molecular detection method.

1.2.3 Prevalence of Vancomycin Non-Susceptible *S. aureus* in Hong Kong

After the first report of vancomycin non-susceptible phenotypes in Japan, these phenotypes were reported from many countries including Hong Kong (Wong et. al. 1999). Although, 5.8% of blood cultures has been reported to VISA in Hong Kong hospital (Wong et. al. 1999), but the study was performed in 1999 and methods used for detection may not be accurate as the methods used are suitable for detection of VISA but fail to detect hVISA. Thus, there is need to investigate the

current prevalence rate of hVISA/VISA in Hong Kong hospital. Determining the prevalence of hVISA/VISA will help heighten awareness of these infections to the health department for implementation of appropriate control measures and possible treatment for elimination of carriage and infection.

1.2.4 Treatment of Vancomycin Non-Susceptible *S. aureus*

Rapid resistance development in *S. aureus* and limited treatment options for VISA infections are of global concern. Development of only two new anti-MRSA agents in last few decades suggests that antimicrobial drug development is not keeping pace with the emergence and spread of resistant phenotypes. This condition is promoting research for development of new antimicrobial agents with novel mechanism of action through natural products.

Although, several drug candidates are in pipeline, the prospective new agents may have possible unknown side effects, may be expensive and may not be available for some time. In order to improve treatment options, it may be possible to extend the usefulness of current antimicrobial agents by combining them with other agents including plant extracts. Few studies have reported synergistic effects of phytochemcials and antibiotics on VISA (Hu et. al. 2001). Currently, biochemicals from insects, humans, animals and plants are being investigated for anti-microbial agents (Zucca and Savoia 2010). Numerous plants have been reported to be effective in treatment of various infections. TCM plants used in treatment of various infections are very likely to possess phytochemicals effective against VISA/hVISA.

The aim and objectives as set out in the succeeding chapter were formulated to address the above mentioned research gaps.

CHAPTER 2: RESEARCH OBJECTIVES

2.1 AIM

The aim of this research study was structured in perspective to the above mentioned research gaps, to provide significant and original contributions to the knowledge base of vancomycin non-susceptibility in *S. aureus*. Specifically, the aim of this research is to provide a framework for effective detection, appropriate antimicrobial selection and effective dosage regimen in treatment of MRSA and hVISA/VISA, as well as to improve the clinical outcomes and infection control guidelines. In achieving the above aim, the proposed research has the following objectives:

1. Evaluation of the spiral gradient endpoint technique as a detection tool for hVISA and VISA.
2. Tracking development and loss of vancomycin non-susceptibility with genotypic and phenotypic evidence.
3. Determining the prevalence rate of hVISA/VISA among clinical MRSA isolates from a local hospital.
4. To study the anti-MRSA and anti-VISA effect of selected TCM herbs alone and in combination with vancomycin.

2.2 SIGNIFICANCE OF THE RESEARCH

Since the first report of VISA in 1997, efforts have been made to understand the mechanism and studies on control strains suggest that mutations in *VraS/GraR* and *RpoB* are involved in the development of VISA. However, more work is required to investigate changes during development and loss of non-susceptibility to identify a consistent marker that confers resistance in all VISA phenotypes to allow for development of a rapid molecular detection method.

Considering the high prevalence of MRSA in Hong Kong and continued use of vancomycin as a preferred drug in treatment of MRSA infection, there is likely to be considerable prevalence of VISA in Hong Kong. There have been case reports of VISA, but the current level of VISA or hVISA among clinical *S. aureus* isolates has not been investigated in local hospitals. In order to accurately identify the prevalence of vancomycin non-susceptible *S. aureus*, there is an urgent need for a simple, reliable and inexpensive method for VISA detection, as well as an alternative treatment to extend the usefulness of current antibiotics, if treatment failure with vancomycin as to avoided. Achieving the above objectives of this research work can help to address the concerns as listed below.

❖ Development of a simple, rapid, reliable and economical detection method will provide an alternative to available CLSI methods and this will help in providing more rapid appropriate therapy to the patients, and determine clinical prevalence of these phenotypes.

❖ Development and loss of non-susceptibility study will further help understand the mechanism and support the design of dosage regimen for vancomycin in treatment of MRSA to help prevent development of hVISA/VISA.

❖ Determination of VISA / hVISA local prevalence among MRSA clinical isolates will help to formulate strategies in antibiotic therapy and infection control.

❖ Knowledge of prevalence levels aids management of healthcare and infection control in the local population, thereby reducing health care expenses.

❖ Investigation of effects of TCM herbs may help in development of therapies useful in combating further resistance development in *S. aureus*.

❖ The combination of effective herb and antibiotics may be useful as an alternative treatment in treatment for *S. aureus* infection.

❖ Improved knowledge of hVISA/VISA can help guide evidence based planning for prevention and control of resistance development.

CHAPTER 3: EVALUATION OF SPIRAL GRADIENT ENDPOINT TECHNIQUE FOR RAPID DETECTION OF VANCOMYCIN NON-SUSCEPTIBLE *STAPHYLOCCOUS AUREUS*

3.1 INTRODUCTION

Vancomycin has been widely used for almost five decades for treatment of MRSA infection as these organisms are resistant to all β-lactam antibiotics. High rates of vancomycin use has paved the way for emergence of strains with various degrees of non-susceptibility including a limited number of highly resistant strains (VRSA) and the more common hVISA (MIC >2-<4mg/L with or without resistant sub-population and VISA (MIC >4mg/L). Reduced vancomycin susceptibility has been associated with sub-inhibitory doses, prolonged exposure to vancomycin, and inability to accurately detect hVISA isolates in clinical settings, resulting in vancomycin clinical failure and sometimes poor clinical outcome (Sakoulas et. al 2006, Rose et. al. 2008, Howden et. al. 2010).

Since the first report of VISA, the rapid and correct detection of vancomycin non-susceptibility has been a global concern, and several detection methods have been developed. However, there is still a need for a simple, cost-effective and reliable method for detecting vancomycin non-susceptible phenotypes. In particular, hVISA detection is difficult as most of the conventional methods fail to accurately identify these phenotypes while reliable methods with higher sensitivity and

specificity are labor-intensive and require 48h of incubation. GRD Etest incorporating vancomcyin and teicoplanin test strips is reported to have both high specificity and sensitivity, but is expensive and requires 48h incubation for good sensitivity (Yusof et. al. 2008, Leonard et. al. 2009). PAP-AUC is the gold standard method for detection of hVISA, in which the area under the curve (AUC) is calculated for each isolate and compared to that of the original hVISA isolate Mu3 (Wootton et. al. 2001). Although, this method is reliable, it is time consuming and labor-intensive, thus unsuitable for routine screening in clinical laboratories.

Currently, CLSI recommends a combination of PAP with an MIC method (CLSI 2012). Performing a combination of methods is time-consuming, labor-intensive and open to error in interpretation of results and may discourage preemptive vancomycin MIC determination before treatment begins.

Single point population analysis using 6mg/L vancomycin supplemented BHA may miss phenotypes with a lower degree of non-susceptibility and therefore has a low level of sensitivity and specificity (Voss et. al. 2007, Wootton et. al. 2007). Five mg/L teicoplanin supplemented agar has been reported to have better specificity, but requires 48h of incubation (Fitzgibbon et. al. 2007). Other variations of the PAP method including BHA supplemented with 5% blood or 20% serum (Horne et. al. 2000, Howden et. al. 2004), BHA supplemented with 4mg/L vancomycin and 4% NaCl *(Jung et. al. 2002),* low level of vancomycin supplementation (3 g/L) with (Burnham et. al. 2010) and without non-inhibitory dye (Kosowska-Shick et. al. 2008) are all reported to have low levels of specificity.

Dilution methods have limitations and lack the ability to detect strains with resistant sub-populations (Prakash et. al. 2008, Swenson et. al. 2009). Whilst, automated methods are rapid they are also poor predictors of non-susceptibility (Swenson et. al. 2009). The limitations associated with the above detection methods have restricted ability to determine the exact proportion of MRSA showing reduced vancomycin susceptibility. Nevertheless, it is important to determine the prevalence of vancomycin non-susceptible MRSA to prevent further resistance development and to establish infection control guidelines. To achieve this, there is a need for a simple, rapid, reliable, and inexpensive detection method, especially for the difficult to detect hVISA phenotypes.

Although, SGE has been reported to be reproducible for the accurate MIC determination of both aerobic and anaerobic organisms (Hill 1990, James 1990, Paton 1990, James 1991, Wexler 1991), this technique did not gain wide acceptance, possibly due to the complex calculation involved in MIC determination and introduction of E-test and automated methods. A recent report incorporated improvements in the spiral plater and availability of software for calculation of drug stock solution concentration and MIC. This report also indicated that SGE is suitable for determining MIC of fastidious organisms (Pong et. al 2010) but there appears to be no report on evaluating its use for the detection of hVISA/VISA.

The absence of a cost-effective, rapid and reliable method for detection of hVISA / VISA coupled with developments to SGE and considering the principle on which SGE is based, it was hypothesized that SGE would be effective in detection of

resistant sub-populations. Therefore, this work aimed to evaluate SGE for rapid detection of hVISA/VISA in comparison to AD which is the gold standard for MIC determination and is a CLSI recommended method for MIC determination (CLSI 2010).

3.2 EXPERIMENTAL DESIGN

3.2.1 Strains

A total of thirty strains, including three control strains [MSSA: NRS149; MRSA: NRS100 and VISA: NRS1], two clinical MRSA isolates, two clinical hVISA strains, one clinical VISA strain, and laboratory induced strains (5 hVISA and 17 VISA), were used in this study. VSSA and VISA were defined based on CLSI criteria; whereas, hVISA were defined as strains having an MIC >2 - <4 mg/L and displaying a resistant sub-population. Bacterial suspensions were prepared by incubating the strains at 37^0C for 2h in BHI broth and adjusted to a turbidity equivalent to that of a 0.5 McFarland.

3.2.2 Storage of Isolates

Isolates were sub-cultured onto BHI agar plates and incubated overnight at 37^0C. After assessing their purity, single colony was inoculated into BHI broth in eppendorf tube incubated for 3h at 37^0C, followed by addition of 20% glycerol (v/ v) and storage at -80^0C.

3.2.3 Preparation of vancomycin stock solutions

Vancomycin (Sigma-Aldrich, St Louis, MO, USA) stock solutions for both AD and SGE were prepared in milli-Q water and sterilized by filtration (Opticap XL 10 Capsule Filters, Millipore). The stock solution was prepared using the following formula:

$$1000 \times V \times C / P = Wt \quad \text{……. (Equation 3.1)}$$

P= Potency given by the manufacturer.

V = Volume in ml required

C = Final concentration (multiples of 1000)

Wt = Weight of antibiotic in mg

The vancomycin concentration required for SGE to produce the exponential concentration gradients (0.5-8mg/L and 2.5-20mg/L) was calculated using the SGE software (Spiral Biotech, Norwood, MA, USA). Stock solutions of 1072 mg/L (low range) and 2452 mg/L (high range) were prepared and 1ml aliquots of stock solutions stored in 1.5ml Eppendorf tubes at -80^0C. The vancomycin stock solution of 800mg/L for AD was calculated using equation 3.1 and used to prepare vancomycin supplemented plates as shown in Table 3.1.

TABLE 3.1: Media preparation for agar dilution method

Vol. of vancomycin stock solution of 800 mg/L (µl)	Vol. of BHA (ml)	Final conc. (mg/L)
0.0	20.0	0.0
12.5	19.9875	0.5
25	19.9750	1.0
50	19.9500	2.0
75	19.9250	3.0
100	19.9000	4.0
125	19.8750	5.0
150	19.8500	6.0
175	19.8250	7.0
200	19.8000	8.0
225	19.7750	9.0
250	19.7500	10.0
275	19.7250	11.0
300	19.7000	12.0
325	19.6750	13.0
350	19.6500	14.0
375	19.6250	15.0
400	19.6000	16.0
425	19.5750	17.0
450	19.5500	18.0
475	19.5250	19.0
500	19.5000	20.0

3.2.4 Media Preparation for Spiral Gradient Endpoint

Three media MHA, BHA (Oxoid, Basingstoke, UK) and Glucose BHA (GBH) were evaluated. GBH was prepared by adding 5.0% D-Glucose anhydrous (Riedel-de Haen, Germany) to BHA and was prepared by dispensing 20ml of GBH into a universal bottle, which was sterilized and plated manually into a 10cm plate. BHA and MHA were prepared as per the manufacturer's instructions and agar plates of 20.0ml were prepared using media clave and Technomat line (INTEGRA Biosciences Holding, Switzerland). Necessary care was taken to produce plates of uniform level

3.2.5 Media Preparation for Agar Dilution

A calculated volume of stock solution was added to sterile BHA media (table 3.1) at 50-60^{0}C and poured into 10cm plates. In order to cover a more complete range of MICs, dilutions at 1mg/L increments were used rather than the standard agar dilution method which suffers from large increments.

3.2.6 Performance of Spiral Gradient Endpoint

Using a spiral plater (Auto plate 4000-Spiral Biotech) 50µl of stock solution was deposited onto a 10cm agar plate in a spiral pattern to produce an exponential concentration gradient. After one hour, suspensions of three strains were swabbed in duplicate on each plate across the spiral to expose them to the concentration gradient and the plates were incubated at 37^{0}C for 24h, as these conditions have been previously shown to be optimal (Pong et. al. 2010).

3.2.7 Performance of Agar Dilution

Using a multi-point inoculator (Mast Co. Ltd., Bootle, UK), 0.5 McFarland bacterial suspensions were deposited onto BHA plates containing vancomycin 0-19 mg/L at 1 mg/L increments which were incubated at 37^0C for 24h. The MIC was determined by presence of growth on the screening plates. BHA was used rather than MHA, as it is recommended by CLSI for detection of hVISA/VISA and has been used in several detection methods with better performance (Voss et. al. 2007, Wootton et. al. 2007), as possibly the additional glucose facilitates optimal growth of vancomycin non-susceptible *S. aureus.*

3.2.8 Reproducibility

In order to determine reproducibility, MIC determination was performed on eight test strains (2 VSSA, 2 hVISA, 2 VISA, 2 highly resistant VISA) that were chosen to include all susceptibility categories from the thirty strains. Intra-batch reproducibility was determined by both SGE and AD on eight batches for eight days. For inter-batch reproducibility MICs were determined by both methods on a batch for eight days on eight test strains; and to observe the inter-observer reproducibility both intra-batch and inter-batch SGE plates were used and the results interpreted by three observers (one experienced and two fresh personnel-inexperienced in using SGE).

3.2.9 Evaluation

SGE was evaluated for correlation of MIC results with those obtained by AD by testing 30 strains as described above. As mentioned above stock solutions of 1072 mg/L L (low level) and 2452 mg/L (high level) were used to produce SGE MIC ranges of 0.5-8 mg/ and 2.5-20 mg/L respectively. Each strain tested by SGE and AD, differences between the results obtained by both the methods were recorded and analyzed for agreement.

3.2.10 Reading and Interpretation of Results of Spiral Graident Endpoint

MIC was determined by measuring the radial advance (RA) i.e the distance between the confluent growth end point (EP) and the commencement of antibiotic deposition by using an SGE template (Figure 3.1a and 3.1b). The concentration of drug at EP as determined using SGE software program serves to compute the MIC. The software provided from the manufacturer of the spiral plater allows the determination of end point concentration at EP (EC). In addition the trailing end point concentration (TEC) was determined. The trailing end point (TP) is the area of the growth that extends beyond confluent growth and includes the outlying colonies. Determining TEC is suitable for identification of hVISA which displays isolated colonies growing at higher concentrations of vancomycin, whereas EC is representative of the overall MIC and was used in detection of VISA in comparison with AD.

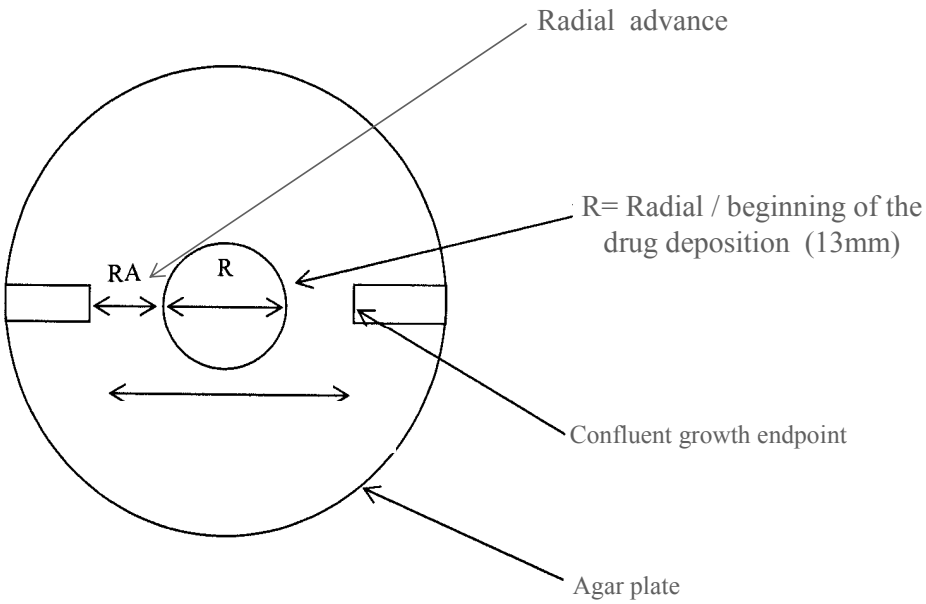


FIGURE 3.1a: Diagrammatic representation of MIC measurement

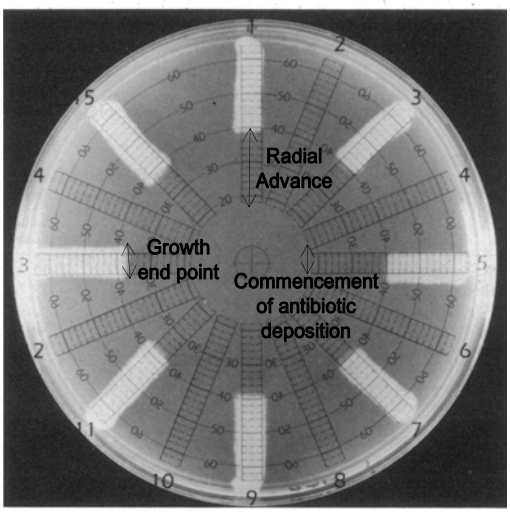


FIGURE 3.1b: Representation of MIC measurement on SGE template

3.2.11 Statistical Analysis

In order to determine the degree of agreement between AD and SGE, results were compared and statistical analysis was performed. Agreement between the two methods was assessed by preparation of a Bland and Altman plot and by determining the distribution of differences in MIC results obtained by AD and SGE and the percentage of isolates yielding results within one dilution. Differences in proportions of isolates in each susceptibility category were determined by the χ^2 test. Regression analysis and Pearson correlation coefficients were calculated to determine association between MICs by SGE and AD. The trend of MICs obtained by SGE was determined by Wilcoxon signed rank test.

3.3 RESULTS

Three growth patterns were displayed by SGE and were used to determine susceptibility. A clear endpoint or minimal growth below the breakpoint MIC (2 mg/L) was defined as VSSA. Strains exhibiting confluent growth above the breakpoint (4 mg/L) with or without a trailing endpoint were defined as VISA and strains with MIC between 2–4 mg/L and / or a trailing endpoint were categorized as hVISA (see Figure 3.2).

3.3.1 Reproducibility of SGE test

Mueller Hinton Agar

The results of the intra-batch, inter-batch and inter-observer reproducibility are as summarized in Tables 3.2a – 3.2c, with full details shown in appendix. The standard deviation (SD) ranged between 0 - 1.1223 and 0 - 1.16 for intra-batch and inter-batch respectively. The % coefficient of variation (CV) ranged between 0 - 22.06 and 0 - 31.4 for intra-batch and inter-batch respectively. For inter-observer variation, the SD ranged between 0 - 0.57 and 0 – 1.54 for intra-batch and inter-batch respectively. The % CV ranged between 0-34.0 and 0-173.20 for intra-batch and inter-batch respectively.

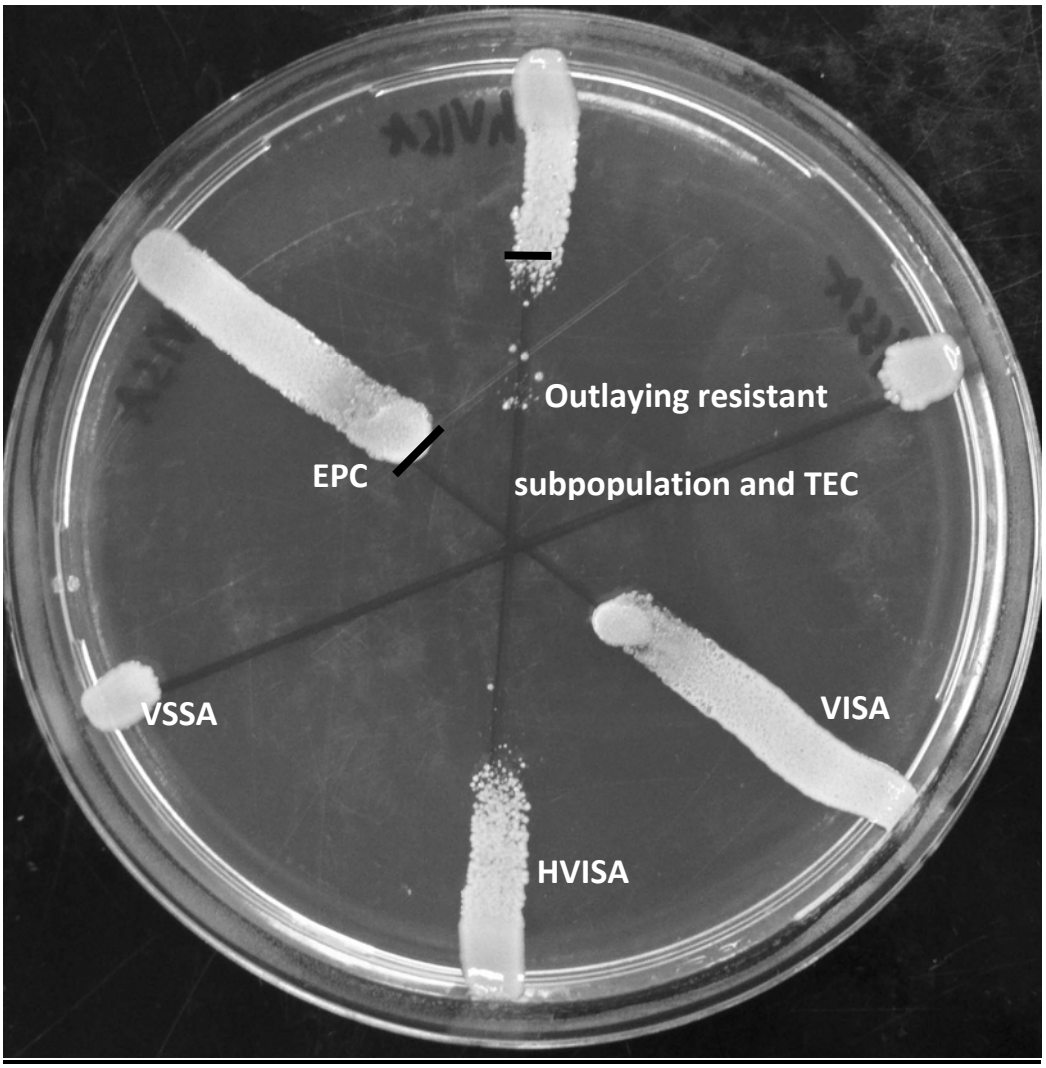


FIGURE 3.2: Detection of vancomycin-non-susceptible *S. aureus* by SGE

SGE is effective in detecting both VISA and hVISA, as the clear confluent growth endpoint provides endpoint concentration (EPC = MIC), while its ability to detect the resistant sub-population (hVISA)

TABLE 3.2a: Mueller Hinton Agar inter-batch and intra-batch data

MHA- Intra- batch											
Day	**Stats.**	**NRS 70**	**B25**	**h20 (EC)**	**h 20 (TEC)**	**h40 (EC)**	**h 40 (TEC)**	**V3**	**NRS1**	**E23**	**E32**
1 (n=8)	**Mean**	1	1	2.494	3.886	3.206	4.69225	4.67	5.29	13.17	10.253
	SD	0	0	0	0.8547	0	0.9538	0	0	0	0
	CV %	0	0	0	21.994	0	20.3289	0	0	0	0
2 (n=8)	**Mean**	1	1	2.494	3.786	2.828	5.001375	4.12	5.29	14.938	10.253
	SD	0	0	0	0.5767	0	1.0810	0	0	0	0
	CV %	0	0	0	15.2325	0	21.6150	0	0	0	0
3 (n=8)	**Mean**	1	1	2.494	3.5557	2.828	4.7565	4.12	6.002	14.938	10.253
	SD	0	0	0	0.5308	0	0.9322	0	0	0	0
	CV %	0	0	0	15.1833	0	19.6003	0	0	0	0
4 (n=8)	**Mean**	1	1	2.828	4.0385	3.206	4.99575	4.12	5.29	11.623	10.253
	SD	0	0	0	0.6721	0	1.1223	0	0	0	0
	CV %	0	0	0	16.6428	0	22.0653	0	0	0	0
5 (n=8)	**Mean**	1	1	2.828	4.338	2.828	4.53	4.12	5.29	11.623	14.938
	SD	0	0	0	0.9208	0	0.8620	0	0	0	0
	CV %	0	0	0	21.2251	0	19.0306	0	0	0	0
6 (n=8)	**Mean**	1	1	2.494	4.8135	3.206	4.764	4.12	4.67	10.253	14.938
	SD	0	0	0	0.8820	0	0.9501	0	0	0	0
	CV %	0	0	0	18.3250	0	19.9441	0	0	0	0
7 (n=8)	**Mean**	1	1	2.828	4.4651	3.206	4.671	4.67	6.804	11.623	13.17
	SD	0	0	0	0.8964	0	0.8694	0	0	0	0
	CV %	0	0	0	20.0775	0	18.6132	0	0	0	0
8 (n=8)	**Mean**	1	1	2.494	4.3345	2.828	4.7245	4.12	4.67	14.938	10.253
	SD	0	0	0	0.9375	0	0.7743	0	0	0	0
	CV %	0	0	0	21.6299	0	16.3905	0	0	0	0
MHA- Inter- batch											
1-8	**Mean**	1	1	2.760	4.436	2.556	4.179	4.55	6.520	10.960	9.516
	SD	0	0	0.3519	1.3931	0.3700	1.1609	0.515	0.63397	1.0864	1.01698
	%CV	0	0	12.7475	31.4012	14.4748	27.7756	11.34	9.7227	9.9126	10.6869

TABLE 3.2b: Mueller Hinton Agar inter-observer (Intra-batch) data

Observer / Strain No	1 Overall Mean (n=8) (SD/CV)	2 Overall Mean (n=8) (SD/CV)	3 Overall Mean (n=8) (SD/CV)	Inter-observer (n=8) (SD range / CV range)
NRS70	1 (0/0)	1 (0/0)	1 (0/0)	(0/0)
B25	1 (0/0)	1.01 (0.12/12.30)	1.01 (0.12/12.30)	(0- 0.57 / 0-34.0)
h20 (EC)	2.62 (0.16/6.22)	2.62 (0.16/6.22)	2.62 (0.16/6.22)	(0/ 0)
h20 (TEC)	4.15 (0.8/20.40)	4.15 (0.8/20.40)	4.15 (0.8/20.40)	(0/ 0)
h40 (EC)	3.01 (0.19/6.31)	3.01 (0.19/6.31)	3.01 (0.19/6.31)	(0/ 0)
h40 (TEC)	4.76 (0.90/19.04)	4.76 (0.90/19.04)	4.76 (0.90/19.04)	(0/ 0)
V3	4.25 (0.24/5.63)	4.25 (0.24/5.63)	4.25 (0.24/5.63)	(0/0)
NRS1	5.41 (0.66/12.22)	5.41 (0.66/12.22)	5.41 (0.66/12.22)	(0/0)
E23	12.88 (1.76/13.66)	12.88 (1.76/13.66)	12.88 (1.76/13.66)	(0/0)
E32	11.78 (2.06/17.50)	11.78 (2.06/17.50)	11.78 (2.06/17.50)	(0/0)

TABLE 3.2c: Mueller Hinton Agar inter-observer (Inter-batch) data

Observer / Strain No	1 Overall Mean (n=8) (SD/CV)	2 Overall Mean (n=8) (SD/CV)	3 Overall Mean (n=8) (SD/CV)	Inter-observer (n=8) (SD range / CV range)
NRS70	1 (0/0)	1 (0/0)	1 (0/0)	(0/0)
B25	1 (0/0)	1 (0/0)	1 (0/0)	(0/0)
h20 (EC)	2.762 (0.35/12.74)	2.762 (0.35/12.74)	2.762 (0.35/12.74)	(0/0)
h20 (TEC)	4.43 (1.39/31.40)	4.43 (1.39/31.40)	4.77 (1.61/33.78)	(0-1.54 / 0-28.37)
h40 (EC)	2.55 (0.37/14.47)	2.28 (0.98/43.08)	2.28 (0.98/43.08)	(0-0.27 / 0-173.20)
h40 (TEC)	4.17 (1.16/22.77)	4.17 (1.16/22.77)	4.17 (1.16/22.77)	(0/0)
V3	4.55 (0.51/11.34)	4.55 (0.51/11.34)	4.55 (0.51/11.34)	(0/0)
NRS1	6.52 (0.63/9.722)	6.52 (0.63/9.722)	6.52 (0.63/9.722)	(0/0)
E23	10.96 (1.08/9.91)	10.96 (1.08/9.91)	10.96 (1.08/9.91)	(0/0)
E32	9.51 (1.01/10.7)	9.51 (1.01/10.7)	9.51 (1.01/10.7)	(0/0)

Brain Heart Infusion Agar

The results of the intra-batch, inter-batch and inter-observer as summarized in Tables 3.3a – 3.3c, with full details shown in appendix. The standard deviation (SD) ranged between 0 – 0.3712 and 0 – 0.5295 for intra-batch and inter-batch respectively. The % coefficient of variation (CV) ranged between 0 – 9.57 and 0 – 8.0464 for intra-batch and inter-batch respectively. For inter-observer the SD ranged between 0 - 0.58 and 0 – 0.58 for intra-batch and inter-batch respectively. The % CV ranged between 0-10.81 and 0-13.3 for intra-batch and inter-batch respectively.

Glucose Brain Heart Infusion Agar

The results of the intra-batch, inter-batch and inter-observer as summarized in Tables 3.4a – 3.4d, with full details shown in appendix. The standard deviation (SD) ranged between 0 – 5.5608 and 0 – 4.1585 for intra-batch and inter-batch respectively. The % coefficient of variation (CV) ranged between 0 – 44.9458 and 40.4329 – 151.4875 for intra-batch and inter-batch respectively. For inter-observer the SD ranged between 0 - 0.57 and 0 – 1.73 for intra-batch and inter-batch respectively. The % CV ranged between 0-17.3 and 0-24.790 for intra-batch and inter-batch respectively.

TABLE 3.3a: Brain Heart Infusion Agar inter-batch and intra-batch data

BHA- Intra- batch											
Day	**Stats.**	**NRS 70**	**B25**	**h20 (EC)**	**h 20 (TEC)**	**h40 (EC)**	**h 40 (TEC)**	**V3**	**NRS1**	**E23**	**E32**
1 (n=8)	**Mean**	1	1	2.828	4.61	2.828	6.10225	4.67	6.804	17	17
	SD	0	0	0	0.3704	0	0.2835	0	0	0	0
	CV %	0	0	0	8.0356	0	4.6466	0	0	0	0
2 (n=8)	**Mean**	1	1	2.828	4.6012	2.828	6.9177	4.67	6.804	17	17
	SD	0	0	0	0.1944	0	0.3217	0	0	0	0
	CV %	0	0	0	4.2261	0	4.6508	0	0	0	0
3 (n=8)	**Mean**	1	1	2.828	4.0555	2.828	6.6035	4.67	6.9252	17	17
	SD	0	0	0	0.1824	0	0.3712	0	0.3429	0	0
	CV %	0	0	0	4.4984	0	5.6220	0	4.9521	0	0
4 (n=8)	**Mean**	1	1	2.828	4.06725	2.828	3.8235	4.7481	6.002	17	17
	SD	0	0	0	0.3279	0	0.3465	0.220	0	0	0
	CV %	0	0	0	8.0619	0	9.0637	4.653	0	0	0
5 (n=8)	**Mean**	1	1	2.828	4.1887	2.828	3.9468	4.67	6.804	17	14.938
	SD	0	0	0	0.1944	0	0.3777	0	0	0	0
	CV %	0	0	0	4.6423	0	9.5707	0	0	0	0
6 (n=8)	**Mean**	1	1	2.7495	4.6787	2.828	4.6787	4.67	6.804	17	14.938
	SD	0	0	0.2220	0.3131	0	0.3131	0	0	0	0
	CV %	0	0	8.0753	6.6922	0	6.6922	0	0	0	0
7 (n=8)	**Mean**	1	1	2.828	4.6787	2.828	4.61	4.67	6.804	17	17
	SD	0	0	0	0.3131	0	0.3704	0	0	0	0
	CV %	0	0	0	6.6922	0	8.0356	0	0	0	0
8 (n=8)	**Mean**	1	1	2.828	5.135	2.828	5.824	4.67	6.002	17	17
	SD	0	0	0	0.2870	0	0.3295	0	0	0	0
	CV %	0	0	0	5.5891	0	5.6592	0	0	0	0
BHA- Inter- batch											
1-8	**Mean**	1	1	2.661	4.9025	3.017	7.7291	4.98	6.1135	17	17
	SD	0	0	0.1785	0.3208	0.2020	0.5295	0.331	0.4919	0	0
	%CV	0	0	6.7091	6.5452	6.6970	6.7214	6.654	8.0464	0	0

TABLE 3.3b: Brain Heart Infusion Agar (Intra-batch) data

Observer / Strain No	1 Overall Mean (n=8) (SD/CV)	2 Overall Mean (n=8) (SD/CV)	3 Overall Mean (n=8) (SD/CV)	Inter-observer (n=8) (SD range / CV range)
NRS70	1 (0/0)	1 (0/0)	1 (0/0)	(0/0)
B25	1 (0/0)	1 (0/0)	1 (0/0)	(0/0)
h20 (EC)	2.818 (0.078/2.78)	2.818 (0.078/2.78)	2.818 (0.078/2.78)	(0/0)
h20 (TEC)	4.53 (0.45/10.03)	4.52 (0.45/10.10)	4.53 (0.47/10.4)	(0-0.46 / 0-10.81)
h40 (EC)	2.828 (0/0)	2.828 (0/0)	2.828 (0/0)	(0/0)
h40 (TEC)	5.31 (1.17/22.16)	5.33 (1.17/22)	5.28 (1.20/22.7)	(0-0.58 / 0-9.37)
V3	4.68 (0.07/1.67)	4.68 (0.07/1.67)	4.68 (0.07/1.67)	(0/0)
NRS1	6.61 (0.37/5.72)	6.61 (0.37/5.72)	6.61 (0.37/5.72)	(0/0)
E23	17 (0/0)	17 (0/0)	17 (0/0)	(0/0)
E32	16.484 (0.899/5.46)	16.484 (0.899/5.46)	16.456 (0.972/5.908)	(0/0)

TABLE 3.3c: Brain Heart Infusion Agar inter-observer (Inter-batch) data

Observer / Strain No	1 Overall Mean (SD/CV)	2 Overall Mean (SD/CV)	3 Overall Mean (SD/CV)	Inter-observer (SD range / CV range)
NRS70	1 (0/0)	1 (0/0)	1 (0/0)	(0/0)
B25	1 (0/0)	1 (0/0)	1 (0/0)	(0/0)
h20 (EC)	2.66 (0.18/6.7)	2.66 (0.18/6.7)	2.585 (0.22/8.56)	(0-0.35 / 0-13.3)
h20 (TEC)	4.90 (0.32/6.545)	4.83 (0.42/8.71)	4.98 (0.33/6.65)	(0-0.58 / 0-12.47)
h40 (EC)	3.01 (0.20/6.7)	3.01 (0.20/6.7)	3.01 (0.20/6.7)	(0/0)
h40 (TEC)	7.729 (0.52/6.72)	7.729 (0.52/6.72)	7.729 (0.52/6.72)	(0/0)
V3	4.98 (0.33/6.65)	4.98 (0.33/6.65)	4.98 (0.33/6.65)	(0/0)
NRS1	6.11 (0.49/8.046)	6.11 (0.49/8.046)	6.11 (0.49/8.046)	(0/0)
E23	17 (0/0)	17 (0/0)	17 (0/0)	(0/0)
E32	17 (0/0)	17 (0/0)	17 (0/0)	(0/0)

TABLE 3.4a: Glucose Brain Heart Infusion Agar inter-batch and intra-batch data

GBH- Intra- batch											
Day	**Stats.**	**NRS 70**	**B25**	**h20 (EC)**	**h 20 (TEC)**	**h40 (EC)**	**h 40 (TEC)**	**V3**	**NRS1**	**E23**	**E32**
1 (n=8)	**Mean**	0.875	0.9387	1.9718	2.2007	1.9422	2.196	2.7385	2.3492	7.4543	8.0713
	SD	0.3535	0.4191	0.3806	0.4047	0.3748	0.3695	0.7095	0.6397	3.2756	3.5135
	CV %	40.4061	44.6548	19.3029	18.3902	19.2983	16.8275	25.9087	27.2341	43.9425	43.5315
2 (n=8)	**Mean**	0.9211	1.1052	2.1078	2.5982	1.459	1.6921	2.3765	4.2688	3.5967	7.3556
	SD	0.3894	0.2006	0.4221	0.3963	0.6425	0.7574	1.0634	1.9245	1.6178	3.3066
	CV %	42.2822	18.1504	20.0292	15.2556	44.0377	44.7647	44.7492	45.0821	44.9804	44.9537
3 (n=8)	**Mean**	1	0.8796	1.9345	2.1938	1.8097	1.9631	3.0846	2.624	6.8295	6.2767
	SD	0	0.3556	0.5101	0.3791	0.5418	0.5022	0.9013	0.9656	3.04841	2.6671
	CV %	0	40.4329	26.3690	17.2827	29.9402	25.5819	29.2193	36.8020	44.6360	42.4925
4 (n=8)	**Mean**	1.2398	1	1.6655	1.8798	1.6816	1.8403	2.6271	2.2837	9.2517	9.9695
	SD	0.4329	0	0.7481	0.8225	0.7262	0.7944	0.8874	0.5126	0.6707	3.2469
	CV %	34.9173	0	44.9208	43.756	43.1893	43.1656	33.7807	22.4460	7.2503	32.5684
5 (n=8)	**Mean**	1	1.037	1.8336	2.0443	1.5693	1.8622	2.0766	2.3657	6.2548	6.924
	SD	0	0	0.5182	0.4347	0.3646	0.4116	0.6261	0.3537	2.2869	3.1111
	CV %	0	0	28.2637	21.2650	23.2329	22.1071	30.1539	14.9533	36.5624	44.9330
6 (n=8)	**Mean**	0.916	0.875	1.228	1.4286	1.7225	2.078	2.4406	2.5413	4.3725	12.1332
	SD	0.3881	0.3535	0.5520	0.6055	0.4616	0.4284	0.7432	0.4987	1.9131	5.5608
	CV %	42.3477	40.4061	44.9458	42.3881	26.7984	20.6093	30.4548	19.6236	43.7540	45.8314
7 (n=8)	**Mean**	1	1	1.9333	2.0831	2.0446	2.332	2.7225	2.6838	7.3208	6.3087
	SD	0	0	0.5588	0.4546	0.5922	0.5110	1.1489	0.6290	3.2406	2.3279
	CV %	0	0	28.9058	21.8230	28.9641	21.9150	42.2035	23.4389	44.2658	36.9011
8 (n=8)	**Mean**	0.8842	0.875	1.6953	1.9681	1.2282	1.461	2.8373	2.5967	7.6786	7.6752
	SD	0.3576	0.3535	0.3541	0.3578	0.5520	0.6426	0.5937	1.1435	3.0651	3.4440
	CV %	40.45	40.4061	20.89	18.1820	44.9458	43.9877	20.9266	44.0378	39.9177	44.8725
GBH- Inter- batch											
1-8	**Mean**	0.625	0.8796	1.6362	1.6728	1.383	1.5678	1.5102	1.843	2.6608	5.564
	SD	0.5175	0.3556	1.1289	1.1816	0.9560	1.1133	1.0541	1.2740	4.0308	4.1585
	%CV	82.807	40.4329	68.9936	70.6372	69.1302	71.0121	69.7986	69.1307	151.4875	74.7395

TABLE 3.4b: Glucose Brain Heart Infusion Agar inter-observer (Intra-batch) data

Observer / Strain No	1 Overall Mean (n=8) (SD/CV)	2 Overall Mean (n=8) (SD/CV)	3 Overall Mean (n=8) (SD/CV)	Inter-observer (n=8) (SD range / CV range)
NRS70	0.96 (0.33/34.36)	0.96 (0.33/34.36)	0.98 (0.35/36.3)	0-0.57 / 0-17.3
B25	0.93 (0.31/34.07)	0.93 (0.31/34.07)	0.93 (0.31/34.07)	(0/0)
h20 (EC)	1.8 (0.55/30.74)	1.8 (0.55/30.74)	1.8 (0.55/30.74)	(0/0)
h20 (TEC)	2.04 (0.56/27.83)	2.04 (0.56/27.83)	2.04 (0.56/27.83)	(0/0)
h40 (EC)	1.68 (0.57/33.92)	1.68 (0.57/33.92)	1.68 (0.57/33.92)	(0/0)
h40 (TEC)	1.92 (0.60/31.13)	1.92 (0.60/31.13)	1.92 (0.60/31.13)	(0/0)
V3	2.61 (0.85/32.82)	2.61 (0.85/32.82)	2.61 (0.85/32.82)	(0/0)
NRS1	2.7 (1.09/40.40)	2.7 (1.09/40.40)	2.7 (1.09/40.40)	(0/0)
E23	6.6 (2.96/44.86)	6.6 (2.96/44.86)	6.6 (2.96/44.86)	(0/0)
E32	8.89 (3.82/47.21)	8.89 (3.82/47.21)	8.89 (3.82/47.21)	(0/0)

TABLE 3.4c: Glucose Brain Heart Infusion Agar inter-observer (Inter-batch) data

Observer / Strain No	1 Overall Mean (n=8) (SD/CV)	2 Overall Mean (n=8) (SD/CV)	3 Overall Mean (n=8) (SD/CV)	Inter-observer (n=8) (SD range / CV range)
NRS70	0.62 (0.51/82.2)	0.62 (0.51/82.2)	0.62 (0.51/82.2)	(0/0)
B25	0.88 (0.35/40.43)	0.88 (0.35/40.43)	0.88 (0.35/40.43)	(0/0)
h20 (EC)	1.63 (1.12/69.0)	1.60 (1.09/68.21)	1.60 (1.09/68.21)	(0-0.58 / 0-24.79)
h20 (TEC)	1.67 (1.18/70.63)	1.67 (1.18/70.63)	1.67 (1.18/70.63)	(0/0)
h40 (EC)	1.38 (0.95/69.13)	1.38 (0.95/69.13)	1.38 (0.95/69.13)	(0/0)
h40 (TEC)	1.56 (1.11/71.01)	1.56 (1.11/71.01)	1.56 (1.11/71.01)	(0/0)
V3	1.51 (1.05/69.8)	1.51 (1.05/69.8)	1.51 (1.05/69.8)	(0/0)
NRS1	1.84 (1.27/69.13)	1.84 (1.27/69.13)	1.84 (1.27/69.13)	(0/0)
E23	2.66 (4.03/151.48)	2.66 (4.03/151.48)	2.66 (4.03/151.48)	(0/0)
E32	5.6 (4.16/74.7)	5.73 (4.4/76.7)	5.3 (3.88/73.14)	(0-1.73 / 0-17.25)

Comparison of MICs obtained by SGE using the different media showed that 100% (BHA), 70% (MHA) and 56.7% (GBH) of strains to match the MIC obtained by AD (Table 3.5). BHA based SGE provided the best correlation with AD in categorizing the susceptibility and non-susceptibility, with no discrepancies in interpretation (r^2 = 0.95). Both MHA and GBH correctly identified all susceptible strains. But their performance was less impressive with categorization of hVISA and VISA strains.

3.3.2 Statistical Analysis

The Bland and Altman plot (Figure 3.3a, 3.3b, 3.3c) showed the best agreement in MICs obtained by both AD and BHA based SGE as 8/9 spots were with in limit i.e $\pm$ 1, as illustrated in figure 3.3a. Although, all the spots were within the limit for MHA based SGE and 19/20 were within the limit for GBH based SGE the range of variation was wider than BHA based SGE. The Pearson correlation coefficients r^2 was found to be 0.95 and the p for ANOVA was found to be 0.001 indicating that there was significant association between the values obtained by the two methods (Appendix-I). Wilcoxon signed rank test suggested that the MICs obtained by SGE on BHA were slightly higher than those obtained by AD method (P=0.015), but if results were rounded off or deemed equivalent if within +1 mg/L dilution there was no difference between the MICs obtained by the test and the control methods (Appendix-I). The Pearson correlation coefficients r^2 were found to be 0.561 and 0.198 for MHA and GBH based SGE method indicating poor correlation between the values obtained by the two methods (Appendix-I).

TABLE 3.5: Comparison of MICs (SGE vs AD) and agreement between categories

Media	category	Difference in MIC (SGE vs AD) (% agreement within)						Strains within 1 dilution
		>-2.0	-2.0	-1.0	0.00	1.00	2.00	
MHA	VSSA	-	-	-	4 (100)	-	-	4 (100)
	hVISA	-	1 (14.3)	3 (42.9)	3 (42.9)	-	-	6 (85.7)
	VISA	-	8 (42.1)	2 (10.5)	7 (36.8)	2 (10.5)	-	11 (57.9)
	Total	-	9 (30)	5 (16.7)	14 (46.7)	2 (6.7)	-	21 (70)
BHA	VSSA	-	-	-	4 (100)	-	-	4 (100)
	hVISA	-	-	-	7 (100)	-	-	7 (100)
	VISA	-	-	-	19 (100)	-	-	19 (100)
	Total	-	-	-	30 (100)	-	-	30 (100)
GBH	VSSA	-	-	-	4 (100)	-	-	4 (100)
	hVISA	-	-	5 (71.4)	1 (14.3)	1 (14.3)	-	7 (100)
	VISA	6 (31.6)	7 (36.8)	2 (10.5)	4 (21)	-	-	6 (31.6)
	Total	6 (20)	7 (23.3)	7 (23.3)	9 (30)	1 (3.3)	-	17 (56.7)

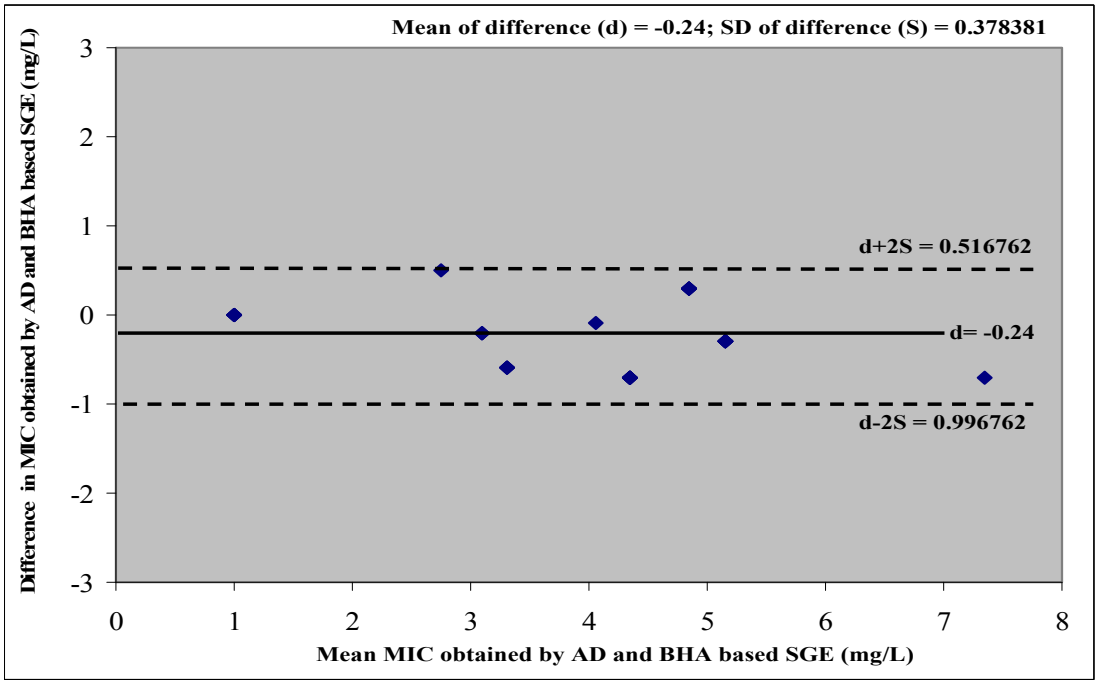


FIGURE 3.3a: Bland and Altman plot of evaluation of MIC values of BHA based SGE with respect to AD

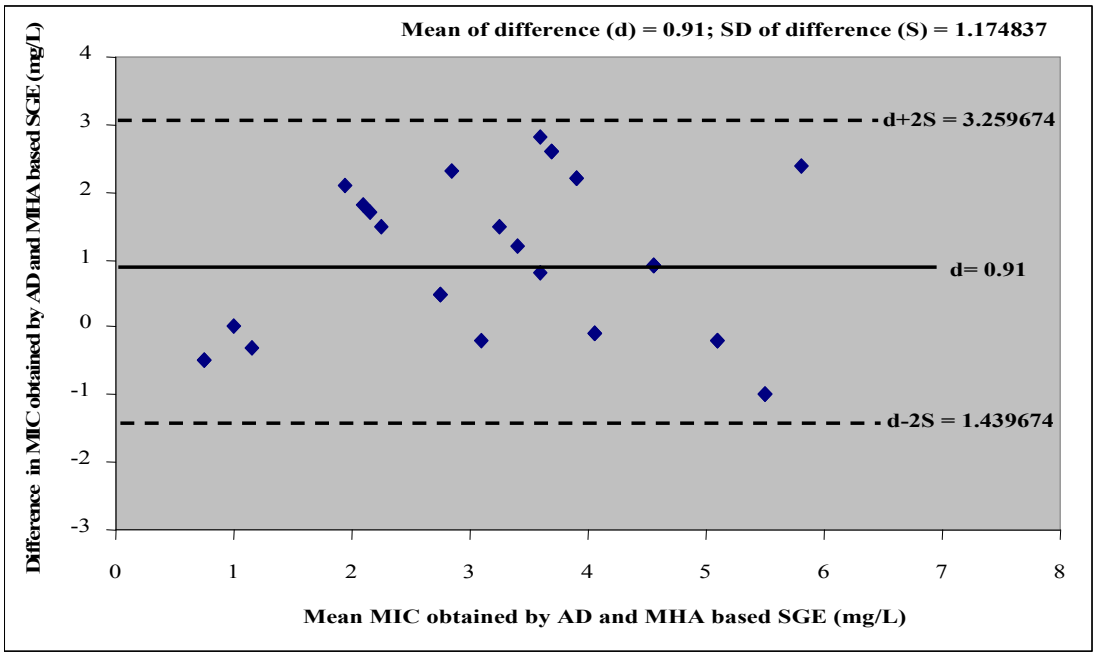


FIGURE 3.3b: Bland and Altman plot of evaluation of MIC values of MHA based SGE with respect to AD

Continued in following page

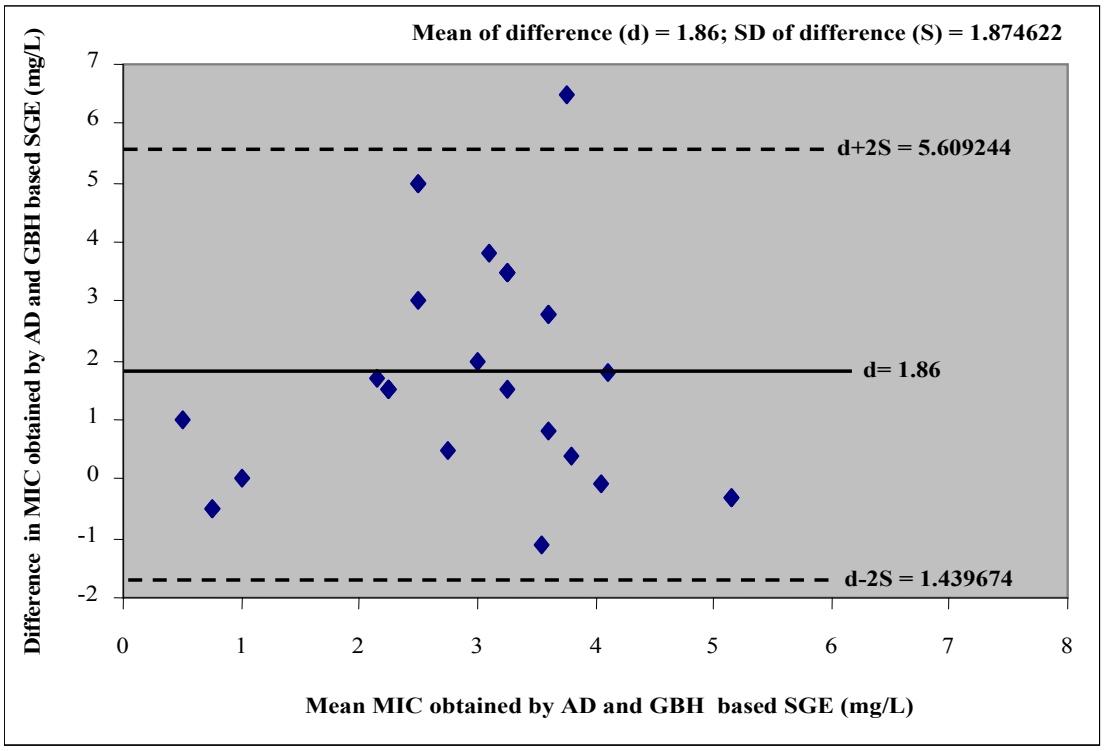


FIGURE 3.3c: Bland and Altman plot of evaluation of MIC values of GBH based SGE with respect to AD

Note: Isolates with identical mean MIC and difference in MIC on the test will group together when Bland and Altman test is performed, so the number of spots appearing on the plot does not match with the actual sample size of 30.

3.4 DISCUSSION

The increasing concern about the use of vancomycin therapy since the emergence of non-susceptibility has been compounded by lack of rapid and accurate detection of vancomycin non-susceptible phenotypes, particularly hVISA. The heterogeneous resistant sub-population expressed within the susceptible population is not detectable by most of the available screening methods and therefore a rapid, accurate and cost-effective detection tool is essential.

The BHA based SGE allowed rapid and accurate detection of vancomycin non-susceptible strains with excellent correlation of MICs with those obtained by AD with 1 mg/L increments between two plates. BHA is the CLSI recommended media for vancomycin non-susceptibility detection and has been used in several detection methods including the single point population analysis method (Hiramatsu et. al. 1997a, Jung et. al. 2002) and in PAP / AUC method (Wootton 2001). The better performance of BHA compared to MHA, is suggested to be beacuse BHA is more nutritious than MHA and is able to meet the higher glucose requirement for bio-synthesis of a thicker cell wall in vancomycin non-susceptible phenotypes (Cui et. al. 2003). In order to evaluate the effect of further increase of glucose concentration, GBH was investigated, but this media yeilded unacceptbale results. This inaccuaracy may be due to reduced water activity between test organism and the agar created by the high level of glucose in media, leading to reduced growth rates and poor accuracy. This phenomenon associated with high level of glucose has not been reported in earlier publications, but a report indicated the *S. aureus* inhibitory effect of sucrose and salt (Scott 1953).

Reproducibility of BHA based SGE was excellent with significant agreement with AD as indicated by Bland and Altman plot and the correlation met the requirements of FDA and the International Organization for Standardization for the evaluation of the performance of antimicrobial susceptibility test devices for susceptibility test systems including procedure for reference method and test method, organism selection, method of inoculation, media use, and incubation conditions (US Food and Drug and Administration 2003, International Organization for Standardization 2007).

Current CLSI recommendations for screening of vancomycin non-susceptibility requires a combination of methods (CLSI 2012), which is rarely acutally practised in the laboratory. However, all these methods lack the ability to detect resistant sub-population (Walsh et. al. 2001, Voss et. al. 2007, Wootton et. al. 2007), combination of methods are open to error, leading to delayed results and even may result in unfavourable patient outcome. Previously clinical laboratories relied heavily on disc diffusion alone, but disk diffusion alone is a poor indicator of vancomycin non-susceptibility and was removed as a recommended method by CLSI (CLSI 2010). Dilution methods whilst inexpesive in nature, but are labour-intensive, cumbersome, and inherent errors of the test results in number of ambiguous results. While the dilution test is difficult to perfom, but automated methods are unsuitable for detection of hVISA/VISA. Etest seem to offer good alternative to standard MIC method. Although, the method of Etest is simple and very few parameters need to be controlled by the user. But, the preformance of Etest entirely depends on the quality of Etest strip, which may be subjected to storage and batch variations. Moreover, the Etest strips are relatively expensive

and both standard Etest and macro Etest lacks the ability to detect heterogenecity. While GRD is effective in detecting heteroresistance but needs 48h of incubation and may delay the appropriate therapy to the patient (Walsh et. al 2001, Swenson et. al. 2009) and is reported to have poor reproducibility and poor specificity in hVISA detection (Richter et. al. 2011). It is suggested that only 1/3rd of laboratories in UK monitered vancomycin MIC routeinly mostly by Etest and vancomycin is commonly used as emperical therapy in treatment of *S. aureus* infections (Hussain et. al. 2010) as determining vancomycin MIC is either laborious or expensive. This study has demonstrated that the results obtained by SGE was compariable to AD, SGE is effective in detecting the hVISA/VISA effectively in comparision to AD and SGE is simple to perform.

Single-point population analysis and modifications of this method lack sensitivy or specificity as these may miss strains with low range of non-susceptibility (Voss et. al. 2007, Wootton et. al. 2007, Kosowska-Shick et. al. 2008, Burnham et. al. 2010). But, SGE is able to provide greater sensitivity due to the continuous scale of concentrations. Although, 5 mg/L teicoplanin-supplemented MHA, GRD and PAP-ACU have been deomstrated to have better specificity, these method requires 48h of incubation and are laborious (Leonard et. al. 2000, Fitzgibbon et. al. 2007, Yusof et. al. 2008). However, this study indicates that SGE is effective in detection of hVISA/VISA with 24h of incubation, as well as being simple and cost-effective as three strains can be tested on a single plate. Commercial automated methods such as Microscan and Phoenix are rapid but tend to categorize VISA as VSSA and VSSA as VISA, therefore are poor predictors of hVISA/VISA (Swenson et. al. 2009) in contrast SGE was able to accurately

categorize the susceptibility of the strains tested. Most of the methods effectively detecting hVISA/VISA need 48h of incubation and this may delay the change to appropriate therapy for a patient harbouring hVISA/VISA strains. If SGE is performed using a suspension prepared from a pure culture in sterile normal saline or by incoculating a single colony in BHI and incubating for 2h and adjusting to the correct concentration (turbidity equivalent to that of a 0.5 McFarland standard), this can save 24h and allows the test to be completed earlier, providing more useful feedback for therapy to clinicians.

In order to assess the clinical prevalence of vancomycin non-susceptible phenotypes, a suitable detection method that can overcome the above listed limitations is essential. This work has demonstrated that vancomycin non-susceptibility, and in particularly hVISA can be rapidly and accuratley detected using SGE. The confluent endpoint result helps in determining exact MIC as well as demonstrating the presence of VISA, whilest the outlying resistant sub-population allows identification of hVISA, which can be easily missed by other detection methods. Regular tracking of vancomycin MIC is important in patients receiving vancomycin therapy, as Soriano et. al. (2008) have reported treatment failure in isolates with MIC levels above 1.0 mg/L, although both CLSI and EUCAST define isolates with MIC $\leq$ 2mg/L as VSSA. Therefore for early and accurate detection of hVISA/VISA, determination of exact vancomycin MIC and preferably demonstration of resistanat sub-population is essential. Considering the vancomycin treament failure in strains with MIC $\leq$ 2mg/L, EUCAST recently updated the breakpoint for vancomycin non-susceptibility to MIC >2 mg/L to avoid confusion and clinical failures (EUCAST 2009). Whilst, SGE method needs

an initial investment for procuring a spiral plater for laboratories not performing the PAP-AUC method, the initial investment can be recovered quickly as SGE is cost-effective compared with other effective detection methods, as GRD costs 55 Hong Kong Dollars to test one isolate, while SGE costs only six Hong Kong Dollars / plate and three isolates can be tested on one plate which would cost only two Hong Kong Dollars. In addition, GRD strips have limited shelflife once opened but plates for SGE can be setup rapidly on demand and results can be obtained in much shorter time scale that GRD. SGE can also be used for detection of reduced susceptibility and resistance for other micro-organisams and antibiotics. This study concludes that SGE is a suitable alternative for accurate, reliable, economical and timely detection of vancomycin non-susceptible phenotype and further large scale evaluation is needed to investigate the clinical implications of SGE.

Publication and Conference Presentation

Doddangoudar VC, O'Donoghue MM, Boost MV, Tsang DNC, Appelbaum PC: Rapid detection of vancomycin-non-susceptible *Staphylococcus aureus* using the spiral gradient endpoint technique. *Journal of Antimicrobial and Chemotherapy.* 2010; 65: 2368–2372.

Doddangoudar VC, Boost MV, O'Donoghue MM, Tsang DNC, Appelbaum PC: Evaluation of spiral gradient endpoint technique (SGE) for rapid detection of vancomycin intermediate-resistant *Staphylococcus aureus*. 20th European Congress of Clinical Microbiology and Infectious Diseases. Vienna, April 2010.

CHAPTER 4:TRACKING THE DEVELOPMENT AND REVERSION OF VANCOMYCIN NON-SUSCEPTIBILITY IN *STAPHYLOCOCCUS AUREUS* WITH GENOTYPIC AND PHENOTYPIC EVIDENCE

4.1 INTRODUCTION

Vancomycin is a clinically important antibiotic because it is both an effective and economical option for the treatment of MRSA infections. It is known to act by preventing the transglycosylation step in peptidoglycan polymerization resulting in a damaged cell wall and bactericidal activity (Reynolds 1989). In recent years, vancomycin non-susceptible strains have emerged with altered cell wall that allows them to trap vancomycin and protect the target site (Cui et. al. 2009). These changes have been associated with treatment failures and increase in morbidity rates. It has been suggested that changes in the cell wall structure are associated with mutations in several genes, including *wal*KR, *yvq*F, *mpr*F, *vra*S *gra*R and *rpo*B (Cui et. al. 2009, Cui et. al. 2010, Kato et. al 2010, Howden et. al 2011). Only a few studies have tracked the genetic changes in a set of iso-genic non-susceptible phenotypes (Cui et. al. 2009) and so far there is no consistent mutation associated with non-susceptibility development and there appears to be no report correlating genetic changes with drug exposure time, MIC increase, and phenotypic changes. More work is necessary to identify a reliable determinant

associated with non-susceptibility development and the time required for these mutations to occur.

Investigation of the whole cycle of vancomycin non-susceptibility development and loss may help uncover consistent mutations conferring resistance in all hVISA/VISA which may aid development of rapid and reliable molecular detection methods. In order to determine if there is a consistent change in all hVISA/VISA isolates, this study compared the patterns of development and loss of non-susceptibility in clinical MRSA isolates and sequenced the *vraS, graR* and *rpoB* genes in selected strains as representatives of each pattern observed. The first part of this study investigated changes in isolates from a patient with MRSA infection, who failed vancomycin therapy and following *in-vitro* vancomycin exposure and a subsequent drug-free growth period. The second part of the study investigated the phenotypic and genotypic changes in six clinical isolates selected from 30 clinical isolates of MRSA exposed to vancomycin followed by a period of drug free growth.

4.1.1 Case Study

An 86-year-old woman was admitted to a local district hospital with persistent fever. An echocardiogram revealed a small mass attached to the anterior mitral leaflet, the blood cultures were positive for MRSA with a vancomycin MIC 1.0 mg/L. Initial dosage regimen included vancomycin 500 mg/24 h (i.v) followed by an increased vancomycin dose of 500 mg/8h. Further cultures remained positive for MRSA and the MIC reached 6 mg/L (GRD Etest) by day 10. When vancomycin MIC reached 6mg/L on day 10, vancomycin was replaced by

daptomycin 300 mg/48 h (i.v) for 6 days, which was increased to 350 mg/48 h for 2 days, and then continued with addition of 300 mg of rifampin. Unfortunately, the patient's condition deteriorated and she died 6 weeks after admission. The day zero, four, five, six and ten isolates of MRSA were collected for further investigation as described in following section (see Figure 4.1).

4.2 EXPERIMENTAL DESIGN

4.2.1 Vancomycin MIC Determination

Case study: The MRSA isolates from day zero to day ten were collected and their vancomycin MICs determined by AD and SGE method as validated and published in Chapter 3, SGE was performed as it would indicate both the confluent growth endpoint (EC) which is equivalent to the MIC and the extended trailing endpoint (TEC) which would help in identification of hVISA.

Induction study: Thirty clinical MRSA isolates were randomly selected from the isolates collected from a local district hospital before the commencement of therapy, but did not include repeat isolates from any individual patient. The initial vancomycin MICs were determined by AD and SGE method as described in Chapter 3.

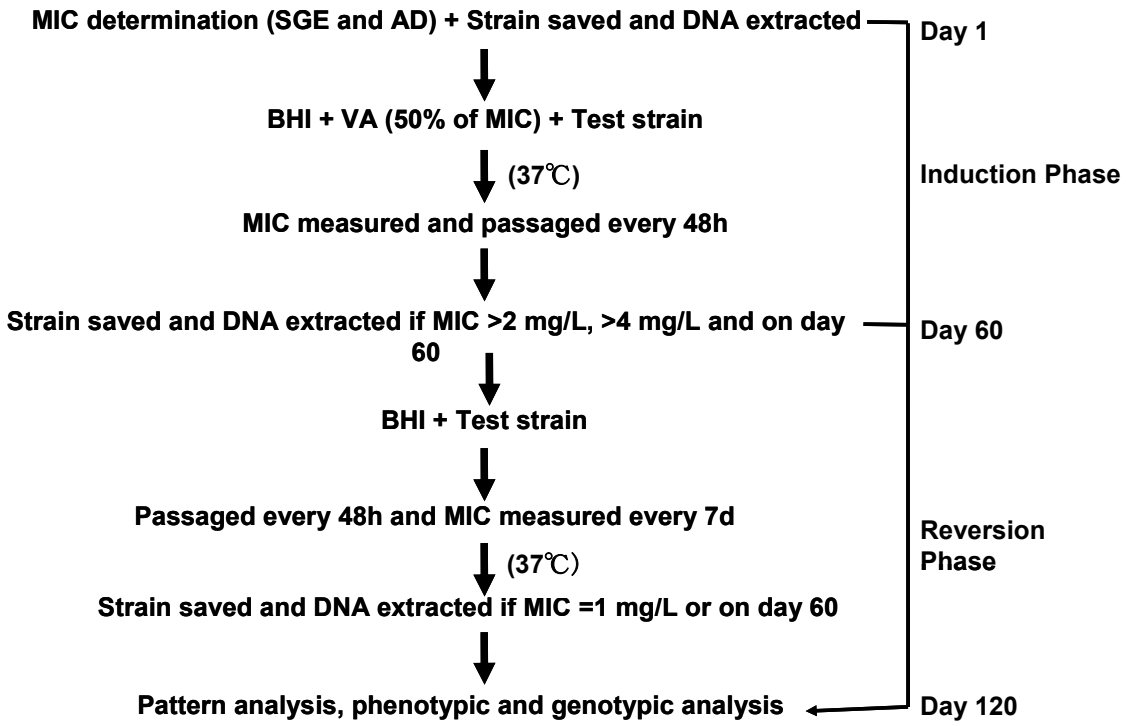


FIGURE 4.1: Flowchart of experimental tracking of vancomycin non-susceptibility development and loss

Note: Although, this figure indicates 60 days of induction phase, in case of case study isolate the induction period was 50 days as the day 10 clinical VISA isolate was used for induction.

4.2.2 Vancomyin Non-Susceptibility Induction Phase

Preparation of vancomycin stock solutions: Vancomycin (Sigma-Aldrich, St. Louis, MO, USA) stock solution of 800 mg/L was prepared as described in Chapter 3. The calculated volume of stock solution (Table 4.1) was added to sterile BHI Broth (Oxoid, Basingstoke, UK) and used in the induction phase.

Case study: In order to determine if continued vancomycin pressure would lead to further increase in the vancomycin MIC, the day ten isolate was incubated in BHI broth supplemented with a vancomycin concentration equivalent to 50% of the initial MIC as shown in Figure 4.1 and Table 4.1 at 37^0C for 48 h., the strains were passaged to fresh media and MIC determined every 48h. If the MIC had increased, the vancomycin concentration of the next passage was increased to the newly calculated 50% MIC. This process was carried out for a period of 50 days. This was also performed on Mu50 a control VISA strain that was isolated from the cardiac surgery wound of a 4 month old patient. This isolate initially had an MIC of 8mg/L and has been previously studied for genetic changes (Cui et. al 2009). However, the effect of continued vancomycin pressure has not been determined in this isolate, and hence it was included in this study.

Induction study: All 30 isolates were incubated as described above for the case study isolate. The process of induction was carried out for a period of 60 days. As a control, six selected strains were also passaged and MIC determined at 48h intervals for 60 days in the absence of vancomycin.

TABLE 4.1: Volume of vancomycin stock solution required to achive the required MICs for induction study

Vol. of vancomycin stock solution of 800 mg/l (µl)	Vol. of BHI Broth (ml)	Final conc. (mg/L)
0.0	20.0	0.0
12.5	19.9875	0.5
25	19.9750	1.0
50	19.9500	2.0
75	19.9250	3.0
100	19.9000	4.0
125	19.8750	5.0
150	19.8500	6.0
175	19.8250	7.0
200	19.8000	8.0
225	19.7750	9.0
250	19.7500	10.0

4.2.3 Vancomyin Non-Susceptibility Stability Phase

Case study: The stability of non-susceptibility was determined by further incubation of day 60 strains in vancomycin-free broth at 37^0C and passaging into fresh medium every 48h. The MIC was determined at weekly intervals by AD and SGE. The passage was discontinued when the MIC had dropped to 1mg/L or on day 60 of stability phase. MICs obtained during non-susceptibility development and loss were plotted over the time.

Induction study: The stability study was performed for the induced strains as described above for the case study isolate and MICs obtained during non-susceptibility development and loss were plotted for each isolate to compare patterns of development and loss of non-susceptibility over time. Six patterns of non-susceptibility development and loss were observed. After observation, six strains were selected, each one as a representative of each pattern, for further genotypic and phenotypic analysis.

Isolates from both the case study and the induction study were saved as described in Chapter 3 from the isolates at five stages in the cycle: VSSA (initial strain with MIC >2mg/L), hVISA (MIC >2-<4mg/L), VISA (MIC >4mg/L), VISA-max (highest MIC attained by day 60), VISAΩ (MIC ≤ 1 mg/L or MIC after 60 days in absence of vancomycin) and control strains of day 60.

4.2.4 Protocol for DNA Extraction

DNA was extracted whenever there was change in susceptibility category for strains of both case study and induction study. Five hundred µl of bacterial suspension was aliquoted into 1500 µl eppendorf tube, which was centrifuged at 10,000 rpm for 5 mins. and the pellet re-suspended in 400 µl of lysis buffer, (table 4.2) and the DNA extracted by incubating at 37^0C for 45mins in a rotary incubator, followed by suspending the eppendorf tubes in boiling water for 10 mins and finally the eppendorf tubes were placed on ice for 10 mins to stop the reaction. After cooling the lysis buffer was centrifuged at 10,000 rpm for 10 mins and 200 µl of the supernatant solution was withdrawn. The DNA concentration was measured and then adjusted to 50ng/µl before storage at -20^0C.

4.2.5 Molecular Characterization of the Isolate (Both case study and induction study samples)

4.2.5.1 Sequencing

Both genes *vraS* (1041bp) and *graR* (672bp), were amplified using the PCR protocol as described in Table 4.3a and 4.3b (Personal communication - Prof. Hiramatsu, Juntendo University, Japan), 5 µl volumes of each amplicon was deposited into a well of 1.2% agarose gel, run along with a 10,000bp ladder at 100V for the required time. The gel was stained in 0.5% ethidum bromide for 15 mins in a covered container and destained in approximately 500 ml distilled water for 10 mins.

TABLE 4.2: Lysis buffer master mix for DNA extraction

Reagents	**Vol (µl)**
Lysostaphin	20
Lysozyme	20
Tris Hcl	4
EDTA (0.5mM)	8
Water	348

The agarose gel image was captured using G BOX Chemi Imaging System (Syngene, Cambridge, UK) to confirm DNA amplification. The amplicons were then sent to the Genome Research Centre, The University of Hong Kong, Hong Kong for sequencing followed by sequence analysis in comparison to N315 sequences to identify possible mutations contributing towards non-susceptibility development and loss. The mutations were identified using the software as described previously (Corpet 1988). The *RpoB* (932bp) gene was amplified using the PCR protocol as described in table 4.4a and 4.4b (Matsuo et. al. 2011). The amplification, sequencing and sequence analysis were performed as described for *vraS and graR* genes.

4..2.5.2 *mec*A PCR

*mec*A (448bp) PCR was performed as described in Table 4.5a and 4.5b (Sakoulas et. al 2001) on isolates of day 0, day 60 and NRS 22 (control strain) and amplification was confirmed as described above.

4..2.5.3 SCC*mec* PCR

SCC*mec* typing was performed as described in Table 4.6a and 4.6b (Zhang et. al 2005) on case study isolate, day 0 isolates of induction study and control strains (SCCmec I: NRS 100, SCCmec II: NRS 22, SCCmec III: NRS65, SCCmec IVa: NRS123, SCCmec IVb: ATCC-1762, SCCmec IVc and SCCmec IVd were kindly donated by Prof. H de Lencastre, University of Lisbon, SCCmec V: ATCC-2094). The amplicons were run as described above in 4.2.5.1.

TABLE 4.3a: Master mix for amplification of *vraS* and *graR* genes

Reagents	Vol. (µl)
10X Buffer	2.5
dNTP (2mM)	2.5
MgCl2 (25mM)	4.5
Primer (10mM)	
vraS-F 5′-ATGGAAGTACTTACGTGAATGA-3′	0.5
vraS-R 5′-TCGACACTGCATCTAATGCACGA-3′	0.5
OR	
graR-F 5′-GGCCGATTTATTACTTTATACAAGCACC-3′	0.5
graR-R 5′-ATCATAATCGATTAGACTAATGCCTAACAT-3′	0.5
DNA (50ng/µl)	5.0
Taq polymerase	0.5
Sterile water	makeup to 25

TABLE 4.3b: PCR conditions for for amplification of *vraS* and *graR* genes

Temp. (^{0}C)	Time	No. Cycles
94.0	3.0 mins	1
94.0	15.0 Sec	10
53.0	30.0 Sec	
72.0	2.0 mins	
94.0	15.0 Sec	20
53.0	30.0 Sec	
72.0	2.0 mins	
72.0	7.0 mins	1
4.0	∞	

TABLE 4.4a: Master mix for amplification of *rpoB* gene
(Matsuo et. al. 2011)

Reagents	Vol. (µl)
10X Buffer	5.0
dNTP (2mM)	4.0
MgCl2 (25mM)	2.0
Primer (10mM)	
RpoB-F 5′-CTGTAATTGGTAATGCTTTCCCTGACTC-3′	0.5
RpoB-R 5′-CCAGAATCACGTGCTGCAACGTGTTCCA-3′	0.5
DNA (50ng/µl)	3.0
Taq polymerase	0.5
Sterile water	Make upto 50

TABLE 4.4b: PCR conditions for for amplification of *rpoB* gene
(Matsuo et. al. 2011)

Temp. (^{0}C)	Time	No. Cycles
98.0	3.0 mins	1
96.0	15.0 Sec	30
53.0	30.0 Sec	
72.0	4.0 mins	
72.0	4.0 mins	1
4.0	∞	

TABLE 4.5a: Master mix for amplification of *mecA* gene (448bp)
(Sakoulas et. al 2001)

Reagents	Vol. (µl)
10X Buffer	3.0
dNTP (2mM)	3.0
$MgCl_2$ (25mM)	6.0
Primer (10mM)	
mecA-F 5′-CTCAGGTACTGCTATCCACC-3′	1.0
mecA-R 5′-CACTTGGTATATCTTCACC -3′	1.0
DNA (50ng/µl)	3.0
Taq polymerase	0.1
Sterile water	Make upto 25µl

TABLE 4.5b: PCR condtions for for amplification of *mecA* gene
(Sakoulas et. al 2001)

Temp. (^{0}C)	Time	No. Cycles
95.0	5.0 mins	1
94.0	30.0 Sec	30
51.5	30.0 Sec	
72.0	30.0 Sec	
72.0	10.0 mins	1
4.0	∞	

TABLE 4.6a: PCR master mix for SCC*mec* typing
(Zhang et. al 2005)

Reagents	Vol. (µl)	Product bp
10X Buffer	2.5	-
dNTP	2.3	-
$MgCl_2$ (25mM)	2.3	-
Primer (5mM)		
Sccmec-I-F 5′-GCTTTAAAGAGTGTCGTTACAGG -3′	0.2	613
Sccmec-I-R 5′-GTTCTCTCATAGTATGACGTCC -3′	0.2	
Sccmec-II-F 5′-CGTTGAAGATGATGAAGCG -3′	0.2	398
Sccmec-II-R 5′-CGAAATCAATGGTTAATGGACC -3′	0.2	
Sccmec-III-F 5 ′CCATATTGTGTACGATGCG- -3′	0.2	280
Sccmec-III-R 5′-CCTTAGTTGTCGTAACAGATCG -3′	0.2	
Sccmec-IVa-F 5′-GCCTTATTCGAAGAAACCG -3′	0.5	776
Sccmec-IVa-R 5′-CTACTCTTCTGAAAAGCGTCG -3′	0.5	
Sccmec-IVb-F 5′-TCTGGAATTACTTCAGCTGC -3′	0.5	493
Sccmec-IVb-R 5′-AAACAATATTGCTCTCCCTC -3′	0.5	
Sccmec-IVc-F 5′-ACAATATTTGTATTATCGGAGAGC-3′	0.4	200
Sccmec-IVc-R 5′- TTGGTATGAGGTATTGCTGG-3′	0.4	
Sccmec-IVd-F 5′-CTCAAAATACGGACCCCAATACA -3′	1.4	881
Sccmec-IVd-R 5′-TGCTCCAGTAATTGCTAAAG -3′	1.4	
Sccmec-V-F 5′-GAACATTGTTACTTAAATGAGCG -3′	0.3	325
Sccmec-V-R 5′-TGAAAGTTGTACCCTTGACACC -3′	0.3	
DNA (50ng/µl)	2.0	-
Taq polymerase	0.2	-
Sterile water	Make upto 25µl	-

TABLE 4.6b: PCR condition for SCC*mec* typing
(Zhang et. al 2005)

Temp. (^{0}C)	**Time**	**No. Cycles**
94.0	5.0 mins	1
94.0	45.0 Sec	10
65.0	45.0 Sec	
72.0	1.5 mins	
94.0	45.0 Sec	25
55.0	45.0 Sec	
72.0	1.5 mins	
72.0	10.0 mins	1
4.0	∞	

4.2.5.4 *vanA* PCR

vanA (1030bp) PCR was performed as described in Table 4.7a and 4.7b (Clark et. al. 1993) for isolates with MIC ≥16 mg/L and a *vanA* control strain (ATCC 700221) and the amplicons were visualized as described above in sequencing section.

4.2.5.5 *agr* PCR

agr typing was performed as described in Table 4.8a and 4.8b (Shopsin et. al 2003) on day 0 isolates and control strains (*agr* I: NRS22, *agr* II: NRS 149, *agr* III: NRS 123, *agr* IV: NRS165) and amplification was confirmed.

TABLE 4.7a: Master mix for amplification *vanA* gene (1030 bp)
(Clark et. al. 1993)

Reagents	Vol. (µl)
10X Buffer	5.0
dNTP (2mM)	2.0
MgCl2 (25mM)	2.0
Primer (10mM)	
van-A -F 5′-CATGAATAGAATAAAAGTTGCAATA-3′	1.0
van-A -R 5′-CCCCTTTAACGCTAATACGATCAA-3′	1.0
DNA (50ng/µl)	1.0
Taq polymerase	1.0
Sterile water	makeup to 25

TABLE 4.7b: PCR conditions for amplification of *vanA* gene
(Clark et. al. 1993)

Temp. (^{0}C)	Time	No. Cycles
95.0	10.0 mins.	1
94.0	30.0 Sec	30
58.0	30.0 Sec	
72.0	30.0 Sec	
72.0	10.0 mins.	1
4.0	∞	

TABLE 4.8a: PCR master mix for *agr* typing
(Shopsin et. al 2003)

Reagents	Vol. (µl)	Product bp
10X Buffer	5.0	-
dNTP 2mM	5.0	-
$MgCl_2$ 25mM	10	-
Primer (100mM)		
Pan agr-F 5′-ATGCACATGGTGCACATGC -3′	2	-
agr-I-R 5′-GTCACAAGTACTATAAGCTGCGAT-3′	2	440
agr-II-R 5′-GTATTACTACTTGAAAAGTGCCATAGC-3′	2	572
OR		
agr-III-R 5′-CTGTTGAAAAGTCAACTAAAAGCTC-3′	2	406
agr-IV-R 5′-CGATAATGCCGTAATACCCG-3′	2	508
DNA (50ng/µl)	1.0	-
Taq polymerase	0.6	-
Sterile water	Make upto 50	-

TABLE 4.8b: PCR conditions for *agr* typing
(Shopsin et. al 2003)

Temp. (^{0}C)	Time	No. Cycles
94.0	4.0 mins	1
94.0	30.0 Sec	30
53.0	30.0 Sec	
72.0	1.0 mins	
72.0	4.0 mins	1
4.0	∞	

Pulse Field Gel Electrophorosis (PFGE) Typing: The PFGE was modified and performed (Bannerman et. al. 1995) as described below on all isolates of induction study on day 0 to determine if there was a correlation of the PFGE pattern and non-susceptibility development and loss. It was also performed for the selected six strains on day 0, day 60 and day 120 to check that the strains remained consistent through out the study without contamination.

On day 1 the test strains were plated on blood agar and incubate overnight at 37^0C.

Following day a single colony was selected from each isolate and inoculated in 10ml BHI broth and incubated overnight at 37^0C.

On day 3, two grams of low melting agarose was accurately weighed into a conical flask, 100ml of TEB (10mM Tris-HCl + 100mM EDTA) (Table 4.9) was added, heated until dissolved, stored at 60^0C until used in plug making.

500µl of bacterial suspension was transferred into an eppendorf tube, centrifuged at 4000rpm for 10mins and the supernatant discarded. These bacterial pellets were used to make the plugs by adding 490µl of TEB (10:100) (Table 4.9), 10µl of lysostaphin to each pellet which was vortexed to re-suspend. To this suspension 500µl of molten agarose was added and mixed by pipetting, quickly a 200µl aliquot of the mixture was gently dropped into each plug blocks and refrigerated at 4^0C for 10mins.

For lysis of the bacteria in the pellets lysis solution containing RNase (1.5µl/tube of 32.5mg/ml solution), lysozyme (1.0µl/ tube of a 10mg/ml solution), lysostaphin

(5µl/tube of 2000U/ml solution) and lysis buffer (1ml/tube) was used and prepared. The ingredients were added into to a clean 10ml tube and mixed well.

For lysis, 1ml of the lysis solution was added to each eppendrof tube, the top of the plugs were cleaned with scalpel and pushed into the lysis solution. The plugs were incubated at 37^0C for 2h, the lysis solution was discarded. The plugs were than washed with 1.0ml TEB (10:1) (Table 4.9) at room temperature for 15mins, after 15mins the TEB was discard. 1ml of EBS buffer and 50µl of 20mg/ml proteinaseK solution was added to each tube and incubated overnight at 50^0C in water bath.

On day 4 to wash the plug, the buffer was removed, 1.0ml of distilled water was added and the mixture incubated at room temperature for 15mins before discarding the water. One ml of TEB (10:1) was added (Table 4.9) and incubated at room temp. for 30mins. This washing step was repeated for a total of 4 times and plugs stored in 1ml of TEB (10:1) in the refrigerator or continue with restriction digestion of DNA.

TABLE 4.9: Preparation of buffer solution used in PFGE (Stored at 4^0C)

Reagents	Quantity
1M Tris-HCl	
TRIS base	121.1gm
Conc. HCl	Quantity sufficient to adjust pH 8
Distilled water	Quantity sufficient to make 1000ml
0.5M EDTA	
EDTA	202.2gm
NaoH pellets	Quantity sufficient to adjust pH 8
Distilled water	Quantity sufficient to make 1000ml
Tris EDTA Buffer (TEB) - pH 8 (10:100)	
1M Tris-Hcl (pH 8)	5.0ml
0.5M EDTA (pH 8)	100.0 ml
Distilled water	Quantity sufficient to make 500ml
TEB- pH 8 (10:1)	
1M Tris-Hcl (pH 8)	5.0ml
0.5M EDTA (pH 8)	1.0 ml
Distilled water	Quantity sufficient to make 500ml
Electrolyte Buffer Solution (EBS) Buffer- pH 8	
Na-laurylsarcosine	1.0gm
0.5M EDTA (pH 8)	100.0 ml
Distilled water	Quantity sufficient to make 500ml
Lysis buffer- pH 8 (10:1)	
1M Tris-Hcl (pH 8)	3.0ml
0.5M EDTA (pH 8)	100.0ml
NaCl	29.2gms
Na-deoxycholate	1.0gm
Na-laurylsarcosine	2.5gms
Distilled water	Quantity sufficient to make 500ml
0.5X TBE Buffer	
TRIS-HCl	12.15gm
Boric Acid	6.187gm
EDTA	1.046gm
Distilled water	Quantity sufficient to make 4500ml

For restriction digestion of the DNA in agarose plugs with *SmaI,* first the plug was cut into small bit to clean the edge. 1mm size plug was cut and the rest of the plug was stored in 1ml TEB (10:1) (Table 4.9) at 4^0C. The 1mm plug was placed into a PCR tube containing 50µl of following solution (Distilled water 43µl/tube + reaction buffer 5µl/tube + *SmaI* 2µl/tube) and incubated at room temperature overnight.

On day 5, two thousand five hundred ml of 0.5X TBE buffer was prepared (Table 4.9), 2.0L pored into PFGE chamber and cooled to 14^0C for approximately an hour.

Accurately 1.6gm Seakem gold agarose gel of agarose was weighed and added to 200ml 0.5X TBE buffer, melted and kept at 60^0C till use.

The plugs with ladder were arranged on the comb and fixed using agarose, gently 180ml of agarose was poured into the casting set and the comb with plugs was slowly positioned and allowed for 45-60mins for the gel to set. The comb was gently removed and the wells covered with agarose and allowed to set. The gel was gently removed from the casting set and placed into the PFGE chamber and ran at conditions indicated in Table 4.10.

TABLE 4.10: Running conditions for PFGE

Block 1 Initial time Final time Run time Volts	 3.0 mins 9.0 mins 12.0 h 6	 Stage 1 Angle 53^0 Stage 2 Angle -53^0
Block 2 Initial time Final time Run time Volts	 8.0 mins 45.0 mins 9.0 h 6	 Stage 1 Angle 60^0 Stage 2 Angle -60^0

Finally on day 6, twenty µl of ethidium bromide stock solution (10mg/ml) was diluted with 800ml of distilled water and the gel stained for 30mins in a covered container. The gel was destained in approximately 500ml distilled water for 30mins, and the image captured. If the background interfered with resolution, the gel was destained for an additional 20-30mins. The buffer was drained from PFGE chamber and discarded.

4.2.6 Phenotypic Characteriation

Phenotypic changes such as growth rate, lysostaphin lysis and % loss of viability were determined in order to verify if there was a relationship between phenotypic and genotypic changes.

4.2.6.1 Growth rate

The growth rate was determined for initial, VISA-max and VISAΩ for the case study isolate and the selected six isolates. Fresh bacterial culture was adjusted to 0.5 McFarland concentrations in 10 ml of fresh BHI broth and incubated at 37°C with agitation of 25rpm for 12h. Samples were drawn every hour to measure the absorbance at 600 nm as an indicator for growth rate. To determine growth curve and doubling time the absorbance was plotted against time for each strain. Doubling time was calculated as described below (Cui et. al. 2010).

Doubling time = [($t2$ - $t1$) x log 2] / [log OD 600 at $t2$ - log OD 600 at $t1$].

4.2.6.2 Lysostaphin lysis test

A colony of selected samples was incubated overnight in 5 ml BHI at 37^0C. After incubation, the bacterial suspension was centrifuged at 1008g for 10 mins and the supernatant discarded. The pellet was re-suspended in 5 ml PBS (pH7.2) and centrifuged at 1008g for 10 mins and the supernatant discarded. This step was repeated once again. Finally, the pellet was re-suspended in PBS (pH 7.2) and the concentration to adjusted 0.5 McFarland. 0.025 ml lysostaphin was added to 0.475 ml of 0.5 McFarland bacterial suspension and incubated at 37^0C for 60 mins; the samples were drawn and plated at 0 mins, 40 mins and 60 mins, incubated overnight at 37^0C. The number of colonies grown were counted using a colony counter (aCOLYTE, Symbiosis UK) to determine the % loss of viability at 40 mins and 60 mins of incubation in lysostaphin (Moreira et. al. 1997).

4.2.6.3 Time for tube coagulase positivity

A colony of selected samples was incubated overnight in 5 ml BHI at 37^0C. After incubation, the bacterial suspension was centrifuged at 1008g for 10 mins and the supernatant discarded. The bacterial pellet was re-suspended in 5 ml normal saline and centrifuged at 1008g for 10 mins and the supernatant discarded and washed. The pellet was re-suspended in normal saline and the concentration adjusted to 0.5 McFarland. The bacterial suspension was added to plasma to obtain mixture in ratio of 1:1, 1:10, 1:100, 1:1000, 1:10000, and 1:100000 and incubated at room temperature for 24h. The mixtures were checked for clot formation every 10 mins for first 4 h and subsequently every 30 mins up to 24h (Moreira et. al. 1997).

4.2.6.4 Susceptibility testing

Susceptibility testing was performed on induction study isolates for a range of antibiotics including chloramphenicol, ciprofloxacin, clindamycin, co-trimoxazole, erythromycin, fusidic acid, gentamicin, linezolid, quinopristin-dalfopristin, and rifampin, (Oxoid, Basingstoke, UK) before, after development and loss of non-susceptibility by disc diffusion (CLSI 2010).

4.2.6.5 MIC for other anti-MRSA agents

MICs for oxacillin, linezolid, tigecycline, and daptomycin were determined at various stages for case study isolate and induction study isolates selected for genotypic analysis by Etest as per manufacturer's instructions. A bacterial suspension corresponding to a 0.5 McFarland standard was swabbed on a MHA (Oxoid, Basingstoke, UK). An Etest strip (AB Biodisk, Solna, Sweden) was then applied to the MHA plate, incubated at 37^0C for 48h and the MIC concentration were read at 24h.

4.3 RESULTS

4.3.1 MIC Determination

Case study: The SGE method indicated the presence of a subpopulation in the initial isolate with MIC for vancomycin >2 mg/L (table 4.11). The day 6 isolate had attained an MIC >4mg/L and by day 10 the isolate had an MIC 6.2mg/L. E-test revealed that oxacillin resistance was observed throughout and only VISAΩ was sensitive to daptomycin. VSSA, VISA and VISAΩ were susceptible to tigecycline and linezolid, but VISA-max was resistant to all drugs (Figure 4.2 & 4.3, Appendix II).

Induction study: All 30 isolates had an initial vancomycin MIC of 1mg/L. E-test revealed that oxacillin resistance was observed throughout in all the tested isolates. All VSSA isolates were susceptible to daptomycin, linezolid, and tigecycline. All six VISA-max isolates were resistant to daptomycin. Four of these isolates exhibited linezolid resistance, but all remained sensitive to tigecycline (Table 4.12, Appendix II). All but one of the isolates reverted back to their initial susceptibility, upon loss of vancomycin non-susceptibility. Only one isolate, P6 retained the altered resistance against other anti-MRSA agents (Table 4.12, Appendix II).

4.3.2 Vancomyin Non-Susceptibility Induction Phase

Case study: Over the period of extended vancomycin exposure of the day 10 isolate, the MIC reached 20 mg/L by 30 days and remained unchanged until 60 days (Figure 4.2). Exposure of Mu50 resulted in an increase in MIC

TABLE 4.11 MICs to vancomycin and other agents, time of coagulase positivity and % loss of viability following lysostaphin treatment of case study isolate

Stage	Vancomycin MIC (mg/L)			TGC	DAP	LZD	OXA	Tube coagulase avtivity (Detection Time in hours)						Lysostaphin test (% loss of viability)	
	AD	SGE						Dilution ratio						40 mins	60 mins
		EC	TEC					1:1	1:10	1:100	1:1000	1:10000	1:100000		
Day 0 (VSSA)	1	1.2	2.8	0.25	1.5	0.50	>128	0.45	1.0	2.0	8.0	18.0	23.30	58.83	84.60
Day 4 (hVISA)	2	2.8	3.2					6.0	15.0	21.30	NC	NC	NC	44.14	70.70
Day 5	3	3.7	4.2												
Day 6 (VISA)	4	4.2	6.2	0.38	2.0	0.75	>128	16.0	23.30	NC	NC	NC	NC	30.76	56.92
Day 10	6	6.2	7.0												
Day 60 (VISA-max)	20	20	20	2.50	3.0	16.0	>128	23.0	NC	NC	NC	NC	NC	8.08	20.10
Day 81 (VISAΩ)	2	1.8	1.8	0.38	0.75	0.75	>128	2.0	3.0	5.0	NC	NC	NC	50.50	77.30

TGC: Tigecycline; DAP: Daptomycin; LZD: Linezolid;OXA: Oxacillin; NC: No coagulation

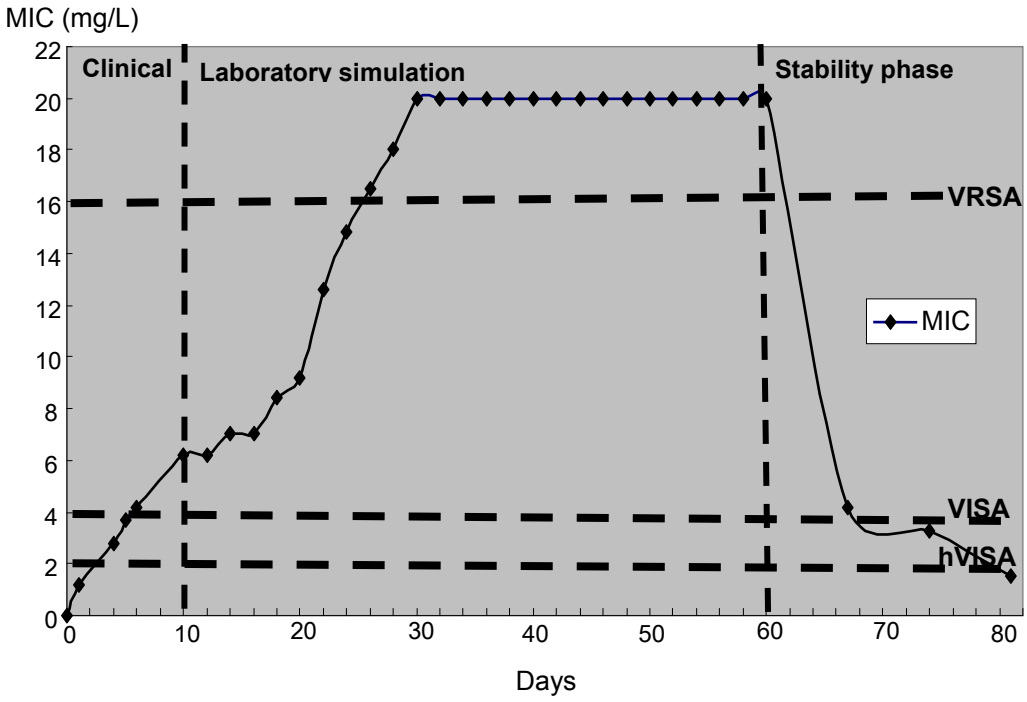


FIGURE 4.2: Vancomycin MIC during development of non-susceptibility and loss

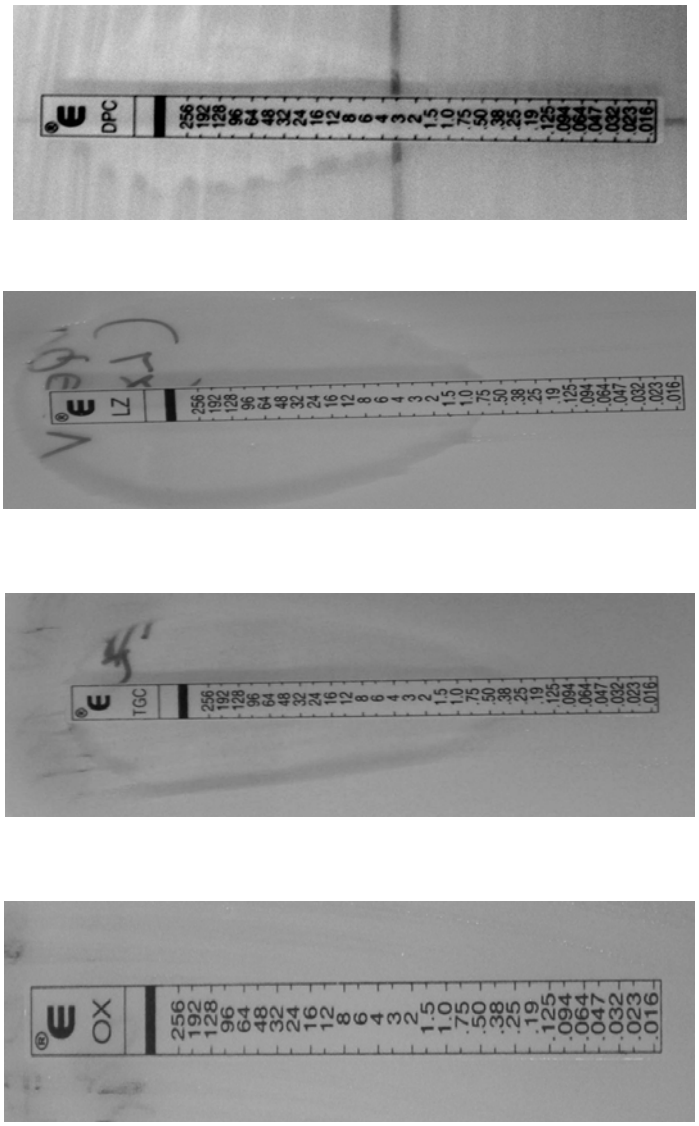


FIGURE 4.3: Etest images of case study isolate at VSSA stage
(See appendix II)

TABLE 4.12: Coagulation positivty time, change in lysostaphin resistance and activity against non-glycopeptide agents

Pattern (n)	Stage	Tube coagulase test						Lyostaphin test		E-test VISA-ax (MIC mg/L)			
		Dilution ratio											
		1:1 (DT)	1:10 (DT)	1:100 (DT)	1:500 (DT)	1:1000 (DT)	1:10000 (DT)	% loss of viability (40 mins)	% loss of viability (60 mins)	OXA	DAP	LZD	TGC
P1 (1)	VSSA	0.5	1.0	1.5	6.0	13.0	23.0	40.0	60.2	>128	0.75	0.50	0.38
	VISA	16.5	21.5	-	-	-	-	28.8	42.2				
	VISA-max	18.0	23.5	-	-	-	-	12.6	24.8	>128	4	1.5	0.38
	VISA Ω	1.0	2.0	5.0	10.5	16.5	-	39.1	58.6	>128	0.75	0.50	0.38
P2 (5)	VSSA	0.5	1.0	2.0	7.0	14.0	23.0	41.5	60.6	>128	0.50	0.50	0.38
	hVISA	7.0	16.5	23.0	-	-	-	36.4	49.3				
	VISA	17.0	23.0	-	-	-	-	24.3	33.8				
	VISA-max	20.0	-	-	-	-	-	12.25	22.25	>128	4	16	0.38
	VISA Ω	1.0	2.0	5.5	10.5	16.0	-	38.4	56.3	>128	0.50	0.50	0.38
P3 (18)	VSSA	0.5	1.0	1.5	7.0	14.0	23.0	46.30	70.7	>128	0.50	0.50	0.38
	hVISA	7.5	17.0	23.0	-	-	-	31.8	58.3				
	VISA	17.0	23.0	-	-	-	-	20.3	36.9				
	VISA-max	19.0	-	-	-	-	-	14.75	21.5	>128	4	16	0.38
	VISA Ω	1.0	2.0	5.5	10.0	17.0	-	42.1	65.3	>128	0.50	0.50	0.38
P4 (1)	VSSA	0.5	1.5	2.0	7.0	14.0	23.0	52.5	70.7	>128	0.75	0.50	0.38
	hVISA	7.0	16.5	23.5	-	-	-	43.0	58.6				
	VISA	17.5	23.5	-	-	-	-	36.7	41.8				
	VISA-max	23.5	-	-	-	-	-	7.6	16.3	>128	4	32	1.0
	VISA Ω	1.0	2.0	6.0	10.5	17.0	-	50.0	66.3	>128	0.75	0.50	0.38
P5 (4)	VSSA	0.5	1.5	2.0	7.0	13.0	23.0	50.5	65.9	>128	0.50	0.50	0.38
	hVISA	7.0	16.5	23.0	-	-	-	43.6	51.9				
	VISA	17.0	23.0	-	-	-	-	29.6	34.5				
	VISA-max	23.5	-	-	-	-	-	6.2	13.8	>128	4	0.75	0.38
	VISA Ω	1.0	2.0	6.0	10.5	17.0	-	50.0	64.3	>128	0.75	0.50	0.38
P6 (1)	VSSA	0.5	1.5	2.0	7.0	14.0	23.0	41.4	62.6	>128	0.50	0.50	0.38
	hVISA	7.0	16.0	23.5	-	-	-	32.9	49.8				
	VISA	17.0	23.0	-	-	-	-	19.5	31.4				
	VISA-max	23.5	-	-	-	-	-	5.8	9.7	>128	4	48	1.0
	VISA Ω	23.5	-	-	-	-	-	6.0	9.3	>128	4	48	1.0

P1-P6 selected strains from each of the pattern of non-susceptibility development and loss described in text

reaching 15 mg/L by day 54 (Mu50-max). During the stability study the VISA-max isolate reverted to susceptibility 21 days after withdrawal of drug pressure. The EC values were generally similar to MICs obtained by AD, but the TEC MIC was considerably higher except for VISA-max (Table 4.11).

Induction study: Although the pace of development varied among the strains, all isolates developed non-susceptibility over the 60 days induction period. The MIC max ranged between 6-20 mg/L. Elevation of MIC above 2 mg/L required between 4-22 days (mean 13 days, median 14 days) and to ≥4 mg/L, ranged from 8-54 days (mean 30 days, median 29 days). A maximum MIC of 20 mg/L was achieved in 6/30 strains and further induction did not result in further increase. Reversion to an MIC in the susceptible range occurred in 29/30 strains within 57 days of removal of vancomycin pressure. However, one isolate retained an MIC of 20 mg/L, forming a stable phenotype. There were no changes in MICs of the control strains over the 60 days drug-free period of passage.

Six patterns of development and loss of non-susceptibility were observed (Figure 4.3). One isolate (P1) only became non-susceptible after 54 days of exposure. At this point, it rapidly lost susceptibility, achieving an MIC >4 mg/L over a 48h period. The isolate that retained non-susceptibility in the absence of vancomycin, (P6), had a gradually increasing MIC over the induction period. Isolates with pattern P4 rapidly gained and lost vancomycin non-susceptibility, also reaching a maximum of 20mg/L (Figure 4.4).

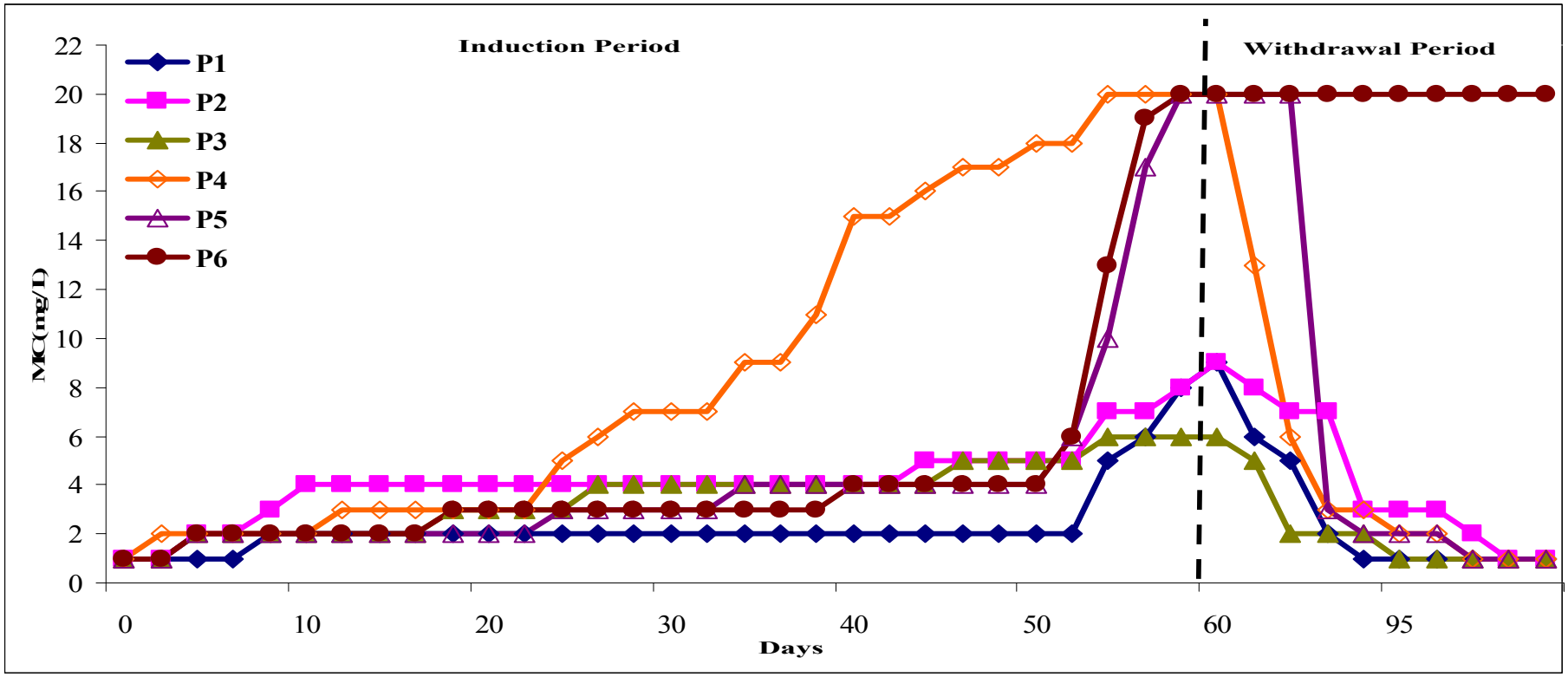


FIGURE 4.4: Vancomycin non-susceptibility development and loss curves

P1-P6 selected strains from each of the pattern of non-susceptibility development and loss described in text.

All isolates were initially sensitive to chloramphenicol, co-trimoxazole, daptomycin, fusidic acid, linezolid, quinopristin-dalfopristin, rifampin and tigecycline, and resistant to ciprofloxacin and erythromycin. Clindamycin sensitivity was only displayed by P1 and gentamicin sensitivity by P1, P5, and P6 isolates. After induction all strains remained sensitive to chloramphenicol and tigecycline, while P1 developed resistance to fusidic acid, quinopristin-dalfopristin and co-trimoxazole, P2 and P5 displayed no change in comparison to initial testing, P3 and P4 exhibited resistance to fusidic acid, and P6 developed resistance to fusidic acid, rifampin, and co-trimoxazole (Table 4.13). Many strains (P2, P3, P4, and P6) developed resistance to linezolid and daptomycin in VISA-max stage and revereted to susceptibility. However, P6 retained the altered resistance as it retained the vancomycin non-susceptibility.

TABLE 4.13: Results of susceptibility testing of all induced VISA isolates against a range of antibiotics

Pattern 1

No.	DA			SXT			FD			CN			LZ			QD			RA		
	α	x	Ω	α	x	Ω	α	x	Ω	α	x	Ω	α	x	Ω	α	x	Ω	α	x	Ω
3	S	S	S	S	R	S	S	R	S	S	S	S	S	S	S	S	R	S	S	S	S

Pattern 2

No.	DA			SXT			FD			Gen			LZ			QD			RA		
	α	x	Ω	α	x	Ω	α	x	Ω	α	x	Ω	α	x	Ω	α	x	Ω	α	x	Ω
1	R	R	R	S	S	S	S	S	S	R	R	R	S	S	S	S	S	S	S	S	S
2	R	R	R	S	S	S	S	S	S	R	R	R	S	S	S	S	S	S	S	I	S
12	R	R	R	S	S	S	S	S	S	R	R	R	S	R	S	S	S	S	S	S	S
20	R	R	R	S	S	S	S	S	S	R	R	R	S	S	S	S	S	S	S	S	S
21	R	R	R	S	S	S	S	S	S	R	R	R	S	S	S	S	S	S	S	S	S

Pattern 3

No.	DA			SXT			FD			CN			LZ			QD			RA		
	α	x	Ω	α	x	Ω	α	x	Ω	α	x	Ω	α	x	Ω	α	x	Ω	α	x	Ω
4	R	R	R	S	s	S	S	S	S	R	R	R	S	S	S	S	S	S	S	S	S
7	S	S	S	S	S	S	S	S	S	R	R	R	S	S	S	S	S	S	S	S	S
8	R	R	R	S	S	S	S	S	S	R	R	R	S	S	S	S	S	S	S	S	S
9	R	R	R	S	S	S	S	S	S	R	R	R	S	S	S	S	S	S	S	I	S
10	R	R	R	S	S	S	S	R	S	R	R	R	S	R	S	S	S	S	S	S	S
11	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
14	R	R	R	S	S	S	S	R	S	R	R	R	S	R	S	S	S	S	S	S	S
16	R	R	R	S	S	S	S	S	S	R	R	R	S	S	S	S	S	S	S	I	S
17	R	R	R	S	S	S	S	R	S	R	R	R	S	R	S	S	S	S	S	S	S
25	S	S	S	R	R	R	S	S	S	R	R	R	S	S	S	S	S	S	S	I	S
26	S	S	S	R	R	R	S	S	S	R	R	R	S	S	S	S	S	S	S	S	S
29	R	R	R	S	S	S	S	R	S	R	R	R	S	S	S	S	S	S	S	I	S
30	R	R	R	R	R	R	S	R	S	R	R	R	S	S	S	S	S	S	S	S	S
35	R	R	R	S	S	S	S	S	S	R	R	R	S	S	S	S	S	S	S	S	S
36	R	R	R	S	S	S	S	R	S	R	R	R	S	S	S	S	S	S	S	I	S
38	R	R	R	S	S	S	S	R	S	R	R	R	S	S	S	S	S	S	S	S	S
39	R	R	R	S	S	S	S	S	S	R	R	R	S	S	S	S	S	S	S	S	S
40	S	S	S	S	S	S	S	S	S	R	R	R	S	S	S	S	S	S	S	I	S

Pattern 4

No.	DA			SXT			FD			CN			LZ			QD			RA		
	α	x	Ω	α	x	Ω	α	x	Ω	α	x	Ω	α	x	Ω	α	x	Ω	α	x	Ω
23	R	R	R	S	S	S	S	R	R	R	R	R	S	R	S	S	S	S	S	S	S

Pattern 5

No.	DA			SXT			FD			CN			LZ			QD			RA		
	α	x	Ω	α	x	Ω	α	x	Ω	α	x	Ω	α	x	Ω	α	x	Ω	α	x	Ω
24	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
28	R	R	R	S	S	S	S	S	S	R	R	R	S	S	S	S	S	S	S	S	S
31	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	I	S
34	R	R	R	S	S	S	R	R	R	R	R	R	S	S	S	S	S	S	S	I	S

Pattern 6

No.	DA			SXT			FD			CN			LZ			QD			RA		
	α	x	Ω	α	x	Ω	α	x	Ω	α	x	Ω	α	x	Ω	α	x	Ω	α	x	Ω
32	R	R	R	S	R	R	S	R	R	S	S	S	S	R	R	S	S	S	S	I	I

α:VSSA, x:VISA-max, Ω:VIS Ω, DA: clindamycin, SXT: co-trimoxazole, FD: fusidic acid, CN: gentamicin, LZD: linezolid, QD: quinopristin-dalfopristin, RA: rifampin, S: sensitive and R: resistant

4.3.3 Molecular Characterization

Sequencing of *vraS, graR* and *rpoB* genes: Only silent mutations were observed in the *rpoB* in all isolates but amino acid changes and presence of stop codons in *vraS* and *graR* genes were observed.

Case study: The sequence analysis of *vraS* and *graR* genes revealed changes in amino acids at F278L and N279I in *vraS* during hVISA development. A mutation V15A occurred at VISA, but reverted at VISA-max, whilst another five silent mutations occurred in *vraS* during the period of MIC increase. In *graR*, a change, N197S, occurred at MIC 4 mg/L, paralleling the change in Mu50. A mutation, L92V, was present in the VSSA isolate, but reverted during non-susceptibility development. Another change D148Q was observed in *graR* when the MIC reached 20 mg/L and remained after reversion. There were four further silent mutations at VISA-max. Loss of non-susceptibility was accompanied by formation of stop codons in both *vraS* and *graR*. Only silent mutations were observed in RpoB at 630AA at all five stages of this strain (Table 4.14).

Induction study: Sequence analyses indicated that mutations in *vraS* and *graR* matched the development and loss of non-susceptibility, although mutation points varied between the strains. An additional change D148Q was observed in *graR* in isolates with MIC 20 mg/L (Table 4.15 - 4.20). The sequences of the control strains matched the genetic conformation of initial stage.

TABLE 4.14: Genetic changes in *vraS, graR, rpoB* genes in the case study strain in comparison to N315

Gene / Stage	***VraS***	***GraR***	***RpoB***
VSSA	Same as N315	CTT301-303GTT (L92V)	Silent mutation at AAC1888-1890AAT (N630N)
hVISA	TTT802-04TTA(F278L), AAT835-837ATA (N279I)	CTT301-303GTT (L92V)	
VISA	TTT802-04TTA(F278L), AAT835-837ATA (N279I) GTA43-45GCA (V15A)	GTT301-303CTT (V92L) ATT589-591AGT (N197S)	
VISA-max	TTT802-04TTA(F278L), AAT835-837ATA (N279I) TTT832-834TTA (F278L)	GTT301-303CTT (V92L) ATT589-591AGT (N197S) GAT442-444CAG(D148Q)	
VISAΩ	*TTA832-834TAA (L278SC), ATA835-837TAA (I279SC)	#AGT628-630TAG (S210SC), GAA670-672TGA (E224SC)	

* Due to deletion of T at 830th NP, # Due to addition of G at 626th NP

Pattern 1 isolates (P1): Only one isolate exhibited this pattern of non-susceptibility development and loss, this strain was characterized by a very slow pace of non-susceptibility development. Sequence analysis of the initial isolate disclosed the presence of stop codons in both *vraS* and *graR* due to the addition and deletion of base C at nucleotide positions (NP) 25 and 27 respectively. On day 54, this isolate developed non-susceptibility with replacement of stop codons by sequences for amino acids in both *vraS* and *graR* and a point mutation resulting in S172L change in GraR (Table 4.15). However, no missense mutations were observed in *vraS*. This strain reverted to susceptibility 21 days after withdrawal of vancomycin by reverting to its initial sequence, including the stop codons.

Pattern 2 isolates (P2): Five strains displayed this pattern of non-susceptibility development and loss, strain number twelve was selected as the representative of this pattern. The initial isolate of the selected strain possessed several silent mutations in both VraS and in GraR (Table 4.16). It developed into hVISA after 6 days of vancomycin exposure and this change in susceptibility was supported by a mutation in VraS with a change of S268P (Table 4.16). By day 10, the strain had developed into a VISA with mutations resulting in changes D182G, D183L, and D189Y in GraR (Table 4.16). An additional mutation, P187L, was observed in VraS at the VISA-max stage. The isolate lost its non-susceptibility and this change in susceptibility was accompanied by formation of stop codons in both *vraS* and *graR* with deletion of A at NP 693 and T at NP 24 respectively.

TABLE 4.15: Genetic changes in *vraS, graR, rpoB* genes in P1 in comparison to N315

Gene / Stage	*VraS*	*GraR*	*RpoB*
VSSA	*AGC49-51TAG (S17SC)	#TTA58-60TAG (L20SC), GTA106-108TAA (V36SC)	Silent mutation at AAC1888-1890AAT (N630N), GGA1971-1974GGT (G658G)
VISA	Same as N315	TCG514-516TTG (S172L)	
VISA-max			
VISAΩ	Reversion to VSSA	Reversion to VSSA	

*Due to addition of C at 25th NP, # Due to deletion of C at 27th NP

TABLE 4.16: Genetic changes in *vraS, graR, rpoB* genes in P2 in comparison to N315

Gene / Stage	*VraS*	*GraR*	*RpoB*
VSSA	Silent mutations at CTA67-69CTG (L23L), GTC142-144GTT (V48V), GCA202-204GCT (A68A), GCT454-456GCA (A152A), CTT508-510CTA (L170L)	Silent mutations at CCT301-303CCG (P101P), GAC439-441GAT (D147D), GAT442-444GAC (D148D), TTT451-453TTC (F151F), CTA454-456CTG (L152L)	Silent mutation at AAC1888-1890AAT (N630N), GGA1971-1974GGT (G658G)
hVISA	Silent mutations as at VSSA, TCA802-804 CCA (S268P)	Same as above	
VISA		Silent mutations as at VSSA, GAT544-546GGA (D182G), GAT547-549TTA (D183L), GAT565-567TAT (D189Y)	
VISA-max	Silent mutations as at VSSA, CCG559-561CTG (P187L), TCA802-804 CCA (S268P)		
VISAΩ	Silent mutations as at VSSA CCG559-561CTG (P187L), * ATG700-102TGA (M234SC)	# TTA58-60TAG (L20SC), GTA106-108TAA (V36SC)	

*Due to deletion of A at 693th NP, # Due to deletion of T at 24th NP

Pattern 3 isolates (P3): Eighteen isolates demonstrated this susceptibility change pattern and strain seventeen was selected as the representative of this pattern. The initial isolate possessed several silent mutations in both VraS and GraR (Table 4.17). Initial non-susceptibility developed within 18 days of drug exposure with a point mutation resulting in F243L in VraS and further increase in MIC to 4mg/L occurred within 26 days of induction with the change of T178P in GraR. Return to susceptibility was accompanied by formation of stop codons by deletion of A at NP 693 and addition of T at NP 142 in *vraS* and *graR* (Table 4.17).

Pattern 4 isolates (P4): Only one isolate exhibited this pattern of change in susceptibility, this strain gained resistance rapidly. The initial isolate possessed additional silent mutations in *vraS* and *graR* along with those noted in P2. This strain developed initial non-susceptibility by 12days of exposure with a change M1K in VraS and attained MIC >4mg/L within 24 days of induction with a change of P45L in GraR, there after gained resistance rapidly reaching an MIC of 20 mg/L by 60 days. Loss of resistance occurred with formation of stop codons by deletion of A at NP 693 in *vraS* and reversion of mutation in *graR* (Table 4.18).

TABLE 4.17: Genetic changes in *vraS, graR, rpoB* genes in P3 in comparison to N315

<table>
<tr><th>Gene / Stage</th><th>VraS</th><th>GraR</th><th>RpoB</th></tr>
<tr><td>VSSA</td><td>Silent mutations at
CTA67-69CTG (L23L),
GTC142-144GTT (V48V),
GCA202-204GCT (A68A),
GCT454-456GCA (A152A),
CTT508-510CTA (L170L),
CCG559-561CCT (P187P)</td><td>Silent mutations at
CCT301-303CCG (P101P),
GAC439-441GAT (D147D),
GAT442-444GAC (D148D),
TTT451-453TTC (F151F),
CTA454-456CTG (L152L)
ATG1-3ACG (M1T)</td><td rowspan="4">Silent mutation at GGA1971-1974GGT (G658G)</td></tr>
<tr><td>hVISA</td><td rowspan="2">Silent mutations as at VSSA,
TTT802-804 TTA(F243L)</td><td>Same as above</td></tr>
<tr><td>VISA
VISA-max</td><td>Silent mutations as at VSSA,
ATG1-3ACG (M1T)
ACT532-534CCT (T178P)</td></tr>
<tr><td>VISAΩ</td><td>Silent mutations as at VSSA,
* ATG700-102TGA (M234SC)</td><td>Silent mutations as at VSSA,
ATG1-3ACG (M1T)
GAT547-549TGA (D183SC),
GAT565-567 (D189SC)</td></tr>
</table>

*Due to deletion of A at 693th NP, # Due to addition of T at 142th NP

TABLE 4.18: Genetic changes in *vraS, graR, rpoB* genes in P4 in comparison to N315

<table>
<tr><th>Gene / Stage</th><th>VraS</th><th>GraR</th><th>RpoB</th></tr>
<tr><td>VSSA</td><td>Silent mutations at
CTA67-69CTG (L23L),
GCA202-204GCT (A68A),
GCT454-456GCA (A152A),
CTT508-510CTA (L170L),
GTG733-735GTA (V245V),
TCA802-804TCG (S268S),
GTA823-825GTC (V275V),
CCG559-561CTG (P187L)</td><td>Silent mutations at
CCT301-303CCG (P101P),
GAC439-441GAT (D147D),
TTT451-453TTC (F151F),
CTA454-456CTG (L152L),
ATG1-3ACG (M1T),
GAT442-444CAG (D148Q)</td><td rowspan="5">Silent mutation at AAC1888-1890AAT (N630N), GGA1971-1974GGT (G658G)</td></tr>
<tr><td>hVISA</td><td rowspan="3">Silent mutations as at VSSA,
ATG1-3AAA (M1K) ,
CCG559-561CTG (P187L)</td><td>Same as above</td></tr>
<tr><td>VISA</td><td rowspan="2">Silent mutations as at VSSA,
ATG1-3ACG (M1T)
CCT133-135CTT (P45L)
GAT442-444CAG (D148Q),</td></tr>
<tr><td>VISA-max</td></tr>
<tr><td>VISAΩ</td><td>Silent mutations as at VSSA,
ATG1-3AAA (M1K) ,
CCG559-561CTG (P187L)
* ATG700-102TGA (M234SC)</td><td>ACG1-3ATG (T1M),
rest reversion to α</td></tr>
</table>

*Due to deletion of A at 693th NP

Pattern 5 isolates (P5): Four strains displayed this pattern of change in susceptibility and strain twenty four was selected as the representative. The initial isolate possessed silent mutations similar to P3. This pattern was typified by a slow increase in the MIC and reaching 3 mg/L by day 24 of exposure with changes I1K and K177R in VraS. By day 34 the strain reached MIC of 5 mg/L with mutation in GraR (D189N), before reaching 20 mg/L by 60 days. An additional mutation was observed in VraS (K1E) at both VISA and VISA-max stage. Loss of resistance was supported by formation of stop codons at 273AA in *vraS* and at AA 20 and AA 36 in *gra*R (Table 4.19).

Pattern 6 isolates (P6): Only one isolate exhibited this pattern of change in susceptibility. The initial isolate possessed silent mutations similar to P3 and retained its non-susceptibility at the end of the study. This isolate was characterized by slow development of non-susceptibility needing 18 days of drug exposure for the change M1K to occur in VraS. Increase in MIC to 4 mg/L was supported by mutations at P181C and S188T in GraR. However, mutation in VraS reverted during VISA-max stage of non-susceptibility development. After vancomycin withdrawal, this isolate did not develop stop codons and retained the maximum MIC of 20 mg/L till the end of stability study (Table 4.20).

TABLE 4.19: Genetic changes in *vraS, graR, rpoB* genes in P5 in comparison to N315

Gene / Stage	*VraS*	*GraR*	*RpoB*
VSSA	Silent mutations at CTA67-69CTG (L23L), GTC142-144GTT (V48V), GCA202-204GCT (A68A), GCT454-456GCA (A152A), CTT508-510CTA (L170L), CCG559-561CCT (P187P), ATG1-3 ATA (M1I), GGT808-810AAG (G270K)	Silent mutations at CCT301-303CCG (P101P), GAC439-441GAT (D147D), TTT451-453TTC (F151F), CTA454-456CTG (L152L) GAT442-444CAG (D148Q)	Silent mutation at AAC1888-1890AAT (N630N), GGA1971-1974GGT (G658G)
hVISA	Silent mutations as at VSSA, ATA1-3AAA (I1K), AAG529-531AGG (K177R)	Same as above	
VISA	Silent mutations as at VSSA, AAA1-3GAA (K1E), AAG529-531AGG (K177R)	Silent mutations as at VSSA, GAT442-444CAG (D148Q), GAT565-567 (D189N)	
VISA-max			
VISAΩ	Silent mutations as at VSSA, AAA1-3GAA (K1E) * GTG817-819TGA (V273SC), GTA823-825TAG (V275SC)	#TTA58-60TAG (L20SC), GTA106-108TAA (V36SC)	

*Due to deletion of A at 781th NP, # deletion of A at 29th and 618th NP

TABLE 4.20: Genetic changes in *vraS, graR, rpoB* genes in P6 in comparison to N315

<table>
<tr><th>Gene / Stage</th><th>VraS</th><th>GraR</th><th>RpoB</th></tr>
<tr><td>VSSA</td><td>Silent mutations at CTA67-69CTG (L23L), GTC142-144GTT (V48V), GCA202-204GCT (A68A), GCT454-456GCA (A152A), CTT508-510CTA (L170L), CCG559-561CCT (P187P)</td><td>Silent mutations at CCT301-303CCG (P101P), GAC439-441GAT (D147D), TTT451-453TTC (F151F), CTA454-456CTG (L152L) GAT442-444CAG (D148Q)</td><td rowspan="5">Silent mutation at AAC1888-1890AAT (N630N), GGA1971-1974GGT (G658G)</td></tr>
<tr><td>hVISA</td><td rowspan="2">Silent mutations as at VSSA, ATG1-3AAA (M1K)</td><td>Same as above</td></tr>
<tr><td>VISA</td><td rowspan="3">Silent mutations as at VSSA, GAT442-444CAG (D148Q), TGG541-543TGC (P181C) AGT-562-564ACT (S188T)</td></tr>
<tr><td>VISA-max</td><td>Reversion to α</td></tr>
<tr><td>VISAΩ</td><td>Silent mutations as at VSSA except at AA 187, CCG559-561CTG (P187L)</td></tr>
</table>

vanA was absent in isolates having an MIC >16 mg/L. *agr-I* was present in 29/30 strains, one was non-typeable. PFGE revealed that all isolates were similar (Type A), strain 24 (A1) and 32 (A2) each differed from type A by one band indicating that these strains were closely related to the type A. Strain 3, differed considerably from the other strains, displaying a pattern unrelated to type A and was designated as type B suggesting no correlation between the PFGE pattern and non-susceptibility development and loss pattern. Each selected set of strains displayed the same macro-restriction lysis pattern of chromosomal DNA with *SamI* at all three stages indicating that the strains were not contaminated (VSSA, VISA-max and VISAΩ) (See appendix II). All isolates were found to harbor *mec*A (VSSA and VISA-max) and SCC*mec*-III.

4.3.4 Phenotypic Analysis

Coagulation time increased with increase in resistance, VISA-max having the greatest delay in coagulation. VISA-max was also more resistant to lysostaphin with low percentage of loss of viability in comparison to VISA and VSSA (Table 4:11 - 4.12). VISA-max isolates displayed very slow growth rates in comparison to their initial isolates (Figure 4.5), with doubling time ranging between 37-200 mins. Although, VISA grew faster then VISA-max, it still grew slower than the VSSA and the doubling time ranged from 30-169 mins, while initial isolates had faster growth rate with doubling time ranged from 21-50 mins, paralleling the genotypic changes.

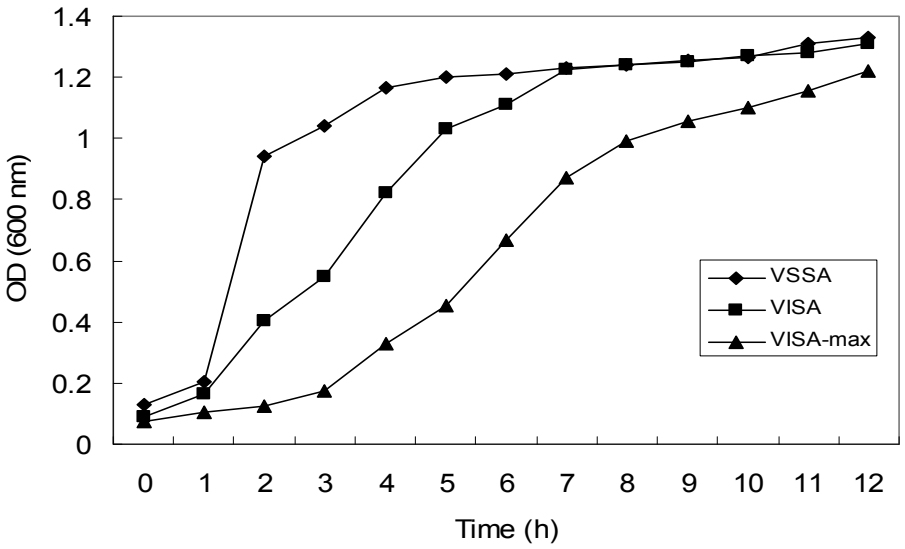


FIGURE 4.5: Comparison of growth rates at different stages of non-susceptibility to vancomycin

4.4 DISCUSSION

Since the first report of VISA, enormous effort has been made to identify a consistent genetic marker in all VISA phenotypes as the lack of a consistent marker has restricted our understanding the complex mechanism associated with vancomycin non-susceptibility and development of a genotypic detection method. This study tracked genotypic and phenotypic changes occurring during the development and loss of vancomycin non-susceptibility in a case study isolate followed by a large scale study of clinical MRSA isolates.

The major findings were: (i) importance of *Vra*S/*Gra*R mutants and (ii) role of stop codons in development and loss of non-susceptibility. In addition, this study has described the role of an additional mutation associated with development of MICs >16 mg/L in the cell wall thickening type of non-susceptibility, which exceeds the CLSI definition of VISA (CLSI 2010).

From most of the case reports, it is evident that whenever a MRSA infected patient fails to respond to vancomycin therapy, a higher dose of vancomycin is prescribed to increase the trough value of the drug as well as to increase the possibility of positive clinical outcome (Denis et. al. 2002). The initial trough value of the drug was low for treatment of endocarditis in this case study and may have contributed to the resistance selection due to the sub-inhibitory dose received by the valve vegetation (Rybak et. al 2009). Replacement of vancomycin with daptomycin followed by addition of rifampin after initial treatment failure was not successful. This treatment as other reports have indicated cross-resistance between vancomycin and daptomycin in *S. aureus* and rifampin is recommended in

combination with an effective anti-MRSA agent (Howden et. al. 2004, Sakoulas et. al. 2006).

Continued *in-vitro* vancomycin exposure resulted in the MIC increasing to a level described as fully resistant (CLSI 2010). Use of SGE allowed for visual identification of a non-susceptible sub-population in the initial isolate of the patient and showed the elimination of heterogeneity of resistance in the VISA-max stage. The sub-population with MIC > 2 mg/L observed in the initial isolate, may have contributed to the rapid development of VISA (Moreillon et. al. 2012).

Tracking vanocmycin MIC in early stage of vancomycin therapy could have revealed the MIC increase and allowed for a timely change to appropriate agent. Failing to change early may severely reduce treatment options and result in negative clinical outcomes. The VISA-max isolates of both the case study isolate and those selected for the induction study were found to be resistant to both linezolid and daptomycin. Daptomycin resistance has been reported in vancomycin non-susceptible strains and has been attributed to a common mechanism leading to cross-resistance between vancomycin and daptomycin in *S. aureus* (Cui et. al. 2006). It is possible this correlation is due to the increase in cell wall thickness affecting the penetration of daptomycin and vancomycin, thus acting as a common obstacle (Cui et. al. 2010). Except for the case study VISA-max isolate, all VISA-max isolates of induction study were sensitive to tigecycline, which may be due to its smaller molecular weight (586Kd) in comparison to vancomycin (1485Kd) and daptomycin (1620Kd), which enabled it to penetrate the thick cell wall, to reach its target 30S ribosome, hinder protein synthesis, and inhibit bacterial growth. Loss of

oxacillin resistance along with deletion of *mecA* has been reported during development of vancomycin non-susceptibility (Adhikari et. al. 2004). However, *mecA* deletion may vary between the strains as the VISA-max isolates remained positive for *mecA,* with no change in oxacillin MIC.

It was observed that some of the VISA-max isolates of induction study developed resistance to some non-glycopeptide antibiotics including co-trimoxazole, fusidic acid, and quinupristin-dalfopristin. This altered susceptibility to other antibiotics may be due to changes in the cell wall thickness affecting permeability rather than the gain of resistance determinants. The lack of alternative therapies for vancomycin non-susceptible strains with high level of vancomycin resistance is of concern (Bhagwat et. al. 2009). As continued exposure to vancomycin after initial VISA development can further increase resistance to other drugs, it is perhaps prudent to change to another effective anti-MRSA agent as soon as the rise in vancomycin MIC is observed to reduce risk of resistance to other possible anti-MRSA agents and negative clinical outcome.

In spite of growing evidence of vancomycin treatment failure, it has been assumed that occurrence of vancomycin non-susceptible *S. aureus* is not widespread (Sadar et. al 2009, Sun et. al. 2009). But an earlier vancomycin non-susceptibility induction study had indicated that 15/18 (3 MSSA/3 MSSA, 12 MRSA/ 15 MRSA) strains developed MIC $\geq$ 4mg/L by the end of the study (Bhateja et. al 2006). In the current study, all isolates developed vancomycin intermediate resistance within 54 days of vancomycin exposure, although the pace of non-susceptibility development varied among isolates. This variation in the development of resistance

has been reported previously in patients failing to respond to vancomycin therapy (Howden et. al. 2006). The median time required to reach an MIC of ≥4 mg/L was found to be 29 days of vancomycin exposure, supporting the association of prolonged clinical exposure with resistance development and treatment failure (Liu et. al. 2011).

Decreased coagulase activity and decreased susceptibility to lysostaphin were uniformaly present in all non-susceptible isolates at different stages of resistance development in comparison to VSSA and VISAΩ isolates. This demonstrates a strong correlation between vancomycin non-susceptibility and phenotypic changes in MRSA (Moreira et. al. 1997). Decreased coagulase activity and decreased susceptibility to lysostaphin in non-susceptible strain may be attributed to increase in the cell wall thickness (Cui et. al. 2003), altered metabolic activity (Avison et. al. 2002) and reduced autolytic avtity (Sieradzki and Tomasz 2003) of hVISA/VISA which may reduce the surface coagulase and also protect the cell from lysotahpin lysis.

Although there were differences in mutations observed between the case study isolate, induced isolates, mu3 and mu50, it appears that mutations in *vraS* is essential for hVISA development and mutations in *graR* for further MIC increment and VISA development. It had been suggested that mutations in RpoB is important in development of vancomycin non-susceptibility (Matsuo et. al. 2011). However, their absence in strains of this study suggests that alternative pathways may exist for resistance development.

Six patterns of non-susceptibility development and loss were observed, with five strains returning to susceptibility and one strain remaining non-susceptible upon withdrawal of vancomycin pressure. Although there were differences in mutation points and sequence configuration among the representative strains from the different non-susceptibility development patterns, it appeared that a mutation / mutations in *vraS* was important for initial development of non-susceptibility and mutations in *graR* contributed to development of VISA. Those isolates which were able to attain a maximum MIC of 20 mg/L had a common mutation at amino acid 1 in VraS. In addition, the initial isolates of selected strains had a mutation at amino acid 148 i.e. aspartic acid was replaced by glutamine. These changes may be important for acquiring high level of resistance to vancomycin with cell wall thickening mechanism). Change D148Q was not observed in Mu50 and Mu50 VISA-max, which had a MIC of 8 mg/L and 15mg/L respectively, but has been reported in other VISA strains (Neoh et. al. 2008) and a similar change was observed in the case study isolate at VISA-max stage that was also able to attain a maximum MIC of 20 mg/L. One strain seemed for some time to be unable to develop non-susceptibility, remaining susceptible with MIC 2mg/L up to day 54. It then rapidly attained non-susceptibility. This sudden change in susceptibility was supported by correction of stop codons to amino acid confirmations in both *vra*S and *gra*R, indicating that the presence of stop codons may delay the cell wall thickening by preventing excess production of the peptidoglycan units associated with intermediate resistance development. This phenomenon in the vraSR/graSR TCS has not been reported in earlier publications. Patients infected with strains having stop codons in *vraS* and *graR* may respond better to vancomycin therapy as

it delays resistance development. However, a further study is needed to confirm this hypothesis.

Other studies have specified the significance of VraSR TCS in modulating cell wall biosynthesis in response to external cell wall stimuli and in resistance development against cell-wall active antibiotics (Kuroda et. al. 2003, Belcheva et. al. 2008). Reports also have suggested that change in *gra*R (S79F or N197S or D148Q) is essential for development of VISA (Neoh et. al. 2008, Cui et. al. 2009). Change of S79F was not found in any of the VISA strains of this study, N197S was observed in the case study isolate, but mutation of D148Q was observed in strains attaining maximum MIC of 20 mg/L. Recently it was reported that mutation in *vra*S is important for development of hVISA followed by mutation in *gra*R being associated with development of VISA (Cui et. al. 2009). This model of resistance development was also observed in the case study isolates as well as in the strains of the induction study, but difference in actual mutations and mutation points was observed.

It was interesting to note that one strain was exceptional in stability of non-susceptibility, all other strains of induction and case study isolate returning to susceptible MIC levels after withdrawal of vancomycin pressure. The rapid return to susceptibility level may be attributed to the fitness cost required to maintain the thick cell wall associated with an intermediate level of resistance. There has been very limited research on stability of non-susceptibility in vancomycin non-susceptible clinical strains other than mu50 (Cui et. al. 2009) and in that case there was a gap of 18 months between isolation of mu50 (VISA) and susceptible mu50Ω

from the same patient. The mu50Ω which was genotypically similar to the initial mu50, but had lost the non-susceptibility with formation of stop codons in *vra*S and reversion of the *graR* gene to the VSSA gene conformation (Cui et. al 2009). Loss of non-susceptibility in strains of the current study was accompanied by novel mutations resulting in formation of stop codons in both *vraS* and *graR*. This may be an attempt for rapid correction of cell wall thickness. In case of mu50Ω, a stop codon was observed only in *vra*S, and not in *gra*R It is possible that a long recovery time may be required for reversion to the initial genetic conformation, as in case of mu50Ω, which was isolated 18 months after isolation of mu50 (Cui et. al 2009). In contrast, mutations resulting in formation of stop codons were absent in the stable strain P6. This suggests that stop codons in *vra*S and *gra*R may delay the development of non-susceptibility as observed in P1 and their formation is important for loss of non-susceptibility. Absence of stop codons upon withdrawal of vancomycin selective pressure may result in stable phenotype formation or strains such as P6 may need longer time to undergo changes resulting in stop codons and loss of non-susceptibility (Cui et. al. 2003) therefore needs further investigation.

It is well accepted that a TCS is based on the delicate balance of kinase and phosphatase activity in histidine kinase (input component). Autophosphorylation from histidine and phosphotransfer to the response regulator (output of the system) and missense mutations in regulatory genes modulate the protein structure and protein-protein interaction resulting in inhibitory / enhanced functioning of TCS (West and Stock 2001, Gao and Stock 2009, Galperin 2010). VraSR GraSR and WalKR TCS are know to regulate cell wall synthesis in response to the stimuli and

mutations in *vra*S and *gra*R genes are known to increase the cell wall biosynthesis and cell wall thickening (Kuroda et. al. 2003, Cui et. al. 2005), while a mutation in *wal*K gene is known to decrease cell wall degradation (Dubrac et. al. 2007, Howden et. al 2011).

Particular amino acid sequence and the resulting protein structure in VraSR and GraSR TCS may increase the pace of cell wall biosynthesis, cell wall thickening and resistance development. Presence of stop codons may decrease the level of response regulator phosphorylation through rapid dephosphorylation resulting in loss of non-susceptibility or delayed resistance development and lack of stop codons in the presence of mis-sense mutation may support the enhanced functioning of TCS resulting in formation of a stable phenotype. This possibly explains the difference in patterns of non-susceptibility development and loss. However, why some strains undergo certain changes of amino acid at a particular position of sequences is unknown and needs further investigation.

In comparison to other studies (Cui et. al. 2010, Matsuo et. al. 2011, Watanabe et. al. 2011), no mutations of importance were observed in *rpo*B during development and loss of vancomycin non-susceptibility. Reports suggest that mutations in *rpoB* were found in most vancomycin non-susceptible phenotypes and may be necessary in non-susceptibility development (Cui et. al. 2010, Matsuo et. al. 2011, Watanabe et. al. 2011). However, absence of such changes in the case study strain as well as in strains of the induction study suggests that either change in *rpoB* is not vital or an alternative pathway can be involved in non-susceptibility development or combinations of changes in selected genetic loci are essential in non-susceptibility

development. A vancomycin non-susceptible strain with mutation in *rpoB* at amino acid 621 has been reported to be resistant to rifampin and the mutation associated with vancomycin non-susceptibility was suggested to be out of rifampin resistance-determining region (RRDR) i.e amino acid 481- 485 (Cui et. al 2010). Other studies have associated the mutations in the RRDR region of *rpoB* with vancomycin non-susceptibility development (Matsuo et. al. 2011, Watanabe et. al. 2011). Intermediate resistance to rifampin was observed in VISA-max stage in the P6 strain, although no mutation was observed in RRDR or at amino acid 621 in any of the stages of non-susceptibility. It is possible that the rifampin intermediate-resistance observed was due to increase in cell wall thickness hindering the penetration of rifampin rather than mutations in *rpoB*; it also appears that mutation in *rpoB* may occur when the strain develops full resistance to rifampin.

A few reports have associated development of vancomycin non-susceptibility with mutations in *wal*KR, *Yvq*F, and *clp*P (Dubrac 2007, Kato et. al. 2010, Shoji et. al. 2011). It is known that WalKR TCS is involved in controlling genes associated with cell wall biosynthesis and degradation. Exhaustion of *wal*KR TCS has been connected with increased peptidoglycan synthesis, lysostaphin resistance, and decreased autolysis (Dubrac et. al. 2007). Kato and coworkers (2010) have associated the development of glycopeptide intermediate resistance with amino acid substitution in both YvqF and VraSR. Recent reports have demonstrated the importance of mutations in TCS *wal*KR, *gra*SR, and *vra*SR (Dubrac et. al 2007, Cui et. al. 2009, Kato et. al. 2010). It is possible that several TCS are involved in vancomycin non-susceptibility development, as genetic changes in *vra*S/*gra*R have

been reported by several researchers; it is likely that changes in these TCS are of important.

Recently, point mutation S337L in *mpr*F has been associated with daptomycin and vancomycin resistance (Boyle-Vavra et. al. 2011). These researchers reported absence of change in cell wall thickness in isolates with reduced susceptibility (Boyle-Vavra et. al. 2011). However, further investigation is required to confirm if low level resistance can occur in strains without associated cell wall thickening. Recent reports suggests *mprF* mutation accompanied with up-regulation of VraSR TCS is involved in modulation of cell wall bio- synthesis in daptomycin resistant strains (Mehta et. al. 2012). Possibly, this substantiates and explains the common mechanism of resistance, as well as the cross-resistance between the two agents. Another recent report indicates that mutation in *mprF* and *dlt* accompanied with mutation in *graR* and *vraS* results in net reduction in surface-positive charge (Cafiso et. al. 2012a), this in turn contributes to reduced susceptibility to cationic antimicrobial peptides and is induced by sub-lethal dose of mammalian platelets, neutrophils and polymyxin B, but not by other cationic agents such as vancomycin, daptomycin and gentamicin (Yang et. al. 2012) suggesting involvement of multiple and interconnected pathways in development of resistance against cationic agents.

Genetic changes in other loci, such as *sig*B, *trf*AB and *tca*A have also been associated with glycopeptide non-susceptibility, particularly with teicoplanin resistance (Singh et. al 2003, Maki et. al. 2004, Renzoni et. al. 2009). Nevertheless, no consistent mutation involved with glycopeptide non-susceptibility development has been identified. Identification of a consistent marker and mutation in all VISA

phenotypes would be beneficial for development of molecular methods for early detection of VISA strains.

So far the genetic changes tracked as well as reported have been conducted on vancomycin non-susceptible *S. aureus* with MIC ≤8mg/L. Although the possibility of strains attaining MIC ≥16mg/L in the absence of *vanA* has been previously reported (Bhateja et. al. 2006), mutations associated with development of MICs ≥16mg/L has not been investigated. An additional mutation, D148Q that was observed in this study was present only in those strains attaining an MIC ≥16mg/L and such change was also observed in case study strain which also reached an MIC of 20mg/L and it appears to be vital for development of high level non-susceptibility. Nonetheless, a detailed investigation of TCS pathways is required to understand what contributes to differences in patterns before reaching the ceiling MIC.

Preliminary studies on vancomycin non-susceptible strains suggested that agr-II was associated with increased risk of development of resistance (Sakoulas et. al. 2003), but later reports have described VISA strains with other agr types. A study has attributed loss of agr to development of non-susceptibility (Sakoulas et. al. 2005). With the exception of one strain which was untypeable, other strains in this study were agr type-I and there was no loss of agr upon development of resistance in contrast to the other reports (Sakoulas et. al. 2003, Sakoulas et. al. 2005).

Phenotypic changes including time to positivity of tube coagulase test and resistance to lysostaphin lysis paralleled the genotypic changes and change in susceptibility category. Similar changes have been observed in these parameters

upon loss of non-susceptibility (Boyle-Vavra et. al. 2001). Investigation of these parameters helped confirm the correlation with increased cell wall thickness and the mutations observed. Notably, the stable phenotype retained phenotypic characteristics of long coagulation time, resistance to lysostaphin lysis and slow growth rate.

In summary, this study confirms the findings that genetic changes in *vraS* are predecessors for development of low level of resistance (hVISA) and is followed by mutations in *graR* to become VISA. This study also suggested that presence of stop codons can delay non-susceptibility development and there is possibility for most MRSA strains to develop vancomycin non-susceptibility as well as formation of a stable phenotype. Genetic changes in VraSR and GraSR TCS appears to be associated with increasing and maintaining non-susceptibility by regulating cell wall bio-synthesis and degradation. Withdrawal of vancomycin selective pressure led to formation of stop codons in both *vraS* and *graR* which seems to be associated with rapid loss of non-susceptibility. The absence of changes in *rpoB* suggests that this pathway is not vital in VISA development. However, it is likely that other consistent genetic targets associated with cell wall biosynthesis and degradation are involved in development of vancomycin non-susceptibility.

Although, findings from this study will help broaden the understanding of mechanism of vancomycin non-susceptibility, establishment of guidelines to prevent further resistance development and design treatment protocols in clinical settings, variation in mutations observed between strains in both development and loss of vancomycin non-susceptibility between this study and other reports

reinforce the need to conduct further studies on TCS pathways to understand determinants associated with different patterns of non-susceptibility development and investigation of other genetic loci associated with non-susceptibility development to identify a consistent genetic marker and to develop molecular detection method.

Publication and Conference Presentation

Doddangoudar VC, Boost MV, Tsang DNC, O'Donoghue MM: Tracking changes in the vraSR and graSR two component regulatory systems during the development and loss of vancomycin non-susceptibility in a clinical isolate. *Clinical Microbiology and Infection.* 2011;17:1268-1272.

Doddangoudar VC, Boost MV, Tsang DNC, O'Donoghue MM: Role of stop codons in development and loss of vancomycin non-susceptibility in MRSA. *Journal of Antimicrobial and Chemotherapy.* doi:10.1093/jac/dks171.

Doddangoudar VC, Boost MV, O'Donoghue MM, Tsang DNC, Appelbaum PC: Development and stability of non-susceptibility to vancomycin in methicillin - resistant *staphylococcus aureus.* 20th European Congress of Clinical Microbiology and Infectious Diseases, Vienna, 2010.

Doddangoudar VC, Boost MV, O'Donoghue MM, Tsang DNC: Tracking development and loss of vancomycin non-susceptibility in methicillin - resistant *staphylococcus aureus*. Western Pacific Congress of Chemotherapy and Infectious Diseases, Singapore, 2010.

CHAPTER 5:DETERMINATION OF THE PREVALENCE OF HVISA/VISA AMONG MRSA IN A LOCAL HOSPITAL

5.1 INTRODUCTION

MRSA has become an important health care burden and a common research theme for various reasons, primarily for its resistance array which severely limits treatment options leaving vancomycin as the only alternative in many cases. Although new agents such as linezolid and daptomycin have been developed, their cost and limited clinical experience means that vancomycin remains the drug of choice. Wide use of vancomycin has paved the way for emergence of strains with altered cell wall and different degree of vancomycin non-susceptibility as described by CLSI (CLSI 2010). In addition, susceptible strains displaying resistant sub-populations (hVISA) have been described (Tenover et. al. 2001). hVISA is a major concern in clinical settings as these are known to be the precursor for VISA (Hiramatsu 2001) and often go undetected with isolate being reported as VSSA if traditional screening methods for non-susceptibility are used. In addition, patients with hVISA infection often receive a sub-inhibitory dose, resulting in chronic, deep-rooted infection, and cross resistance to other anti-MRSA agents, particularly to daptomycin and rifampin (Sakoulas et. al 2006, Rose et. al. 2008). Further, vancomycin treatment failure has been reported even in MRSA strains that are susceptible to vancomycin (MIC <2 mg/L) (Soriano et. al 2008). Thus, timely assessment for non-susceptibility development in patients

receiving vancomycin therapy is important to circumvent negative clinical outcomes.

Since the first report of vancomycin non-susceptible strain in Japan, these phenotypes have been reported from other countries including Hong Kong (Wong et. al. 1999). There has been a growing concern in recent years about increasing prevalence of hVISA, as hVISA has been considered as responsible for vancomycin treatment failure as these strains may progress into VISA in hospitalized vancomycin receiving patients for a prolonged period (Hiramatsu 1998, Wong et. al. 1999). There is a wide variability on the prevalence rate of vancomycin resistant phenotypes in the published literature (<1.0 -65.0%). A recent study has indicated high prevalence level of hVISA, approximately 50% in Australia (Howden 2010). A rate of 9.3% of hVISA/VISA in MRSA isolates were reported at university hospitals in Japan (Hiramatsu et. al. 1997). The prevalence rate of 65.0% of hVISA has been reported in a study carried out at a Spanish hospital in which only MRSA isolates obtained from surgical-site infections of orthopedic operations were included (Ariza et. al 1999). A retrospective study conducted on a *S. aureus* collection over a 22 year period indicated an increase in hVISA prevalence from 2.2 % (1986-1993) to 8.3% (2003-2007) in Detroit (Rybak 2008). A study performed at a French institute indicated 11.0% of hVISA among *S. aureus* and 2.75% of hVISA in MSSA (Garnier et. al. 2006). In Israel 6.0% of patients with MRSA bacteremia were found to be positive for hVISA during 2003-2004 (Maor 2007). There is evidence of increase of hVISA from 1.6% in 1998 to 32.0% in 2001 in a Turkish hospital (Sancak 2005). A low level prevalence of 1.3% has been reported in Canada (Adam et. al 2010) and Korea (Kim et. al 2002).

Although, bacteremia due to hVISA has been reported in Hong Kong at a prevalence level of 5.8% (Wong et. al. 1999), the report was based on the screening of MRSA strains obtained from only blood culture and thus would not provide the accurate prevalence level of hVISA/VISA among *S. aureus* strains. Moreover, the reliability of method used to detect the non-susceptibility is under question. With high prevalence of MRSA strains in Hong Kong (Ip et. al. 2004), it is not surprising that vancomycin MIC creep and hVISA/VISA has been reported in Hong Kong (Wong et. al 1999, Ho et. al. 2010, Ip et. al. 2010). Determining the prevalence of hVISA / VISA will help in formulating guidelines for effective identification and treatment.

Although, both CLSI (2010) and EUCAST (2009) define isolates with vancomycin MIC $\leq$2mg/L as VSSA, vancomycin treatment failure has been reported even in apparently susceptible strains (MIC <2 mg/L) (Soriano et. al 2008). This is attributed to sub-populations of vancomycin non-susceptible cells. Early identification of isolates harboring such sub-populations before and soon after commencement of vancomycin therapy may benefit patients by allowing for change to more appropriate therapy and better clinical outcomes. Thus, this study aimed to determine the prevalence of hVISA / VISA in clinical isolates in Hong Kong, detect presumptive-hVISA isolates likely to rapidly develop non-susceptibility during vancomycin therapy and determine the length of time for these isolates to progress to homogenous resistance in the presence of clinical concentrations of vancomycin.

5.2 EXPERIMENTAL DESIGN

5.2.1 Sample Size

Sample size was determined to give an estimation of the prevalence level of hVISA/VISA that will be representative with current prevalence rate elsewhere.

Sample size determination:

$$n = z^2 \times p\,(1\text{-}p) / m^2$$

Description: Based on the prevalence of hVISA/VISA in Hong Kong

n = Required sample size

z = Confidence level at 95% (standard value of 1.96)

p = Estimated prevalence of hVISA (5.8%)

m = Margin of error at 5% (standard value of 0.05)

The sample size was found to be 84.0 based on the prevalence rate of hVISA/VISA in Hong Kong in 1999. Based on the recent reports of prevalence of hVISA/VISA world wide 1.3% - 50% (lower level and upper level), 25% prevalence was considered for sample size calculation

n = Required sample size

z = Confidence level at 95% (standard value of 1.96)

p = Estimated prevalence of hVISA (25.0%)

m = Margin of error at 5% (standard value of 0.05)

Based on the above formula with p = 25% a sample size of 288 was required but was rounded up to 300. Three hundred and thirty consecutive clinical *S. aureus* isolates from a district hospital and general practitioner clinics that were confirmed to be MSSA and MRSA, were collected during the period of July 2010 - March 2011 and included in this study. One hundred and fifty *S. aureus* (100 MRSA and 50 MSSA) isolates from blood stream infection (BSI), 150 *S. aureus* (100 MRSA and 50 MSSA) isolates from skin and soft tissue infection (SSI) isolated from hospitalized patients and 30 MRSA isolates (SSI) submitted from general practitioners were included to investigate if there was a correlation between the site of infection and vancomycin non-susceptibility as well as between the source and vancomycin non-susceptibility. *S. aureus* from BSI are commonly observed in patients with co-morbidity who would be receiving multiple antibiotics therapy in contrast to patients with SSI, which are caused due to surgical wound or accidents and or due to CA-MRSA among healthy adults. Previous studies have reported hVISA/VISA from MSSA and MRSA (Wong et. al. 1999, Garnier et. al. 2006, Howden et. al. 2006, van Hal et. al. 2011) hence this study included MRSA and MSSA from hospitalized patients as well as MRSA from general practitioner. Duplicate strains for the same patient were excluded.

5.2.2 Screening for Vancomycin Non-Susceptibility

Initial vancomycin MICs were determined by AD and SGE as described in chapter 3. Isolates exhibiting MIC $\leq$2mg/L were defined as VSSA (EUCAST 2009, CLSI 2010) isolates displaying MIC >2mg/L were defined as non-susceptible as these strains are not treatable by a dose within the therapeutic window of vancomycin

(EUCAST 2009) and isolates exhibiting a confluent growth (MIC) below 2mg/L with trailing endpoint (TEC) >2mg/L were defined as presumptive-hVISA.

In order to determine if short term vancomycin exposure would lead to development of resistance, presumptive-hVISA isolates, and as a control, two VSSA strains without resistant sub-populations were incubated in brain heart infusion (BHI) broth (Oxoid, Basingstoke, UK) with vancomycin (Sigma-Aldrich, St. Louis, MO, USA) at clinical concentration of vancomycin (2mg/L) at 37^0C for 48h, passaged to fresh media every 48h. The MIC was determined at weekly intervals by SGE. This process was repeated for a total of 14 days to determine the percentage of presumptive-hVISA that could develop further non-susceptibility to vancomycin over a relatively short treatment period.

In order to correlate the MICs obtained by both SGE and GRD methods as well as to investigate which of these methods are effective in detecting presumptive-hVISA phenotypes. GRD was performed according to manufacturer's instructions. A bacterial suspension corresponding to a 0.5 McFarland standard was swabbed on a MHA (Oxoid, Basingstoke, UK) supplemented with 5% horse blood (Becton, Dickinson and Company, Sparks, MD, USA) (MHB). A GRD E-test strip (AB Biodisk, Solna, Sweden) consisting of vancomycin and teicoplanin was then applied to the MHB plate, incubated at 37^0C for 48h and the plates were read at both 24h and 48h for all presumptive-hVISA on day 0, day 7 and day 14

5.2.3 Susceptibility Testing (ST)

ST was performed for a range of antibiotics including ciprofloxacin, chloramphenicol, clindamycin, co-trimoxazole, erythromycin, fusidic acid, gentamicin, linezolid, quinupristin-dalfopristin, tetracycline and tigecycline (Oxoid, Basingstoke, UK) by disc diffusion method (CLSI 2010) to determine the level of resistance against the above listed antimicrobials as well as to check if there was correlation with ST pattern and SCC*mec* type.

5.2.4 Genotyping

SCC*mec* typing was performed for all MRSA isolates as previously described (Zhang et. al. 2005) to investigate if there was a correlation between vancomycin non-susceptibility prevalence and SCC*mec* type.

agr type was performed for all isolates as previously described in Chapter 4 (Shopsin et. al. 2003) to determine if there was a correlation between vancomycin non-susceptibility prevalence and *agr* type.

5.2.5 Statistical Analysis

Chi square test was performed in order to determine the correlation between the following: 1. vancomycin non-susceptibility and site of infection for MRSA isolates from hospitalized patients, 2. vancomycin non-susceptibility and SCC*mec* type, 3. ST and SCC*mec* type, and 4. vancomycin non-susceptibility and agr type.

5.3 RESULTS

5.3.1 Screening for Vancomycin Non-Susceptibility

All 30 isolates collected from general practitioners were found to be VSSA with MIC ≤1mg/L by both AD and SGE (Table 5.1)

AD: Overall a total of 16.36% (54/330) of isolates had MIC of 1mg/L, 70.90% (234/330) were found to have MIC of 2mg/L, 9.69% (32/330) of isolates had MIC of 3mg/L and 3.03% (10/330) of strains exhibited MIC of 4mg/L (Table 5.1). Overall a total of 12.72% (42/330) of isolates were found to be non-susceptible with MIC >2mg/L by AD method (Table 5.1).

SGE: A total of 15.15% (50/330) of isolates had MIC of 1mg/L, 60.21% (202/330) were found to have MIC of 2mg/L, 10.90% (36/330) of isolates had MIC of 3mg/L, 3.33% (11/330) of strains were found have MIC of ≤4mg/L and 0.30% (1/330) of strains were found to have MIC >4mg/L (Table 5.1). Overall a total of 14.53% (48/330) of isolates were found to be non-susceptible with MIC >2mg/L by SGE method (Table 5.1).

The MIC_{50} and MIC_{90} were found to be 2mg/L and 4mg/L respectively. Statistical analysis indicated that there was no significant correlation between vancomycin non-susceptibility and site of infection for MRSA isolates from hospitalized patients (p= 0.577) (Statistical analysis Appendix III).

TABLE 5.1: Vancomycin MIC of 330 *S. aureus* strains determined by agar dilution and spiral graident endpoint technique

Source of strains	AD-MIC mg/L					SGE-MIC mg/L				
	1	2	3	4	5	≤1	>1 - ≤2	>2 - ≤3	>3 - ≤4	>4 - ≤5
MRSA – Hospitalized patients										
SSI	6/100	81/100	9/100	4/100	0/100	35/100	48/100	13/100	4/100	0/100
BSI	8/100	73/100	16/100	3/100	0/100	5/100	77/100	14/100	4/100	0/100
Total (%)	14/200 (7.0)	154/200 (77.0)	25/200 (12.5)	7/200 (3.5)	0/200 (0.0)	40/200 (21.75)	125/200 (62.5)	27/200 (13.5)	8/200 (4.0)	0/200 (0.0)
MRSA –General practitioner										
SSI (%)	30/30 (100.0)	0/30 (0.0)	0/30 (0.0)	0/30 (0.0)	0/30 (0.0)	0/30 (0.0)	0/30 (0.0)	0/30 (0.0)	0/30 (0.0)	0/30 (0.0)
MSSA – Hospitalized patients										
SSI	7/50	41/50	3/50	2/50	0/50	5/50	38/50	5/50	2/50	0/50
BSI	3/50	39/50	4/50	1/50	0/50	5/50	39/50	4/50	1/50	1/50
Total (%)	10/100 (10.0)	80/100 (80.0)	7/100 (7.0)	3/100 (3.0)	0/100 (0.0)	10/100 (10.0)	77/100 (77.0)	9/100 (9.0)	3/100 (3.0)	1/100 (1.0)
Grand Total (%)	54/330 (16.36)	234/330 (70.90)	32/330 (9.69)	10/330 (3.03)	0/330 (0)	50/330 (15.15)	202/330 (61.21)	36/330 (10.90)	11/330 (3.33)	1/330 (0.30)

BSI: Blood stream infection, SSI: Skin and soft tissue infection

Highlighting represents non-susceptibility.

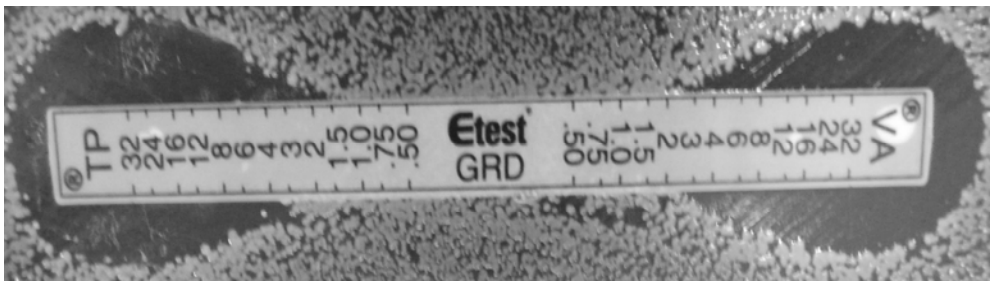
The prevalence rate of hVISA/VISA is approximately 15.0% in a local hospital in Hong Kong and SGE appers to be more sensitive than AD.

In total eight (8/330, 2.42%) isolates were found to be covert resistant, six were from hospitalized MRSA patients of SSI and two from hospitalized MRSA patients of BSI. Following incubation of covert resistant strains with clinical concentrations of vancomycin (2 mg/L), all isolates attained an MIC of 3.2 mg/L (SGE) and showed homogeneous growth by day 7. By day 14 all strains developed further vancomycin non-susceptibility with MIC ranging from 4.12 to 5.29 mg/L (SGE). Initial GRD Etest observations at both 24h and 48h for day 0 strains was 2 mg/L, but resistant sub-populations were observed in only three isolates. MICs obtained by GRD for day 7 and day 14 were slightly lower than those obtained by SGE (Figure 5.1). The control strains with MIC of 1.5mg/L failed to grow after incubation at clinical concentrations (2 mg/L) of vancomycin for 48h (Table 5.2)

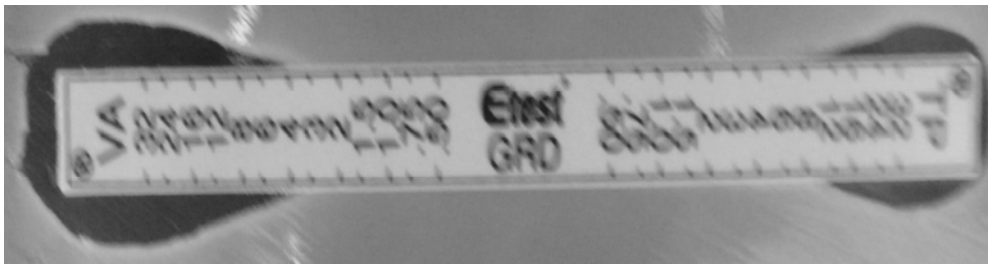
5.3.2 Susceptibility Testing (AST)

All isolates were found to be sensitive to tigecycline and linezolid. Resistance was observed to: quinupristin-dalfopristin: 3.3% (11/330), chloramphenicol: 6.06% (20/330), co-trimoxazole: 9.09% (30/330), clindamycin: 16.69% (56/330), gentamicin: 27.9% (92/330), fusidic acid: 30.0% (99/330), tetracycline: 38.48% (127/330), erythromycin 43.03% (142/330), and ciprofloxacin: 43.63% (144/330) . All MRSA isolates from the community were found to be sensitive to tigecycline, linezolid, quinupristin-dalfopristin, chloramphenicol and clindamycin.

Day 0



Day 7



Day 14

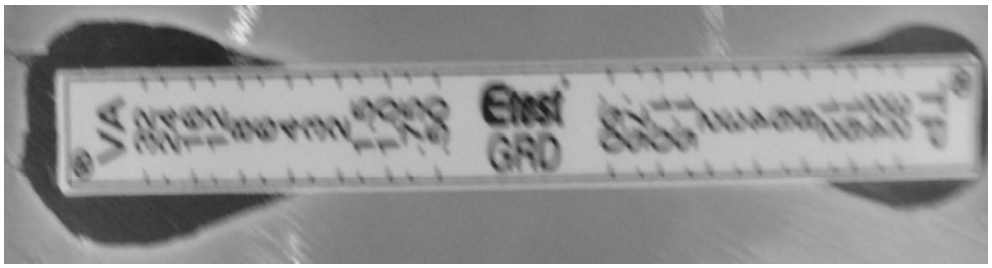


FIGURE 5.1: Representative of GRD Etest images

TABLE 5.2: Vancomycin MIC in MRSA strains with covert resistance by various methods

Test S. No.	Day 0			Day 7			Day 14		
	AD	SGE (EC/TEC)	GRD	AD	SGE (EC/TEC)	GRD	AD	SGE (EC/TEC)	GRD
B53	3	1.51/3.0	*2	4	3.20/3.20	3	4	4.12/4.12	4
B94	3	1.94/3.0	*2	3	3.20/3.20	3	4	4.12/4.12	4
S3	2	1.71/2.5	2	3	3.20/3.20	3	4	4.12/4.12	4
S37	2	1.71/2.2	2	3	3.20/3.20	3	4	4.12/4.12	4
S39	2	1.94/2.2	2	3	3.20/3.20	3	5	5.29/5.29	4
S41	3	1.94/4.12	*2	3	3.20/3.20	3	4	4.12/4.12	4
S50	2	1.94/2.2	2	4	3.20/3.20	3	4	4.12/4.12	4
S72	2	1.51/2.5	2	3	3.20/3.20	3	5	5.29/5.29	4
C1	2	1.51/1.51	2	No growth					
C2	2	1.51/1.51	2	No growth					

*With resistant sub-population.

C1 and C2 are control strains, AD: agar dilution, GRD: GRD Etest, SGE: Spiral gradient endpoint, EC: Endpoint concetration, TEC: Traling endpoint concetration

Note: TEC was absent in SGE in both day 7 and day 14. SGE was found to be sensitive in detecting presumptive-hVISA in comparison to AD and GRD.

Among the community isolates from general practitoner clinics there was considerable resistance to fusidic acid (11/30), erythromycin (12/30), and tetracycline (17/30) with less resistance to co-trimoxazole (2/30), gentamicin (5/30), and ciprofloxacin (8/30) see Figure 5.2. A high percentage of community isolates exhibited resistance to tetracycline in comparison to MRSA and MSSA isolated from hospitalized patient see Figure 5.2. A large percentage of SSI strains were susceptible to most of the drugs when compared to strains from BSI (Figure 5.3).

5.3.3 Genotyping

SCC*mec* Typing

The majority of isolates were found to be SCC*mec* III: 56.08% (129/230), SCC*mec* II: 18.26% (42/230), SCC*mec* IV a: 11.73% (27/230), SCC*mec* IV b: 4.34% (10/230), SCC*mec* IV d: 2.6% (6/230), and 6.95% (16/230) of MRSA isolates were non-typable (Table 5.3). Of the 30 isolates collected from patients from community none were found to be typical HA-MRSA (SCC*mec* Type I, II, III). Only nine MRSA isolates from BSI and four from SSI belong to Scc*mec* Type IV. Statistical analysis indicated correlation between SCC*mec* and ST pattern including clindamycin (p=0.001), gentamicin (p=0.008), and vancomycin (p=0.042) and no correlation was observed between other tested drugs and SCC*mec* (Appendix III).

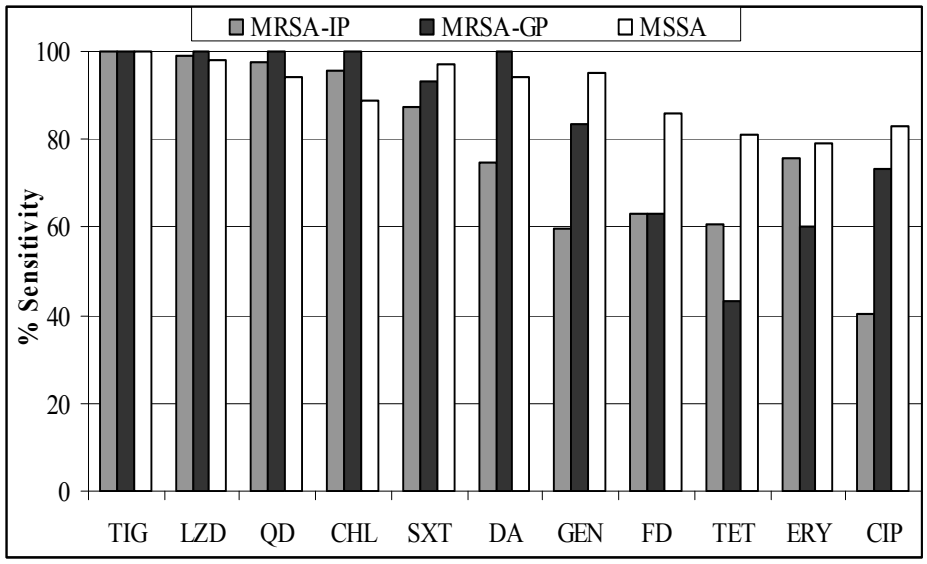


FIGURE 5.2: Percentage sensitivity to a range of antibiotics (Source)

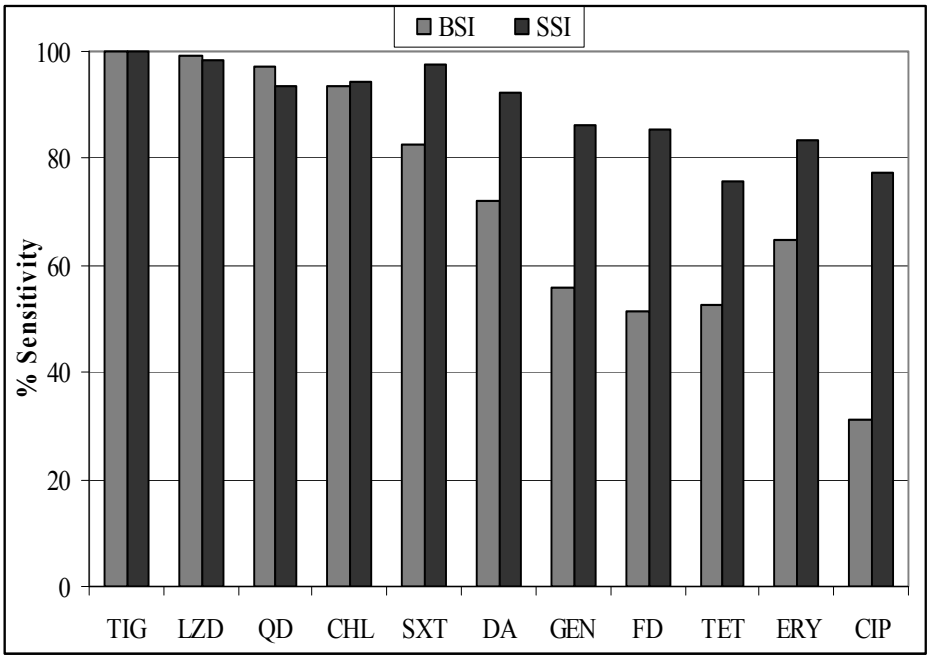


FIGURE 5.3: Percentage sensitivity of *S. aureus* to a range of antibiotics (Site of infection)

Tig=Tigecycline, LZD=Linezolid, QD=Quinupristin-dalfopristin, CHL=Chloramphenicol, SXT=Co-trimoxazole, DA=Clindamycin, GEN=Gentamicin, FD=Fusidic acid, TET=Tetracycline, ERY=Erythromycin, and CIP=Ciprofloxacin, MRSA-IP= MRSA-Hospitalized patients, MRSA-GP= MRSA- General practitioner, BSI= Blood stream infection, SSI= Skin and soft tissue infection

TABLE 5.3: Distribution of SCC*mec* types among clinical isolates

SCC*mec* type / Source	II	III	IV			NT	Total
			a	b	d		
MRSA-Hospitalized							
BSI	19	66	5	3	1	6	100
SSI	23	63	2	1	1	10	100
MRSA-General practitioner							
SSI	0	0	20	6	4	0	30
Total	42	129	27	10	6	16	230

TABLE 5.4: Distribution of *agr* types among clinical isolates

agr type / Source	I	II	III	IV	NT	Total
MRSA- Hospitalized						
BSI	29	32	20	19	0	100
SSI	46	19	14	14	7	100
MSSA- Hospitalized						
BSI	20	17	9	4	0	50
SSI	29	12	3	6	0	50
MRSA- General practitioner						
SSI	9	1	1	19	0	30
Total	133	81	47	62	7	330

agr typing and correlation with vancomycin non-susceptibility

The majority of isolates were found to be *agr* I: 40.30% (133/330), 24.54% (81/330): *agr* II, 14.24% (47/330): *agr* III, 18.78% (62/330): *agr* IV and 2.12% (7/330): were non-typeable as shown in Table 5.4. About of 13.53% (18/133) of *agr* I, 14.81% (12/81) of agr II, 21.27% (10/47) of *agr* III, 8.06% (5/62) of *agr* IV, 42.85% (3/7) of non-typable strains were found to be hVISA/VISA as shown in figure 5.4. Statistical analysis indicated no correlation between vancomycin non-susceptibility and the *agr* (p=0.077) suggesting that vancomycin non-susceptibility could develop among strains with any *agr* type (Ref. Appendix III).

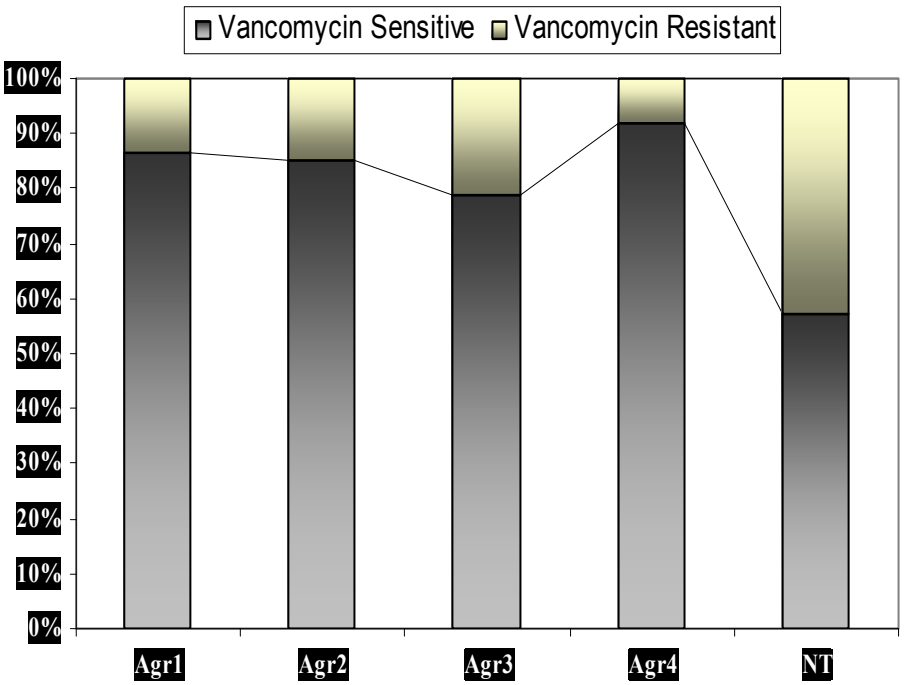


FIGURE 5.4: Correlation between agr type and vancomycin non-susceptibility

5.4 DISCUSSION

This study describes the prevalence rate of vancomycin non-suceptible *S. aureus* in a local district hospital as determined by screening consecutive clinical and community isolates collected druing July 2010 - March 2011. Since VISA strains had been reported previously and MRSA was reported to be prevalent at a high level in Hong Kong (Wong et. al. 1999, Ip et. al. 2004), the study was initiated with the expectation of finding a significant number of strains with reduced susceptiblity. Only 12.72% of isolates were found to be non-susceptible and total of 10 (7 MRSA and 3 MSSA) isolates exhibited an MIC of 4mg/L by AD method. The prevalance level of non-susceptibility was 14.53% by SGE method. As per CLSI (2010) guidelines one MSSA strain with MIC >4mg/L (VISA) was found, but no VISA strain was found among MRSA by this method.

Although the prevalence of vancomycin non-susceptible phenotypes was considerably lower than the prevalence reported elsewhere: 65.0% in Spain (Ariza et. al. 1999), 50.0% in Australia (Howden et. al. 2010) and 32.0% in Turkey (Sancak et. al. 2005), the rate was higher than those reported in Japan (Hiramatsu et. al 1997), Detroit (Raybak et. al. 2008) and Israel (Maor et. al. 2007), comparable to that of France (Garnier et. al. 2006) though considerably higher than those of Canada (1.3%) (Adam et. al. 2010) and Korea (1.3%) (Kim et. al. 2002). These variations in prevalence may be due to differences in sample size, regional differences of the *S. aureus* strains, difference in screening methods used, due to the difference in antibiotics used commonly, and or variation in definition for vancomycin non-susceptible phenotypes. It was observed that SGE had a higher

sensitivity than AD and GRD methods and hence screening methods used to detect reduced vancomycin susceptibility may also account for the differences.

While reports of hVISA strains are increasing considerably, their accurate detection is still controversial as there are no clear guidelines issued. Currently, PAP-AUC method is the gold standard for detection of resistant sub-population, but it is laborious and unsuitable for routine screening in clinical microbiology laboratories (Walsh and Howe 2002). GRD-Etest is a simple test and is known to have good sensitivity and specificity (Yusof et. al. 2008, Leonard and Rybak 2009). The findings from this study indicate that the MICs obtained by both SGE and GRD Etest were similar, but resistant sub-population was observed in teicoplanin in only 3/8 strains after 48hrs of incubation. This suggests that SGE may be a rapid and effective method for the detection of resistant sub-population in presumptive-hVISA. However, a further large scale comparative study is needed to confirm an effective detection method for presumptive-hVISA and to provide a standardized method for rapid and reliable detection of strains with heterogeneous sub-populations.

All eight strains meeting the criteria of possible hVISA (EC < 2mg/L - TEC MIC >2mg/L) attained an MIC of 3.2 mg/L by day 7 and by day 14, all of these isolates had MIC $\geq$4mg/L. The control strains failed to grow at clinical concentrations of vancomycin; these strains had a similar EC to the possible hVISA strains but did not exhibit (TEC) a resistant sub-population in SGE. These data support the hypothesis that strains with heterogeneous sub-population are precursors for VISA development as the resistant sub-population with

MIC >2mg/L would receive a sub-inhibitory dose resulting in selection for resistant portion of the population leading to VISA development and possible vancomycin treatment failure. Frequently, vancomycin therapy lasts for several weeks and this study has deomstrated that a exposure to sub-inhibitory dose for even 7 days promotes resistance selection. Possibly, this explains the rapid development of VISA and also supports the findings of vancomycin treatment failure in strains with vancomycin MIC <2 mg/L as previously reported (Soriano et. al. 2008).

The findings from this study indicate an increase in prevalence rate of vancomycin non-susceptible phenotypes in Hong Kong from 5.8% as reported in 1999 (Wong et. al. 1999) to approximately 15.0%. More recently vancomycin MIC creep has been reported in Hong Kong, showing an increase in percentage of isolates with an MIC equal to 1 mg/L from 10.4% to 38.3% in 12 years (Ho et. al. 2010). Reports on vancomycin MIC creep among MRSA over time have indicated conflicting results between studies performed at various centers. Whilst, some studies have demonstrated MIC creep over time (Rhee et. al. 2005, Robert 2006, Steinkraus et. al. 2007, Wang et. al. 2007, Hawser et. al. 2011, Kehrmann et. al. 2011, Zhao et. al. 2012) but this phenomenon was not been observed by others (Alos et. al. 2008, Sader et. al. 2009, Edwards et. al. 2012).

Various factors have been proposed to explain the inconsistent findings, including the storage conditions of historical isolates, region of origin and susceptibility testing methods used. Edwards et. al. (2012) indicated a significant difference between the MICs obtained for the same samples by different susceptibility testing

methods. Their study has also indicated that both Etest and MBD to be superior to automated methods. Other workers have suggested including AUC-PAP to determine the susceptibility trends in addition to the traditional methods (Steinkraus et. al. 2007). Although, AUC-PAP is laborious reports indicate correlation between vancomycin MIC increase and prevalence of hVISA, since 50.0% of MRSA strains with vancomycin MIC of 2 mg/L are reported to be hVISA by AUC-PAP method (Horne et. al. 2009) demonstrating the effectiveness of AUC-MIC in determining the hVISA. In order to determine if MIC creep is a real phenomenon; susceptibility test need to be performed on both historical samples and the current samples at the same time and with same method, and the method has to be sensitive to show small increments, therefore gradient method should be a preferred to stepwise methods. However, it is reported that strains stored in freezer may loos their non-susceptibility making it difficult to compare the MIC of historical samples.

In the current study, several isolates were resistant to other non-glycopeptide antibiotics incluiding quinopristin-dalfopristin, chloramphenicol, co-trimoxazole, clindamycin, and considerable proportion of isolates were resistant to gentamicin, fusidic acid, erythromycin, tetracycline, and ciprofloxacin. The majority of MRSA (obtained from hospitalized patients) strains with altered susceptibility possessed SCC*mec* II or SCC*mec* III, which may be due to the nature of the SCC*mec* type as these SCC*mec* types are known to be bigger and are able to accommodate a greater number of genes conferring resistance to various antibiotics and also indicates the influence of the environment as these strains will have greater exposure to selective pressure such as antibiotics and disinfectants (Ito et. al. 2001, Ma et. al.

2002). Most of the strains exhibiting vancomycin MIC >2mg/L also displayed resistance to other non-glycopeptide antibiotics and these changes in susceptibility may be associated with changes in the cell wall thickness affecting permeability. In this respect, both tigecycline and linezolid appear to be superior when the vancomycin MIC >2mg/L and further study is required to clarify the effectiveness of other new anti-MRSA agents on strains with vancomycin MIC >2mg/L and the strains would need to be tested for presence of resistant determinants.

Although, CA-MRSA has a greater spectrum of antimicrobial susceptibility including tigecycline, linezolid, quinpristin-dalfopristin, and chloramphenicol, the data from this study suggests that vancomycin and clindamycin are likely to be effective against CA-MRSA as most of the isolates were resistant to tetracycline and some to co-trimoxazole, gentamicin, fusidic acid, ciprofloxacin, and erythromycin. The high level of resistance against tetracycline may be due to frequent use of these antibiotics and the low level of resistance against clindamycin may be due to the fact that it is rarely used mainly due to the adverse effect (pseudomembranous colitis) (Rossi 2006). However this condition is more commonly observed hospitalized patients infected by *Clostridium difficile*, as *C. difficile* is inherently resistant to clindamycin, resulting in the production of a toxin that causes a range of adverse effects incluiding pseudomembranous colitis (Rossi 2006). Considering the fact that vancomycin needs to be administered by i.v route which needs hospitalization, SXT could be considered as an effective alternative for treatment of out-patient CA-MRSA infections as this drug can be administered by both oral and topical route of administration.

Although, reports suggest an association between *agr*-II and vancomycin non-susceptibility (Sakoulas et. al. 2003, Sakoulas et. al. 2005), the findings from this study suggest that strains possessing other *agr* types can develop non-susceptibility and *agr*-III positive isolates were found to have greater level of resistance against vancomycin.

Several reports have described CA-MRSA as a cause of nosocomial infection (Seybold et. al. 2006, Patel et. al 2008). CA-MRSA has caused 34% of healthcare-associated bloodstream infections in USA (Seybold et. al. 2006). In this study around 13.0% of clinical MRSA isolates were SCC*mec* IV, 9.0% of healthcare-associated BSI and 4.0% of healthcare-associated SSI which may be CA-MRSA. It is possible that these strains were introduced to healthcare environment by patients who were hospitalized. Although, the prevalence of CA-MRSA in this study is lower than the prevalence level reported in Brazil (75%) (Trindade et. al. 2005) and USA (34%) (Seybold et. al. 2006, Patel et. al. 2008), these findings demonstrate that CA-MRSA strains are moving into the hospital environment in Hong Kong and are able to cause bacteremia and soft-tissue infections in hospital settings as observed elsewhere (Trindade et. al. 2005, Seybold et. al. 2006, Patel et. al 2008).

Although, strains with MIC $\leq$2mg/L are defined as VSSA by EUCAST and CLSI, it is important to define isolates with resistant sub-population with MIC $\leq$2mg/L as non-susceptible. To select an effective agent and control further resistance development, a reliable screening method for isolates with resistant sub-population needs to be a part of the routine susceptibility testing of clinical strains. Since non-

susceptible strains were found in both MRSA and MSSA, as reported elsewhere (Garnier 2006), it appears that screening all *S. aureus* isolates is practical rather than only MRSA for the following reasons. Firstly, it is more practical to screen all *S. aureus* isolates because phenotypic screening of MRSA would require a pre-testing for oxacillin susceptibility, causing a delay in the final report. Parallel testing for vancomycin non-susceptibility can be performed for epidemiological purposes without delaying the final report. Secondly, to provide an effective dosage regimen for treatment of MSSA infections and finally, vancomycin non-susceptibility in MSSA has been reported (Garnier et. al. 2006) and similar data was observed in this study, suggesting the need for screening for vancomycin non-susceptibility in both MSSA and MRSA.

The vancomycin non-susceptibility among MSSA may be due to use of β-lactam antibiotics (imipenem) to treat *S. aureus* infections and this might be one of the risk factors for development of hVISA/VISA as suggested previously (Katayama et. al. 2009, Kato et. al. 2010) and more recently it is reported that hVISA had emerged before the clinical introduction of vancomycin (Yamakawa et. al. 2012).

This study has demonstrated that the resistant sub-population would grow at the possible attainable plasma drug concentration and this study concludes that vancomycin can be considered as a drug of choice only when the MIC of the isolate is <1 mg/L with no resistant sub-population, as the possibility of attaining the effective PK/PD of 350 index is only 40%-60% when MIC is >1 mg/L and is unattainable when the MIC is 2 mg/L with trough serum concentration of 10-15 mg/L (Soriano et. al. 2008). Considering the findings from this study and the

reports of vancomycin treatment failure in strains with vancomycin MIC ≤2 mg/L and PK/PD parameters of vancomycin (Sariono et. al. 2008), it should be strongly considered to define the strains as resistant if the MIC is <2 mg/L but exhibits a resistant sub-population of MIC >2mg/L, it would appear that these strains are not treatable with the recommended clinical concentration of vancomycin and a clear definition is needed to avoid clinical confusion and vancomycin treatment failure as well as to improve patient management.

In summary, 15% of *S. aureus* isolates from a local hospital were vancomycin non-susceptible with a further 2.4% of strains being presumptive-hVISA. This study suggests that SGE is an effective screening tool for detection of resistant subpopulation of apparently VSSA strains. SGE could be cost-effective in detection of presumptive-hVISA within 24h. The significance of early and accurate detection of such strains has been demonstrated through exposure of presumptive-hVISA strains to clinical concentration of vancomycin which led in rapid development of further resistance resulting in VISA phenotypes. Accurate and early detection of presumptive-hVISA isolates could have significant clinical implications for patient management during treatment of MRSA infections. A further large scale study is needed to confirm the effectiveness of SGE for this use in the clinical laboratory.

Publication and Conference Presentation

Doddangoudar VC, O'Donoghue MM, Tsang DNC, Boost MV: Early detection of vancomycin resistant sub-population in clinical MRSA with MIC <2mg/L. 22nd European Congress of Clinical Microbiology and Infectious Diseases, London, 2012

CHAPTER 6:THE EFFECT OF SELECTED TCM HERBS ON VISA AND MRSA

6.1 INTRODUCTION

Since the introduction of modern chemotherapy, antimicrobial agents have been able to effectively control and treat majority of infectious diseases. However, in recent years the increasing development of antimicrobial resistance has led to reduced efficacy of antibiotics, resulting in reduced availability of effective antibiotics for some infections. Multi-drug resistant bacteria, such as MRSA, have become a major problem worldwide. Wide use of vancomycin has led to reports of gradually increasing MIC, "MIC creep", decreased susceptibility, increasing morbidity and treatment failure (Robert et. al. 2006, Soriano et. al. 2008).

Although, non-susceptibility to vancomycin developed slowly in comparison to that of other antibiotics, such as penicillin, its clinical value is now being increasingly challenged with increasing number of reports of vancomycin non-susceptibility and treatment failure (Lodise et. al. 2008, Sakoulas and Moellering 2008). Therefore, alternative antimicrobial agents need to be developed and employed for effective treatment of MRSA and VISA, as well as to prevent further resistance development. However, the cost and the difficulty involved in identifying, characterizing and licensing new antibiotics have dramatically lowered the number of new antimicrobial agents approved for marketing (Alanis 2005).

In the last three decades, only two new classes of anti-MRSA agents having novel mechanisms of action have been introduced into the market. Daptomycin, a

lipopeptide antibiotic is known to exhibit anti-MRSA activity by depolarizing the cytoplasmic membrane (Schriever et. al. 2005, Hawkey 2008). However, daptomycin non-susceptibility in MRSA has been reported (Mangili et. al. 2005). Moreover, daptomycin cross-resistance with vancomycin has resulted in its reduced usefulness in the treatment of MRSA (Cui et. al. 2006b). Linezolid, an oxazolidinone class of antibiotic exhibits its anti-MRSA effect by inhibiting protein synthesis (Fung et. al. 2001). However, adverse effects for the patients and resistance development during therapy have been reported (Wilson et. al. 2003, Huang et. al. 2008). Although, Motrin et. al. (2007) have reported that linezolid is less effective in comparison to daptomycin in treatment of gram positive infections, cross-resistance between linezolid and other available antibiotics has been reported to be less likely (Wilson et. al. 2003), an advantage in comparison to daptomycin.

Development of only two new classes of anti-MRSA agents in recent years suggests that antimicrobial drug development is much slower than the emergence and spread of resistant phenotypes. This situation is leading to more research in development of antimicrobial agents with novel mechanisms of action through investigation of natural products for the development of novel antimicrobial agent.

A substantial amount of research has been conducted in the field of antimicrobial biochemicals produced by microbes, plants, insects and animals, very few have made it to stage III clinical trial and no molecule has been recently introduced to market, prompting the need of further research to identify new anti-MRSA and anti-VISA agents.

In recent years, several antimicrobial phytochemicals have been isolated and are considered to be possible alternatives for future therapy (Bonjar et. al 2004). Polyphenols from green tea have been reported to exhibit bactericidal activity against various Gram-positive and Gram-negative bacteria (Akiyama et. al. 2001, Friedman 2007, Cho et. al. 2008). Epigallocatechin gallate was reported to act synergistically with various β-lactam antibiotics against MRSA (Akiyama et. al. 2001, Hu et. al. 2001, Hu et. al. 2002). Recently, Chao et. al. (2008) have reported anti-MRSA activity of essential oils including cinnamon, thyme, ginner, clove, coriander and lemon grass. A study has suggested that flavonoids of *Acacia aroma* possess antimicrobial effects mainly on MSSA and MRSA (Mattan et. al. 2010). Kuzma et. al. (2007) have demonstrated the anti-biofilm and antimicrobial effect of diterpenoids from *Salvia sclarea* against gram positive bacteria. Several antimicrobial peptides have been identified and some of them are in phase II or III of clinical trials but may not be available in the market for sometime.

Loss of avaliable antibiotics is a serious concern as vancomycin resistance emerges. A strategy to treat such resistant strains and improve the efficacy of current antibiotics is to use of combination of antibiotics. Antibiotic combinations may reduce the likelihood of developing resistance against more than one drug simultaneously. Also, combination of antibiotics can lead to an additive effect or even antimicrobial synergy (Eliopoulos and Moellering 1996). Several studies have described synergistic effects of herbs when used in combination with current antibiotics (Akiyama et. al. 2001, Hu et. al. 2001, Basri et. al. 2008). Hence, it is reasonable to investigate Traditional Chinese Medicinal (TCM) herbs for their

potential to extend the clinical value of current antibiotics by combining them with plant extracts.

The Fractional Inhibitory Concentration Indices (FICI) is a popular approach to quantify drug interactions. FICI is the sum of concentration of each drug in combination that produces inhibitory effect and is expressed as a fraction of the concentration that inhibits growth when the drug is used alone: FICI = (MIC of drug A in combination/MIC of drug A alone) + (MIC of drug B in combination/MIC of drug B alone). If the sum of the FIC is equal to 1, the interaction is additive; if the sum is less than 1, the interaction is synergistic; and if the sum is greater than 1, the interaction is considered to be antagonistic (Warnock 1989). Considering the conclusions as additive and partial synergy applied to FICI value slightly above or below the critical cut-off value of 1.0 appears to put a positive spin on findings, such as within the limits of experimental error and is rarely indicated as no interaction between the combination of agents (Odds 2003). In order to encourage conservative interpretation of results, recently the description for interaction has been revised and the interactions between the agents has been defined as, 'synergy' (FICI $\leq$ 0.5), 'antagonism' (FICI > 4.0) and 'no interaction' (FICI > 0.5–4.0) (Odds 2003).

There has been increased acceptance of traditional medicine as a complementary system in health care to treat various diseases. *Cortex phellodendri, Rhizoma coptidis* and *Radix scutellariae* have a long history of use in TCM and are used to treat various disorders including infections (Li et al. 2000, Li et al. 2005). Therefore, this study aimed to investigate the anti-MRSA, anti-VISA and

vancomycin resistance modifying potential of *C. phellodendron*, *R. coptidis* and *R.scutellariae.* The next section will briefly describe the experimental design of this study.

6.2 EXPERIMENTAL DESIGN

6.2.1 Strains

A total of fifty *S. aureus* isoaltes, including three control strains [MSSA: NRS149; MRSA: NRS100 and VISA: NRS1], two clinical MRSA isolates (vancomycin MIC 1.5 mg/L), eight clinical MRSA isolates (EC<2 mg/L, TEC>2 mg/L), thirteen clinical hVISA isolates (EC>2 mg/L, TEC>2-4.1 mg/L), Eighteen VISA isolates (MIC 4-16 mg/L) and six laboratory induced VISA-max isolates (MIC >16 mg/L) were tested. Their MIC was detemined by agar dilution and SGE method as described in Chapter 3, VSSA and VISA were defined based on CLSI criteria; whereas hVISA were defined as strains having an MIC >2 but <4 mg/L and / or displaying a resistant sub-population by SGE method and isolates exhibiting MIC >16 mg/L were defined as VISA-max. All the isolates were stored as described in Chapter 3.

6.2.2 Screening Methods for Identification of Antimicrobial Activity of TCM Herbs

The agar dilution method was used as a screening tool to study the inhibitory effects of the individual herbs alone as the method is widely used in the initial stage of a search of new antimicrobial agents.

The spiral gradient endpoint technique was used for MIC determination of the combination of an herb and vancomycin, as the method was found to be valuable in providing exact vancomycin MIC of MRSA and in detection of hVISA/VISA as described in Chapter 3. Most importantly SGE is beneficial in detecting hVISA phenotpyes as the presence of resistant sub-population can be easily observed, while VISA strains can be identfied based on the clear end point with MIC $\geq$ 4 mg/L. Therefore, SGE was considered suitable to investigate the synergistic effect of the test herb extract when combined with vancomcyin as the vancomycin stock solution can be deposited on agar plates containing the herb to be tested.

6.2.3 Preparation of Herb Extract

The commercial preparations of *C. Phellodendri*, *R. coptidis* and *R.scutellariae* were obtained from a Good Manufacturing Practice accredited manufacturer in China (Guangdong Yifang Pharmaceutical Co. Ltd.). Whilest herb content could be guaranteed the level of phytochemicals may vary from batch to batch. In this study the samples of herb tested were all taken from the same batch. Three grams of herb powder was accurately weighed and added into 60.0ml of sterile water to make a 50.0 g/L stock solution. The contents were well mixed and then incubated in a water bath at 80^0C for 2.0h to dissolve completely. The stock solutions were then sterilized by filtration using a 0.22µm syringe filter (Opticap XL 10 Capsule Filters, Millipore) and stored at -80^0C until further use. Vancomycin stock solution of 1072 mg/L was prepared as described in Chapter 3 and used to detemine if the herb extracts were able to restore the effectiveness of vancomycin by reducing the vancomycin MIC of test isolates to <2 mg/L.

6.2.4 Preparation of Herb Agar

For each of the herbs to be tested, agar with different concentrations of herb were prepared by adding a calculated volume of herb extract to molten BHA (50-60^{0}C) and poured into a 10cm petri dish (Table 6.1), allowed to set and used to investigate antimicrobial activity alone and in combination with vancomycin.

If any of the herbs exhibited the desired effect at the lowest concentration then the herb was examined at lower concentrations (0.060 g/L and 0.125 g/L) in order to determine the exact minimum effective concentration for the effect exhibited by the herb.

The interactions between the herbs and vancomycin were defined as synergistic if FICI $\leq$ 0.5 and antagonistic if the FICI > 4.0. A FICI of >0.5-4 was defined as no interaction as described by Odds (2003). FICI were determined by adding the FIC values of vancomycin and herb as described previously by Warnock (1989):

FICA = Vancomycin MIC in combination with herb / MIC Vancomycin

FICB = Herb MIC in combination with vancomycin / MIC Herb

FICI = FICA + FICB

TABLE 6.1: Preparation of agar with herb extract for use in both AD and SGE

Reagents	Concentration of herb agars (g/L)								
	0.0	**0.25**	**0.5**	**1**	**2**	**3**	**4**	**5**	**6**
SS-I (ml)	0.0	0.1	0.2	0.4	0.8	1.2	1.6	2.0	2.4
BHA ml)	20.0	19.90	19.80	19.60	19.20	18.80	18.40	18.0	17.60

6.2.5 Bacterial Suspension

Bacterial suspensions were prepared by incubating the test isolates at 37^0C for 2hrs in BHI broth and adjusting to a turbidity equivalent to that of a 0.5 McFarland standard. In order to study the desired effect of the test herbs by agar dilution method, the 0.5 McFarland standard bacterial suspension was deposited onto the herb agar using multi-point inoculator (Mast Co. Ltd, UK). To investigate any interaction between the herb and vancomycin (synergistic effect), 50 µl of vancomycin stock solution was deposited using spiral plater. After 1h the bacterial suspension was swabbed across the vancomycin gradient of the plate as described in Chapter 3 and the plates were incubated at 37^0C for 24hrs.

6.2.6 Effect of pH

The pH of the most effective herb extract was determined and was adjusted to pH 5.6 and pH 7.0 by using phosphate buffer in order to investigate the effect of pH on antimicrobial activity. In order to eliminate the possibility of the antimicrobial effect being due to its ability to create an acidic or alkaline environment, the pH of the agar was measured for 20 ml of BHA alone and after supplementation with 6 g/L liter of herb extract for all three pH.

6.2.7 Statstical Analysis

One-way repated ANOVA was performed to compare if there was reduction in vancomycin MIC with increase in herb concentration and paired student t-test was performed to find the most effective concentration of the herb.

6.3 RESULTS

6.3.1 Antimicrobial activity of *Cortex phellodendri* alone and in combination with vancomycin

The combination of *C. phellodendri* of 5.0 g/L and vancomycin exhibited complete inhibition of growth of all isolates, while 3.0 g/L of *C. phellodendri* with vancomycin resulted in reduction of vancomycin MIC to susceptible level (<2.0 mg/L) for all isolates (Table 6.2). *C. phellodendri* alone exhibited a strong anti-MRSA effect with MIC_{90} of 2.0 g/L and anti-VISA activity with MIC_{90} of 6.0 g/L. A combination of *C. phellodendri* and vancomycin can restore the efficacy of vancomycin against VISA isolates as shown in Table 6.2 and Figure 6.1 for two representative strains.

One-way repeated ANOVA showed significant reduction in vancomycin MICs in comparison to the initial MIC (P = <0.001). Paired student t-test showed that a concentration of 3.0 g/L of *C. phellodendri* in combination with vancomycin was found to be effective in reducing the vancomycin MIC to susceptible levels (P = <0.001) demonstrating a significant change in vancomycin susceptibility for the isolates (statistical analysis are as shown in Appendix IV). The FICI was found to be 0.55 indicating no interaction between *C. phellodendri* and vancomycin.

TABLE 6.2: Results of antimicrobial activity of *Cortex phellodendri* alone and in combination

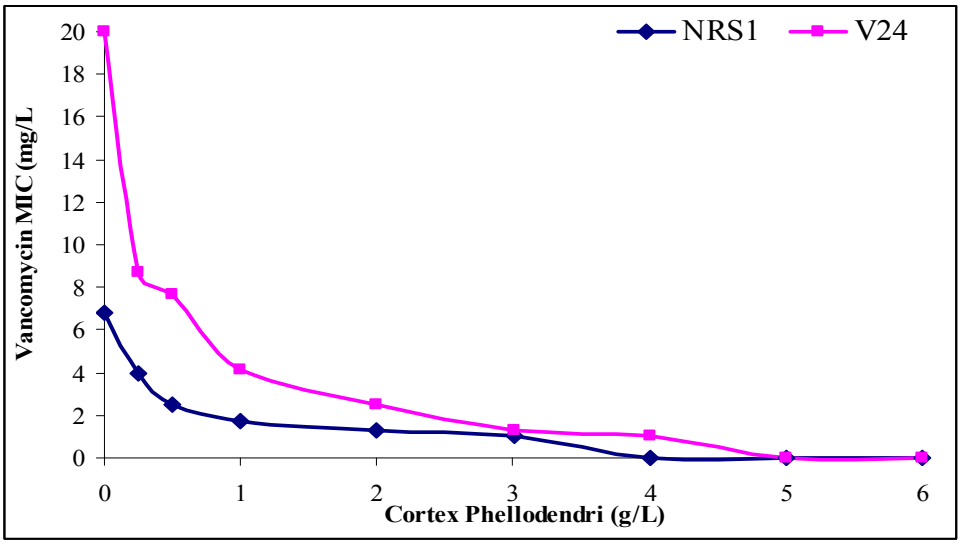
Strain No.	Vancomycin MIC (mg/L)		Herb MIC by AD (g/L)	Vancomycin MIC (mg/L) in combination with herb by SGE								
				Herb extract (g/L)								
	AD	SGE EC/TEC		0.0	0.25	0.5	1	2	3	4	5	6
NRS149	0.5	≤ 1	0.25	≤ 1	NG	NG	NG	NG	NG	NG	NG	NG
NRS100	0.5	≤ 1	0.25	≤ 1	NG	NG	NG	NG	NG	NG	NG	NG
MR1	2.0	1.5	2.0	1.5	1.3	1.1	NG	NG	NG	NG	NG	NG
MR2	2.0	1.5	2.0	1.5	1.3	1.1	NG	NG	NG	NG	NG	NG
MR3	2.0	1.5/2.2	2.0	1.5/2.2	1.3	1.1	NG	NG	NG	NG	NG	NG
MR4	2.0	1.5/2.2	2.0	1.5/2.2	1.3	1.1	NG	NG	NG	NG	NG	NG
MR5	2.0	1.5/2.2	2.0	1.5/2.2	1.3	1.1	≤ 1	NG	NG	NG	NG	NG
MR6	2.0	1.5/2.2	2.0	1.5/2.2	1.3	1.1	≤ 1	NG	NG	NG	NG	NG
MR7	2.0	1.5/2.2	2.0	1.5/2.2	1.3	1.1	≤ 1	NG	NG	NG	NG	NG
MR8	3.0	1.5/2.8	2.0	1.5/2.8	2.0	1.3	≤ 1	NG	NG	NG	NG	NG
MR9	3.0	1.5/2.8	2.0	1.5/2.8	2.0	1.3	≤ 1	NG	NG	NG	NG	NG
MR10	4.0	1.5/4.0	2.0	1.5/4.0	2.0	1.3	≤ 1	NG	NG	NG	NG	NG
HV1	3.0	2.2/2.8	2.0	2.2/2.8	2.0	1.3	≤ 1	NG	NG	NG	NG	NG
HV2	3.0	2.5/2.8	2.0	2.5/2.8	2.0	1.3	≤ 1	NG	NG	NG	NG	NG
HV3	3.0	2.2/3.2	2.0	2.2/3.2	2.0	1.3	≤ 1	NG	NG	NG	NG	NG
HV4	3.0	3.0/3.2	2.0	3.0/3.2	2.0	1.3	≤ 1	NG	NG	NG	NG	NG
HV5	3.0	2.2/2.8	2.0	2.2/2.8	2.0	1.3	≤ 1	NG	NG	NG	NG	NG
HV6	3.0	2.5/2.8	2.0	2.5/2.8	2.0	1.3	1.1	≤ 1	NG	NG	NG	NG
HV7	3.0	2.5/3.2	2.0	2.5/3.2	2.0	1.3	≤ 1	NG	NG	NG	NG	NG
HV8	3.0	2.2/2.8	2.0	2.2/2.8	2.0	1.3	≤ 1	NG	NG	NG	NG	NG
HV9	3.0	3.2/4.1	3.0	3.2/4.1	2.0	1.3	1.1	≤ 1	NG	NG	NG	NG
HV10	3.0	3.0/4.1	3.0	3.0/4.1	2.0	1.3	1.1	≤ 1	NG	NG	NG	NG
HV11	3.0	2.2/3.2	2.0	2.2/3.2	2.0	1.3	≤ 1	NG	NG	NG	NG	NG
HV12	3.0	2.2/3.2	2.0	2.2/3.2	2.0	1.3	≤ 1	NG	NG	NG	NG	NG
HV13	3.0	2.2/2.8	2.0	2.2/2.8	2.0	1.3	≤ 1	NG	NG	NG	NG	NG

Continued on the following page

Table 6.2 Continued

Strain No.	Vancomycin MIC (mg/L)		Herb MIC by AD (g/L)	Vancomycin MIC (mg/L) in combination with herb by SGE								
				Herb extract (g/L)								
	AD	SGE EC/TEC		0.0	0.25	0.5	1	2	3	4	5	6
NRS1	7.0	6.8	3.0	6.8	4.0	2.5	1.7	1.3	≤ 1	NG	NG	NG
V1	6.0	6.0	3.0	6.0	3.2	2.5	1.7	1.3	≤ 1	NG	NG	NG
V2	6.0	6.0	3.0	6.0	3.2	2.5	1.7	1.3	≤ 1	NG	NG	NG
V3	8.0	8.7	3.0	8.7	4.7	3.2	2.5	1.5	≤ 1	≤ 1	NG	NG
V4	10.0	10.2	4.0	10.2	6.8	4.0	2.5	1.5	1.0	≤ 1	NG	NG
V5	8.0	8.7	3.0	8.7	4.7	3.2	1.7	1.3	≤ 1	NG	NG	NG
V6	8.0	7.7	3.0	7.7	4.1	3.2	1.7	1.3	≤ 1	NG	NG	NG
V7	10.0	8.7	4.0	8.7	6.8	4.1	2.5	1.5	≤ 1	NG	NG	NG
V8	5.0	5.3	3.0	5.3	2.8	2.5	1.7	1.3	≤ 1	≤ 1	NG	NG
V9	6.0	6.0	3.0	6.0	3.2	2.5	1.7	1.3	≤ 1	NG	NG	NG
V10	8.0	7.7	3.0	7.7	4.1	2.8	2.0	1.3	≤ 1	NG	NG	NG
V11	8.0	7.7	3.0	7.7	4.7	3.2	1.7	1.3	≤ 1	NG	NG	NG
V12	9.0	8.7	4.0	8.7	5.2	4.7	2.5	1.5	≤ 1	NG	NG	NG
V13	12.0	11.6	5.0	11.6	7.7	6.0	2.5	1.3	≤ 1	≤ 1	NG	NG
V14	12.0	11.6	5.0	11.6	7.7	5.3	2.5	1.3	≤ 1	NG	NG	NG
V15	11.0	10.2	4.0	10.2	7.7	5.3	2.5	1.5	1.0	≤ 1	NG	NG
V16	10.0	10.2	5.0	10.2	7.7	5.3	2.5	1.5	1.0	≤ 1	NG	NG
V17	12.0	11.6	5.0	11.6	7.7	6.8	2.8	1.5	≤ 1	NG	NG	NG
V18	12.0	11.6	5.0	11.6	7.7	6.8	2.8	1.5	≤ 1	NG	NG	NG
V19	20.0	20.0	6.0	20.0	8.7	7.7	3.2	2.5	1.3	≤ 1	NG	NG
V20	20.0	20.0	6.0	20.0	8.7	7.7	4.1	2.5	1.3	≤ 1	NG	NG
V21	20.0	20.0	6.0	20.0	8.7	7.7	4.1	2.5	1.3	≤ 1	NG	NG
V22	20.0	20.0	6.0	20.0	8.7	7.7	4.1	2.5	1.3	≤ 1	NG	NG
V23	20.0	20.0	6.0	20.0	8.7	7.7	4.1	2.5	1.3	≤ 1	NG	NG
V24	20.0	20.0	6.0	20.0	8.7	7.7	4.1	2.5	1.3	≤ 1	NG	NG

MR=MRSA, HV=hVISA, V=VISA, Shading=Significant change in susceptibility



NRS1=VISA control strain, V24= laboratory induced strain with MIC 20mg/L

FIGURE 6.1: Antimicrobial activity of *Cortex phellodendri* and vancomycin on VISA strains

6.3.2 Antimicrobial activity of *Rhizoma coptidis* alone and in combination with vancomycin

The combination of *R. coptidis* of 3.0 g/L and vancomycin exhibited complete inhibition of growth of all isolates, while 2.0 g/L of *R. coptidis* with vancomycin resulted in reduction of vancomycin MIC to susceptible level (<2.0 mg/L) for all isolates (Table 6.3). *R. coptidis* alone exhibited a strong anti-MRSA effect with MIC $_{90}$ of 1.0 g/L and anti-VISA activity with MIC_{90} of 4.0 g/L. A combination of *R. coptidis* and vancomycin can restore the efficacy of vancomycin against VISA isolates as shown in Table 6.3 and Figure 6.2 for two representative strains.

One-way repeated ANOVA showed significant reduction in vancomycin MICs in comparison to the initial MIC (P = <0.001). Paired student t-test showed that a concentration of 2.0 g/L of *R. coptidis* in combination with vancomycin was found to be effective in reducing the vancomycin MIC to susceptible levels (P = <0.001) demonstrating a significant change in vancomycin susceptibility for the isolates (statistical analysis are as shown in Appendix IV). The FICI was found to be 0.555 indicating no interaction between *R. coptidis* and vancomycin.

TABLE 6.3: Results of antimicrobial activity of *Rhizoma coptidis* alone and in combination

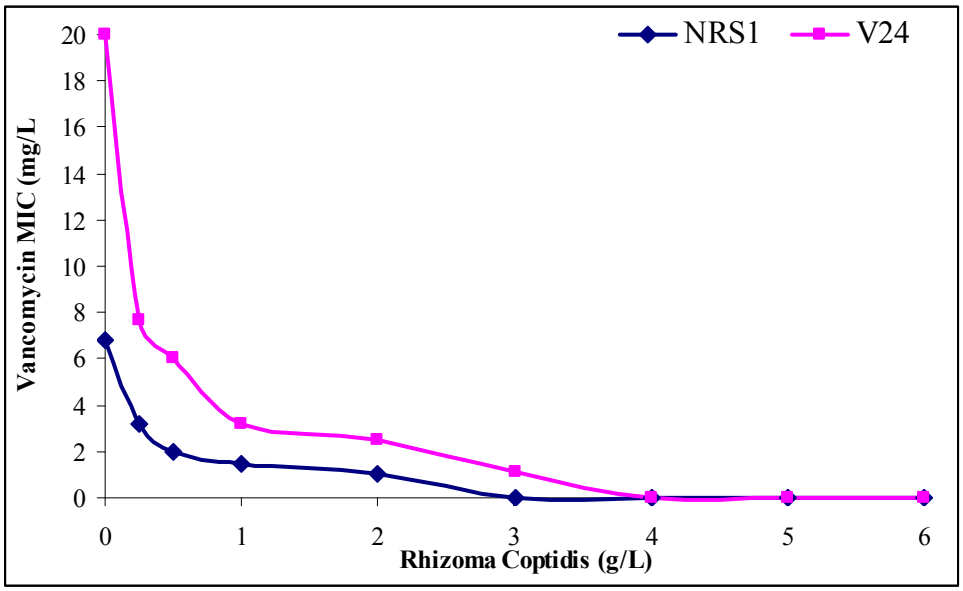
Strain No.	Vancomycin MIC (mg/L)		Herb MIC by AD (g/L)	Vancomycin MIC (mg/L) in combination with herb by SGE								
				Herb extract (g/L)								
	AD	SGE EC/TEC		0.0	0.25	0.5	1	2	3	4	5	6
NRS149	0.5	≤ 1	0.25	≤ 1	NG	NG	NG	NG	NG	NG	NG	NG
NRS100	0.5	≤ 1	0.25	≤ 1	NG	NG	NG	NG	NG	NG	NG	NG
MR1	2.0	1.5	1.0	1.5	1.2	≤ 1	NG	NG	NG	NG	NG	NG
MR2	2.0	1.5	1.0	1.5	1.2	≤ 1	NG	NG	NG	NG	NG	NG
MR3	2.0	1.5/2.2	1.0	1.5/2.2	1.2	≤ 1	NG	NG	NG	NG	NG	NG
MR4	2.0	1.5/2.2	1.0	1.5/2.2	1.2	≤ 1	NG	NG	NG	NG	NG	NG
MR5	2.0	1.5/2.2	1.0	1.5/2.2	1.2	≤ 1	NG	NG	NG	NG	NG	NG
MR6	2.0	1.5/2.2	1.0	1.5/2.2	1.2	≤ 1	NG	NG	NG	NG	NG	NG
MR7	2.0	1.5/2.2	1.0	1.5/2.2	1.2	≤ 1	NG	NG	NG	NG	NG	NG
MR8	3.0	1.5/2.8	2.0	1.5/2.8	1.2	≤ 1	NG	NG	NG	NG	NG	NG
MR9	3.0	1.5/2.8	1.0	1.5/2.8	1.2	≤ 1	NG	NG	NG	NG	NG	NG
MR10	4.0	1.5/4.0	2.0	1.5/4.0	1.2	≤ 1	≤ 1	NG	NG	NG	NG	NG
HV1	3.0	2.2/2.8	1.0	2.2/2.8	1.5	1.2	≤ 1	NG	NG	NG	NG	NG
HV2	3.0	2.5/2.8	1.0	2.5/2.8	1.7	1.2	≤ 1	NG	NG	NG	NG	NG
HV3	3.0	2.2/3.2	1.0	2.2/3.2	1.5	1.2	≤ 1	NG	NG	NG	NG	NG
HV4	3.0	3.0/3.2	1.0	3.0/3.2	1.7	1.5	1.2	NG	NG	NG	NG	NG
HV5	3.0	2.2/2.8	1.0	2.2/2.8	1.5	≤ 1	NG	NG	NG	NG	NG	NG
HV6	3.0	2.5/2.8	1.0	2.5/2.8	2.0	1.7	1.1	NG	NG	NG	NG	NG
HV7	3.0	2.5/3.2	1.0	2.5/3.2	1.5	≤ 1	NG	NG	NG	NG	NG	NG
HV8	3.0	2.2/2.8	1.0	2.2/2.8	1.2	< 1	≤ 1	NG	NG	NG	NG	NG
HV9	3.0	3.2/4.1	2.0	3.2/4.1	2.2	1.5	1.1	NG	NG	NG	NG	NG
HV10	3.0	3.0/4.1	2.0	3.0/4.1	2.0	≤ 1	NG	NG	NG	NG	NG	NG
HV11	3.0	2.2/3.2	1.0	2.2/3.2	2.2	1.3	≤ 1	NG	NG	NG	NG	NG
HV12	3.0	2.2/3.2	1.0	2.2/3.2	2.2	1.3	≤ 1	NG	NG	NG	NG	NG
HV13	3.0	2.2/2.8	1.0	2.2/2.8	2.2	1.3	≤ 1	NG	NG	NG	NG	NG

Continued on the following page

Table 6.3 Continued

Strain No.	Vancomycin MIC (mg/L)		Herb MIC by AD (g/L)	Vancomycin MIC (mg/L) in combination with herb by SGE								
				Herb extract (g/L)								
	AD	SGE EC/TEC		0.0	0.25	0.5	1	2	3	4	5	6
NRS1	7.0	6.8	3.0	6.8	3.2	2.0	1.5	≤ 1	NG	NG	NG	NG
V1	6.0	6.0	3.0	6.0	2.5	2.0	1.5	NG	NG	NG	NG	NG
V2	6.0	6.0	3.0	6.0	2.2	2.0	1.5	NG	NG	NG	NG	NG
V3	8.0	8.7	3.0	8.7	4.1	2.5	2.0	NG	NG	NG	NG	NG
V4	10.0	10.2	3.0	10.2	6.8	3.2	2.0	≤ 1	NG	NG	NG	NG
V5	8.0	8.7	3.0	8.7	4.1	3.0	1.2	NG	NG	NG	NG	NG
V6	8.0	7.7	3.0	7.7	3.6	2.5	1.2	NG	NG	NG	NG	NG
V7	10.0	8.7	3.0	8.7	6.0	3.2	1.5	NG	NG	NG	NG	NG
V8	5.0	5.3	3.0	5.3	2.2	1.7	1.2	NG	NG	NG	NG	NG
V9	6.0	6.0	3.0	6.0	2.2	1.7	1.2	NG	NG	NG	NG	NG
V10	8.0	7.7	3.0	7.7	3.6	2.0	1.2	NG	NG	NG	NG	NG
V11	8.0	7.7	3.0	7.7	3.6	2.5	1.2	NG	NG	NG	NG	NG
V12	9.0	8.7	3.0	8.7	4.7	4.1	2.0	NG	NG	NG	NG	NG
V13	12.0	11.6	4.0	11.6	6.8	4.7	2.0	NG	NG	NG	NG	NG
V14	12.0	11.6	4.0	11.6	6.8	4.7	2.0	NG	NG	NG	NG	NG
V15	11.0	10.2	4.0	10.2	6.0	4.7	2.0	≤ 1	NG	NG	NG	NG
V16	10.0	10.2	4.0	10.2	6.0	4.7	2.0	≤ 1	NG	NG	NG	NG
V17	12.0	11.6	4.0	11.6	6.0	5.2	2.5	≤ 1	NG	NG	NG	NG
V18	12.0	11.6	4.0	11.6	6.0	5.2	2.5	≤ 1	NG	NG	NG	NG
V19	20.0	20.0	4.0	20.0	7.7	6.0	3.2	1.1	NG	NG	NG	NG
V20	20.0	20.0	4.0	20.0	7.7	6.0	3.2	1.1	NG	NG	NG	NG
V21	20.0	20.0	4.0	20.0	7.7	6.0	2.8	1.1	NG	NG	NG	NG
V22	20.0	20.0	4.0	20.0	7.7	6.0	3.2	1.1	NG	NG	NG	NG
V23	20.0	20.0	4.0	20.0	7.7	6.0	3.2	1.1	NG	NG	NG	NG
V24	20.0	20.0	4.0	20.0	7.7	6.0	3.2	1.1	NG	NG	NG	NG

MR=MRSA, HV=hVISA, V=VISA, Shading=Significant change in susceptibility



NRS1=VISA control strain, V24= laboratory induced strain with MIC 20mg/L

FIGURE 6.2: Antimicrobial activity of *Rhizoma Coptidis* and vancomycin on VISA strains

6.3.3 Antimicrobial activity of *Radix scutellariae* alone and in combination with vancomycin

The combination of *R. scutellariae* of 1.0 g/L and vancomycin exhibited complete inhibition of growth of all isolates, while 0.25 g/L of *R. coptidis* with vancomycin resulted in reduction of vancomycin MIC to susceptible level (<2.0 mg/L) for all isolates (Table 6.4a and Table 6.4b). *R. scutellariae* alone exhibited a strong anti-MRSA effect with MIC $_{90}$ of 0.5 g/L and anti-VISA activity with MIC_{90} of 2.0 g/L. A combination of *R. scutellariae* and vancomycin can restore the efficacy of vancomycin against VISA isolates as shown in Table 6.4a and Figure 6.3 for two representative strains. Further studies at two lower concentrations, 0.062 g/L and 0.125 g/L in combination with vancomycin confirmed that *R. scutellariae* at 0.25 g/L was the most effective concentration across a range of non-susceptibility. A combination of 0.062 g/L of *R. scutellariae* and vancomycin was almost similar to the MIC obtained by vancomycin alone and 0.125 g/L was able to restore the effectiveness of vancomycin in hVISA isolates, but not in VISA (Table 6.4b).

One-way repeated ANOVA showed significant reduction in vancomycin MICs in comparison to the initial MIC (P = <0.001). Paired student t-test showed that a concentration of 0.25 g/L of *R. scutellariae* in combination with vancomycin was found to be effective in reducing the vancomycin MIC to susceptible levels (P = <0.001) demonstrating a significant change in vancomycin susceptibility for the isolates (statistical analysis are as shown in Appendix IV). The FICI was found to be 0.21 indicating synergistic interaction between *R. scutellariae* and vancomycin.

TABLE 6.4a: Results of antimicrobial activity of *Radix scutellariae* alone and in combination

Strain No.	Vancomycin MIC (mg/L)		Herb MIC by AD (g/L)	Vancomycin MIC (mg/L) in combination with herb by SGE								
				Herb extract (g/L)								
	AD	SGE EC/TEC		0.0	0.25	0.5	1	2	3	4	5	6
NRS149	0.5	≤ 1	0.25	≤ 1	NG	NG	NG	NG	NG	NG	NG	NG
NRS100	0.5	≤ 1	0.25	≤ 1	NG	NG	NG	NG	NG	NG	NG	NG
MR1	2.0	1.5	0.5	1.5	NG	NG	NG	NG	NG	NG	NG	NG
MR2	2.0	1.5	0.5	1.5	NG	NG	NG	NG	NG	NG	NG	NG
MR3	2.0	1.5/2.2	0.5	1.5/2.2	NG	NG	NG	NG	NG	NG	NG	NG
MR4	2.0	1.5/2.2	0.5	1.5/2.2	NG	NG	NG	NG	NG	NG	NG	NG
MR5	2.0	1.5/2.2	0.5	1.5/2.2	NG	NG	NG	NG	NG	NG	NG	NG
MR6	2.0	1.5/2.2	0.5	1.5/2.2	NG	NG	NG	NG	NG	NG	NG	NG
MR7	2.0	1.5/2.2	0.5	1.5/2.2	NG	NG	NG	NG	NG	NG	NG	NG
MR8	3.0	1.5/2.8	0.5	1.5/2.8	NG	NG	NG	NG	NG	NG	NG	NG
MR9	3.0	1.5/2.8	0.5	1.5/2.8	NG	NG	NG	NG	NG	NG	NG	NG
MR10	4.0	1.5/4.0	0.5	1.5/4.0	NG	NG	NG	NG	NG	NG	NG	NG
HV1	3.0	2.2/2.8	0.5	2.2/2.8	NG	NG	NG	NG	NG	NG	NG	NG
HV2	3.0	2.5/2.8	0.5	2.5/2.8	NG	NG	NG	NG	NG	NG	NG	NG
HV3	3.0	2.2/3.2	0.5	2.2/3.2	NG	NG	NG	NG	NG	NG	NG	NG
HV4	3.0	3.0/3.2	0.5	3.0/3.2	NG	NG	NG	NG	NG	NG	NG	NG
HV5	3.0	2.2/2.8	0.5	2.2/2.8	NG	NG	NG	NG	NG	NG	NG	NG
HV6	3.0	2.5/2.8	0.5	2.5/2.8	NG	NG	NG	NG	NG	NG	NG	NG
HV7	3.0	2.5/3.2	0.5	2.5/3.2	NG	NG	NG	NG	NG	NG	NG	NG
HV8	3.0	2.2/2.8	0.5	2.2/2.8	NG	NG	NG	NG	NG	NG	NG	NG
HV9	3.0	3.2/4.1	0.5	3.2/4.1	NG	NG	NG	NG	NG	NG	NG	NG
HV10	3.0	3.0/4.1	0.5	3.0/4.1	NG	NG	NG	NG	NG	NG	NG	NG
HV11	3.0	2.2/3.2	0.5	2.2/3.2	NG	NG	NG	NG	NG	NG	NG	NG
HV12	3.0	2.2/3.2	0.5	2.2/3.2	NG	NG	NG	NG	NG	NG	NG	NG
HV13	3.0	2.2/2.8	0.5	2.2/2.8	NG	NG	NG	NG	NG	NG	NG	NG

Continued on the following page

Table 6.4a Continued

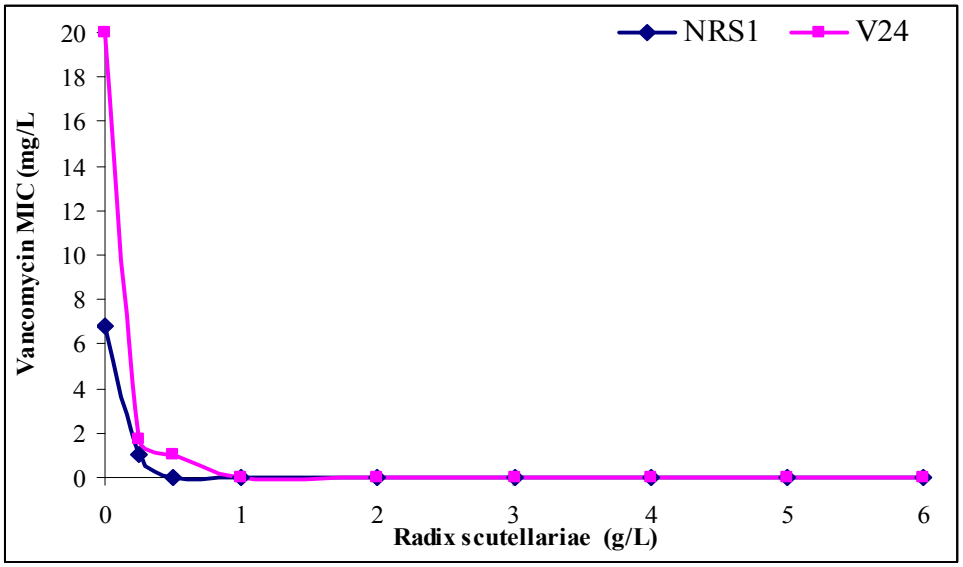
Strain No.	Vancomycin MIC (mg/L)		Herb MIC by AD (g/L)	Vancomycin MIC (mg/L) in combination with herb by SGE								
				Herb extract (g/L)								
	AD	SGE EC/TEC		0.0	0.25	0.5	1	2	3	4	5	6
NRS1	7.0	6.8	2.0	6.8	≤ 1	NG	NG	NG	NG	NG	NG	NG
V1	6.0	6.0	2.0	6.0	≤ 1	NG	NG	NG	NG	NG	NG	NG
V2	6.0	6.0	2.0	6.0	≤ 1	NG	NG	NG	NG	NG	NG	NG
V3	8.0	8.7	2.0	8.7	≤ 1	NG	NG	NG	NG	NG	NG	NG
V4	10.0	10.2	2.0	10.2	1.1	NG	NG	NG	NG	NG	NG	NG
V5	8.0	8.7	2.0	8.7	≤ 1	NG	NG	NG	NG	NG	NG	NG
V6	8.0	7.7	2.0	7.7	≤ 1	NG	NG	NG	NG	NG	NG	NG
V7	10.0	8.7	2.0	8.7	1.5	NG	NG	NG	NG	NG	NG	NG
V8	5.0	5.3	2.0	5.3	≤ 1	NG	NG	NG	NG	NG	NG	NG
V9	6.0	6.0	2.0	6.0	≤ 1	NG	NG	NG	NG	NG	NG	NG
V10	8.0	7.7	2.0	7.7	≤ 1	NG	NG	NG	NG	NG	NG	NG
V11	8.0	7.7	2.0	7.7	≤ 1	NG	NG	NG	NG	NG	NG	NG
V12	9.0	8.7	2.0	8.7	≤ 1	NG	NG	NG	NG	NG	NG	NG
V13	12.0	11.6	2.0	11.6	≤ 1	NG	NG	NG	NG	NG	NG	NG
V14	12.0	11.6	2.0	11.6	≤ 1	NG	NG	NG	NG	NG	NG	NG
V15	11.0	10.2	2.0	10.2	≤ 1	NG	NG	NG	NG	NG	NG	NG
V16	10.0	10.2	2.0	10.2	1.1	NG	NG	NG	NG	NG	NG	NG
V17	12.0	11.6	2.0	11.6	≤ 1	NG	NG	NG	NG	NG	NG	NG
V18	12.0	11.6	2.0	11.6	≤ 1	NG	NG	NG	NG	NG	NG	NG
V19	20.0	20.0	2.0	20.0	1.7	≤ 1	NG	NG	NG	NG	NG	NG
V20	20.0	20.0	2.0	20.0	1.7	≤ 1	NG	NG	NG	NG	NG	NG
V21	20.0	20.0	2.0	20.0	1.7	≤ 1	NG	NG	NG	NG	NG	NG
V22	20.0	20.0	2.0	20.0	1.7	≤ 1	NG	NG	NG	NG	NG	NG
V23	20.0	20.0	2.0	20.0	1.7	≤ 1	NG	NG	NG	NG	NG	NG
V24	20.0	20.0	2.0	20.0	1.7	≤ 1	NG	NG	NG	NG	NG	NG

MR=MRSA, HV=hVISA, V=VISA, Shading=Significant change in susceptibility

TABLE 6.4b: Results of antimicrobial activity of *Radix scutellariae* alone and in combination

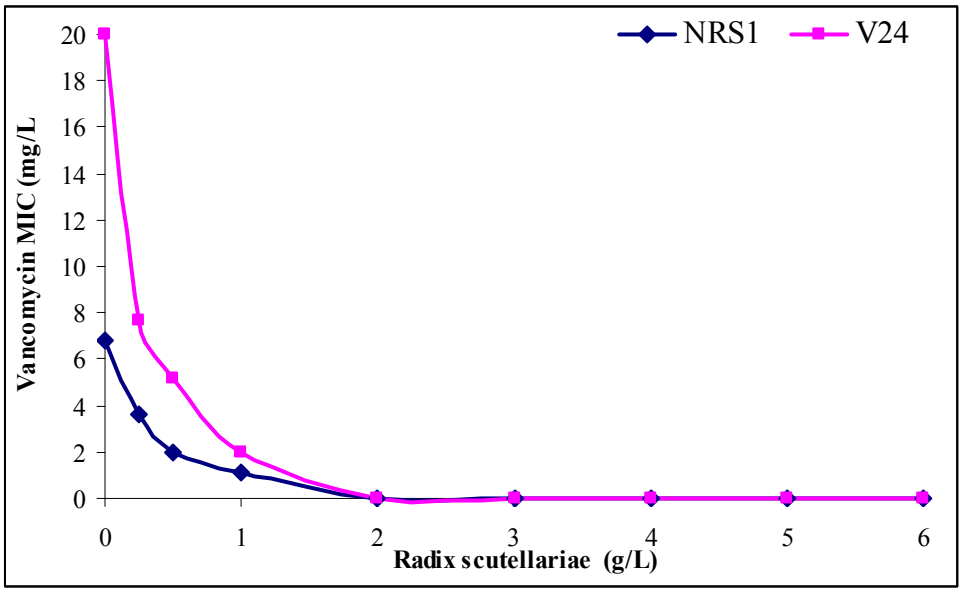
Strain No.	Vancomycin MIC (mg/L)	Vancomycin MIC (mg/L) in combination with herb by SGE		Strain No.	Vancomycin MIC (mg/L)	Vancomycin MIC (mg/L) in combination with herb by SGE	
	SGE (EC/TEC)	Herb extract (g/L)			SGE (EC/TEC)	Herb extract (g/L)	
		0.062	0.125			0.062	0.125
NRS149	≤ 1	NG	NG	NRS1	6.8	6.8	3.2
NRS100	≤ 1	NG	NG	V1	6.0	6.0	3.2
MR1	1.5	≤ 1	NG	V2	6.0	6.0	3.0
MR2	1.5	≤ 1	NG	V3	8.7	8.7	3.2
MR3	1.5/2.2	≤ 1	NG	V4	10.2	10.2	4.7
MR4	1.5/2.2	≤ 1	NG	V5	8.7	8.7	3.2
MR5	1.5/2.2	1.0/2.0	NG	V6	7.7	7.7	3.0
MR6	1.5/2.2	1.5/2.0	NG	V7	8.7	8.7	4.7
MR7	1.5/2.2	≤ 1	NG	V8	5.3	5.3	2.8
MR8	1.5/2.8	≤ 1	NG	V9	6.0	6.0	2.8
MR9	1.5/2.8	1.0/1.5	NG	V10	7.7	7.7	3.0
MR10	1.5/4.0	1.0/2.5	NG	V11	7.7	7.7	3.2
HV1	2.2/2.8	2.2/2.5	NG	V12	8.7	8.7	3.2
HV2	2.5/2.8	2.5/2.2	≤ 1	V13	11.6	11.6	4.7
HV3	2.2/3.2	2.2/2.5	≤ 1	V14	11.6	11.6	4.7
HV4	3.0/3.2	3.0/2.8	1.5	V15	10.2	10.2	4.1
HV5	2.2/2.8	2.2	≤ 1	V16	10.2	10.2	4.7
HV6	2.5/2.8	2.5	≤ 1	V17	11.6	11.6	4.7
HV7	2.5/3.2	2.5/2.8	1.5	V18	11.6	11.6	4.7
HV8	2.2/2.8	2.2	≤ 1	V19	20.0	20.0	11.6
HV9	3.2/4.1	3.2	≤ 1	V20	20.0	20.0	11.6
HV10	3.0/4.1	3.0/3.2	1.5	V21	20.0	20.0	10.2
HV11	2.2/3.2	2.2/2.5	≤ 1	V22	20.0	20.0	11.6
HV12	2.2/3.2	2.2/2.8	≤ 1	V23	20.0	20.0	11.6
HV13	2.2/2.8	2.2	≤ 1	V24	20.0	20.0	11.6

MR=MRSA, HV=hVISA, V=VISA, Shading=Significant change in susceptibility



NRS1=VISA control strain, V24= laboratory induced strain with MIC 20mg/L

FIGURE 6.3: Antimicrobial activity of *Radix scutellariae* and vancomycin on VISA strains



NRS1=VISA control strain, V24= laboratory induced strain with MIC 20mg/L

FIGURE 6.4: Antimicrobial activity of *Radix scutellariae* (pH 5.6) and vancomycin on VISA strains

R. Scutellarie was found to be most effective among the tested herbs and the pH was found to be 5.2. This herb was investigated at two other pH values i.e 5.6 and 7.0 to determine the influence of pH on the desired activity. The increase of pH to 5.6 and 7.0 resulted in precipitation and change of color of herb extract to purple.

6.3.4 Antimicrobial activity of *Radix scutellariae* alone and in combination with vancomycin (pH 5.6)

The combination of *R. scutellariae* of 2.0 g/L and vancomycin exhibited complete inhibition of growth of all isolates, while 1.0 g/L of *R. scutellariae* with vancomycin resulted in reduction of vancomycin MIC to susceptible level (<2.0 mg/L) for all isolates (Table 6.5). *R. scutellariae* alone exhibited a strong anti-MRSA effect with MIC_{90} of 1.0 g/L and anti-VISA activity with MIC_{90} of 3.0 g/L. A combination of *R. scutellariae* and vancomycin can restore the efficacy of vancomycin against VISA isolates as shown in Table 6.5 and Figure 6.4 for two representative strains.

One-way repeated ANOVA showed significant reduction in vancomycin MICs in comparison to the initial MIC (P = <0.001). Paired student t-test showed that a concentration of 1.0 g/L of *R. scutellariae* in combination with vancomycin was found to be effective in reducing the vancomycin MIC to susceptible levels (P = <0.001) demonstrating a significant change in vancomycin susceptibility for the isolates (statistical analysis are as shown in Appendix IV). The FICI was found to be 0.666 indicating no interaction between *R. Scutellarie* and vancomycin.

TABLE 6.5: Results of antimicrobial activity of *Radix scutellariae* alone and in combination (pH 5.6)

Strain No.	Vancomycin MIC (mg/L)		Herb MIC by AD (g/L)	Vancomycin MIC (mg/L) in combination with herb by SGE								
				Herb extract (g/L)								
	AD	SGE EC/TEC		0.0	0.25	0.5	1	2	3	4	5	6
NRS149	0.5	≤ 1	0.25	≤ 1	NG	NG	NG	NG	NG	NG	NG	NG
NRS100	0.5	≤ 1	0.25	≤ 1	NG	NG	NG	NG	NG	NG	NG	NG
MR1	2.0	1.5	1.0	1.5	≤ 1	NG	NG	NG	NG	NG	NG	NG
MR2	2.0	1.5	1.0	1.5	≤ 1	NG	NG	NG	NG	NG	NG	NG
MR3	2.0	1.5/2.2	1.0	1.5/2.2	≤ 1	NG	NG	NG	NG	NG	NG	NG
MR4	2.0	1.5/2.2	1.0	1.5/2.2	≤ 1	NG	NG	NG	NG	NG	NG	NG
MR5	2.0	1.5/2.2	1.0	1.5/2.2	≤ 1	NG	NG	NG	NG	NG	NG	NG
MR6	2.0	1.5/2.2	1.0	1.5/2.2	≤ 1	NG	NG	NG	NG	NG	NG	NG
MR7	2.0	1.5/2.2	1.0	1.5/2.2	≤ 1	NG	NG	NG	NG	NG	NG	NG
MR8	3.0	1.5/2.8	1.0	1.5/2.8	≤ 1	NG	NG	NG	NG	NG	NG	NG
MR9	3.0	1.5/2.8	1.0	1.5/2.8	≤ 1	NG	NG	NG	NG	NG	NG	NG
MR10	4.0	1.5/4.0	1.0	1.5/4.0	≤ 1	≤ 1	NG	NG	NG	NG	NG	NG
HV1	3.0	2.2/2.8	1.0	2.2/2.8	≤ 1	NG	NG	NG	NG	NG	NG	NG
HV2	3.0	2.5/2.8	1.0	2.5/2.8	≤ 1	NG	NG	NG	NG	NG	NG	NG
HV3	3.0	2.2/3.2	1.0	2.2/3.2	≤ 1	NG	NG	NG	NG	NG	NG	NG
HV4	3.0	3.0/3.2	1.0	3.0/3.2	≤ 1	NG	NG	NG	NG	NG	NG	NG
HV5	3.0	2.2/2.8	1.0	2.2/2.8	≤ 1	NG	NG	NG	NG	NG	NG	NG
HV6	3.0	2.5/2.8	1.0	2.5/2.8	≤ 1	NG	NG	NG	NG	NG	NG	NG
HV7	3.0	2.5/3.2	1.0	2.5/3.2	≤ 1	NG	NG	NG	NG	NG	NG	NG
HV8	3.0	2.2/2.8	1.0	2.2/2.8	≤ 1	≤ 1	NG	NG	NG	NG	NG	NG
HV9	3.0	3.2/4.1	1.0	3.2/4.1	1.1	≤ 1	NG	NG	NG	NG	NG	NG
HV10	3.0	3.0/4.1	1.0	3.0/4.1	1.3	≤ 1	NG	NG	NG	NG	NG	NG
HV11	3.0	2.2/3.2	1.0	2.2/3.2	≤ 1	≤ 1	NG	NG	NG	NG	NG	NG
HV12	3.0	2.2/3.2	1.0	2.2/3.2	≤ 1	NG	NG	NG	NG	NG	NG	NG
HV13	3.0	2.2/2.8	1.0	2.2/2.8	≤ 1	NG	NG	NG	NG	NG	NG	NG

Continued on the following page

Table 6.5 Continued

Strain No.	Vanco MIC (mg/L)		Herb MIC by AD (g/L)	Vancomycin MIC (mg/L) in combination with herb by SGE								
				Herb extract (g/L)								
	AD	SGE EC/TEC		0.0	0.25	0.5	1	2	3	4	5	6
NRS1	7.0	6.8	3.0	6.8	3.6	2.0	1.1	NG	NG	NG	NG	NG
V1	6.0	6.0	3.0	6.0	3.0	2.0	1.1	NG	NG	NG	NG	NG
V2	6.0	6.0	3.0	6.0	3.0	2.0	1.1	NG	NG	NG	NG	NG
V3	8.0	8.7	3.0	8.7	3.6	2.0	1.1	NG	NG	NG	NG	NG
V4	10.0	10.2	3.0	10.2	3.6	3.0	1.7	NG	NG	NG	NG	NG
V5	8.0	8.7	3.0	8.7	3.6	2.0	1.5	NG	NG	NG	NG	NG
V6	8.0	7.7	3.0	7.7	3.6	2.0	1.1	NG	NG	NG	NG	NG
V7	10.0	8.7	3.0	8.7	5.2	3.2	1.5	NG	NG	NG	NG	NG
V8	5.0	5.3	3.0	5.3	3.0	2.0	1.1	NG	NG	NG	NG	NG
V9	6.0	6.0	3.0	6.0	3.0	2.0	1.1	NG	NG	NG	NG	NG
V10	8.0	7.7	3.0	7.7	3.0	2.0	1.3	NG	NG	NG	NG	NG
V11	8.0	7.7	3.0	7.7	3.0	2.0	1.3	NG	NG	NG	NG	NG
V12	9.0	8.7	3.0	8.7	3.6	2.0	1.3	NG	NG	NG	NG	NG
V13	12.0	11.6	3.0	11.6	4.6	3.2	1.5	NG	NG	NG	NG	NG
V14	12.0	11.6	3.0	11.6	4.6	3.6	1.5	NG	NG	NG	NG	NG
V15	11.0	10.2	3.0	10.2	4.6	3.2	1.5	NG	NG	NG	NG	NG
V16	10.0	10.2	3.0	10.2	4.0	3.0	1.7	NG	NG	NG	NG	NG
V17	12.0	11.6	3.0	11.6	4.6	3.2	1.7	NG	NG	NG	NG	NG
V18	12.0	11.6	3.0	11.6	4.6	3.2	1.7	NG	NG	NG	NG	NG
V19	20.0	20.0	3.0	20.0	7.7	5.2	2.0	NG	NG	NG	NG	NG
V20	20.0	20.0	3.0	20.0	7.7	5.2	2.0	NG	NG	NG	NG	NG
V21	20.0	20.0	3.0	20.0	7.7	5.2	2.0	NG	NG	NG	NG	NG
V22	20.0	20.0	3.0	20.0	7.7	5.2	2.0	NG	NG	NG	NG	NG
V23	20.0	20.0	3.0	20.0	7.7	5.2	2.0	NG	NG	NG	NG	NG
V24	20.0	20.0	3.0	20.0	7.7	5.2	2.0	NG	NG	NG	NG	NG

MR=MRSA, HV=hVISA, V=VISA, Shading=Significant change in susceptibility

6.3.5 Antimicrobial activity of *Radix scutellariae* alone and in combination with vancomycin (pH 7.0)

The combination of *R. scutellariae* of 4.0 g/L and vancomycin exhibited complete inhibition of growth of all isolates, while 3.0 g/L of *R. scutellariae* with vancomycin resulted in reduction of vancomycin MIC to susceptible level (<2.0 mg/L) for all isolates (Table 6.6). *R. scutellariae* alone exhibited a strong anti-MRSA effect with MIC_{90} of 2.0 g/L and anti-VISA activity with MIC_{90} of 5.0 g/L. A combination of *R. scutellariae* and vancomycin can restore the efficacy of vancomycin against VISA isolates as shown in Table 6.6 and Figure 6.5 for two representative strains.

One-way repeated ANOVA showed significant reduction in vancomycin MICs in comparison to the initial MIC (P = <0.001). Paired student t-test showed that a concentration of 3.0 g/L of *R. scutellariae* in combination with vancomycin was found to be effective in reducing the vancomycin MIC to susceptible levels (P = <0.001) demonstrating a significant change in vancomycin susceptibility for the isolates (statistical analysis are as shown in Appendix IV). The FICI was found to be 0.665 indicating no interaction between *R. coptidis* and vancomycin.

TABLE 6.6: Results of antimicrobial activity of *Radix scutellariae* alone and in combination (pH 7.0)

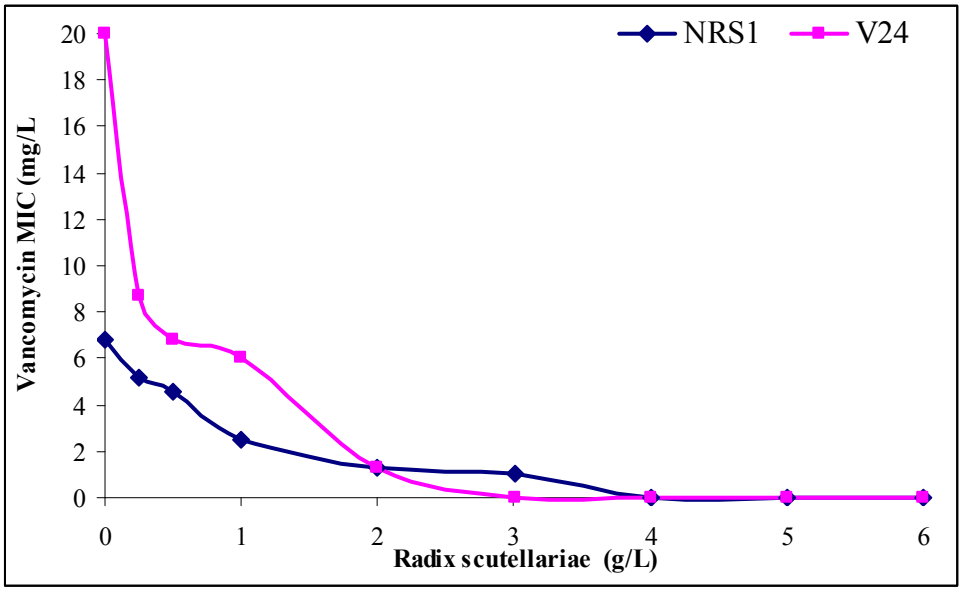
Strain No.	Vancomycin MIC (mg/L)		Herb MIC by AD (g/L)	Vancomycin MIC (mg/L) in combination with herb by SGE								
				Herb extract (g/L)								
	AD	SGE EC/TEC		0.0	0.25	0.5	1	2	3	4	5	6
NRS149	0.5	≤ 1	0.25	≤ 1	≤ 1	NG	NG	NG	NG	NG	NG	NG
NRS100	0.5	≤ 1	0.25	≤ 1	≤ 1	NG	NG	NG	NG	NG	NG	NG
MR1	2.0	1.5	2.0	1.5	1.5	1.3	≤ 1	NG	NG	NG	NG	NG
MR2	2.0	1.5	2.0	1.5	1.5	1.3	≤ 1	NG	NG	NG	NG	NG
MR3	2.0	1.5	2.0	1.5	1.5	1.3	≤ 1	NG	NG	NG	NG	NG
MR4	2.0	1.5	2.0	1.5	1.5	1.3	≤ 1	NG	NG	NG	NG	NG
MR5	2.0	1.5/2.2	2.0	1.5/2.2	1.5	1.3	≤ 1	NG	NG	NG	NG	NG
MR6	2.0	1.5/2.2	2.0	1.5/2.2	1.5	1.3	≤ 1	≤ 1	NG	NG	NG	NG
MR7	2.0	1.5/2.2	2.0	1.5/2.2	1.5	1.3	1.1	≤ 1	NG	NG	NG	NG
MR8	3.0	1.5/2.8	2.0	1.5/2.8	1.5	1.3	1.1	≤ 1	NG	NG	NG	NG
MR9	3.0	1.5/2.8	2.0	1.5/2.8	1.5	1.3	1.1	≤ 1	NG	NG	NG	NG
MR10	4.0	1.5/4.0	2.0	1.5/4.0	1.5	1.3	1.3	NG	NG	NG	NG	NG
HV1	3.0	2.2/2.8	2.0	2.2/2.8	2.5	1.3	≤ 1	NG	NG	NG	NG	NG
HV2	3.0	2.5/2.8	2.0	2.5/2.8	2.5	1.3	1.3	≤ 1	NG	NG	NG	NG
HV3	3.0	2.2/3.2	2.0	2.2/3.2	2.5	1.3	≤ 1	NG	NG	NG	NG	NG
HV4	3.0	3.0/3.2	2.0	3.0/3.2	2.5	1.3	≤ 1	NG	NG	NG	NG	NG
HV5	3.0	2.2/2.8	2.0	2.2/2.8	2.0	2.0	1.3	≤ 1	NG	NG	NG	NG
HV6	3.0	2.5/2.8	2.0	2.5/2.8	2.5	2.0	1.3	1.1	NG	NG	NG	NG
HV7	3.0	2.5/3.2	2.0	2.5/3.2	2.5	2.0	1.3	≤ 1	NG	NG	NG	NG
HV8	3.0	2.2/2.8	2.0	2.2/2.8	2.0	2.0	1.3	1.1	NG	NG	NG	NG
HV9	3.0	3.2/4.1	3.0	3.2/4.1	3.0	2.0	1.3	≤ 1	NG	NG	NG	NG
HV10	3.0	3.0/4.1	3.0	3.0/4.1	2.5	2.5	1.3	1.1	NG	NG	NG	NG
HV11	3.0	2.2/3.2	2.0	2.2/3.2	2.5	2.0	1.3	NG	NG	NG	NG	NG
HV12	3.0	2.2/3.2	2.0	2.2/3.2	2.5	2.0	1.3	≤ 1	NG	NG	NG	NG
HV13	3.0	2.2/2.8	2.0	2.2/2.8	2.5	2.0	1.3	≤ 1	NG	NG	NG	NG

Continued on the following page

Table 6.6 Continued

Strain No.	Vancomycin MIC (mg/L)		Herb MIC by AD (g/L)	Vancomycin MIC (mg/L) in combination with herb by SGE								
				Herb extract (g/L)								
	AD	SGE EC/TEC		0.0	0.25	0.5	1	2	3	4	5	6
NRS1	7.0	6.8	4.0	6.8	5.2	4.6	2.5	1.3	≤ 1	NG	NG	NG
V1	6.0	6.0	4.0	6.0	5.2	4.0	2.5	1.3	NG	NG	NG	NG
V2	6.0	6.0	4.0	6.0	5.2	4.0	2.5	1.3	NG	NG	NG	NG
V3	8.0	8.7	4.0	8.7	5.2	4.6	2.5	1.3	≤ 1	NG	NG	NG
V4	10.0	10.2	4.0	10.2	8.6	5.3	3.6	1.5	≤ 1	NG	NG	NG
V5	8.0	8.7	4.0	8.7	5.2	4.6	2.5	1.3	≤ 1	NG	NG	NG
V6	8.0	7.7	4.0	7.7	5.2	4.6	2.5	1.3	≤ 1	NG	NG	NG
V7	10.0	9.0	4.0	9.0	5.2	5.3	3.6	1.5	≤ 1	NG	NG	NG
V8	5.0	5.3	4.0	5.3	5.2	3.6	2.2	1.1	NG	NG	NG	NG
V9	6.0	6.0	4.0	6.0	5.2	3.6	2.2	1.3	NG	NG	NG	NG
V10	8.0	7.7	4.0	7.7	5.2	4.0	2.5	1.3	≤ 1	NG	NG	NG
V11	8.0	7.7	4.0	7.7	5.2	4.0	2.5	1.3	≤ 1	NG	NG	NG
V12	9.0	8.7	4.0	8.7	5.2	4.6	2.5	1.5	≤ 1	NG	NG	NG
V13	12.0	11.6	4.0	11.6	6.8	5.3	4.6	1.5	≤ 1	NG	NG	NG
V14	12.0	11.6	4.0	11.6	7.7	6.0	4.6	1.5	≤ 1	NG	NG	NG
V15	11.0	10.2	4.0	10.2	6.0	5.3	4.0	1.7	≤ 1	NG	NG	NG
V16	10.0	10.2	4.0	10.2	6.0	5.3	4.0	1.7	≤ 1	NG	NG	NG
V17	12.0	11.6	4.0	11.6	7.7	5.3	4.6	1.7	1.1	NG	NG	NG
V18	12.0	11.6	4.0	11.6	7.7	5.3	4.6	1.7	1.1	NG	NG	NG
V19	20.0	20.0	5.0	20.0	8.7	6.8	6.0	2.5	1.3	NG	NG	NG
V20	20.0	20.0	5.0	20.0	8.7	6.8	6.0	2.5	1.3	NG	NG	NG
V21	20.0	20.0	5.0	20.0	8.7	6.8	6.0	2.5	1.3	NG	NG	NG
V22	20.0	20.0	5.0	20.0	8.7	6.8	6.0	2.5	1.5	NG	NG	NG
V23	20.0	20.0	5.0	20.0	8.7	6.8	6.0	2.5	1.3	NG	NG	NG
V24	20.0	20.0	5.0	20.0	8.7	6.8	6.0	2.5	1.3	NG	NG	NG

MR=MRSA, HV=hVISA, V=VISA, Shading=Significant change in susceptibility



NRS1=VISA control strain, V24= laboratory induced strain with MIC 20mg/L

FIGURE 6.5: Antimicrobial activity of *Radix scutellariae* (pH 7.0) and vancomycin on VISA strains

6.3.6 Effect of pH on Efficacy of *Radix scutellariae* on MRSA and Vancomycin Non-Susceptible Strains

Although, *R. scutellariae* is effective in reducing the MICs of tested MRSA and VISA isolates to susceptible levels at all pH values, the *R. scutellariae* was found to be most effective at the initial pH i.e 5.2.

The pH of the agar was found to be 7.1 for BHA alone, BHA supplemented with 6g/L of *R. scutellariae* pH 5.2 (7.0), BHA supplemented with 6.0 g/L of *R. scutellariae* pH 5.6 (7.0) and BHA supplemented with 6.0 g/L of *R. scutellariae* pH 7.0 (7.0) suggesting that the antimicrobial activity and synergy was not due to the ability of the herb to create the acidic or alkaline environment in the media, but due to the active phytochemical present in it, which is effective at actual pH.

The combination of *R. scutellariae* (Initial pH 5.2) gave FICI <0.5 and increase in pH values resulted in increase in FICIs suggesting that change in pH resulted in reduced efficacy and loss of synergy and of *R. sctellariae*.

In summary, combination of 0.25 gm/L *R. scutellariae* (pH 5.2) and vancomycin was the most effective with synergistic effect against MRSA and VISA.

6.4 DISCUSSION

Emergence of MRSA and VISA has resulted in decreased antimicrobial efficacy, and an increase in frequency of treatment failure and morbidity. This raises the need for structurally new anti-MRSA, anti-VISA agents and / or agents that have synergistic effect. Plant based resources offer a unique pool of chemical substances with therapeutic value and investigation of these phytochemicals is a valid

approach in the search for new antimicrobial agents as they may be chemically different from the traditional antibiotics and may have less propensity to generate non-susceptibility.

In the current study of the three tested herbs, *R. scutellariae* exhibited strongest anti-MRSA, anti-VISA and synergistic effects. Li et. al. (2006) reported the antimicrobial activity of a hot water extract of *R. scutellariae* on *Escherichia coli* and *Bacillus subtilis* at a concentration of 20.0 g/L, in addition *R. scutellariae* has been reported to be effective in inhibiting MSSA. However, in the current study a lower concentration was effective against MRSA and VISA suggesting its effectiveness against resistant *S. aureus.*

The phytochemical baicalin is an abundant constituent of *R. scutellariae* and is recognized as an effective antiviral agent. It is known to halt HIV replication and inhibit both SARS and influenza viruses (Li et al. 2000, Xu et al. 2010). The flavonoid wogonin has a potent antiviral activity. Both *in-vitro* and *in-vivo* studies have shown that wogonin has anti-hepatitis B virus (anti-HBV) activity (Guo et al. 2007).

In addition to being an effective antiviral agent, phytochemicals from *R. scutellariae* are reported to exhibit resistance modulatory effect with antibiotics. Comparing penicillin MIC for *S. aureus* ATCC 29213 indicated that baicalin was able to significantly reduce the MIC from 0.25 g/L to 0.06 gm/L when combined with 128 g/L of baicalin (Meng et. al. 2006). In the current study (combination of vancomycin and *R. scutellariae* extract) similar effects were observed in reducing the vancomycin MIC against a range of vancomycin non-susceptible strains

indicating *R. scutellariae* could be used as a complementary medicine along with vancomycin in treatment of vancomycin non-susceptible MRSA infections.

In the current study *C. phellodendri* was found to exhibit antimicrobial activity alone and in combination with vancomycin. Although, *C. phellodendri* exhibited antimicrobial effect, it is less effective in comparison to *R. sctellariae*, as higher concentraion of *C. phellodendri* was required to exhibit the desired effect. This could be due to the fact that the important phytochemical in *C. phellodendri* and *R. coptidis* is limonin *(*Wagner et al. 2004, Yang et al. 2010) which is known to inhibit HIV replication in the cell (Battinelli et al. 2003). However, limonin has been show to have poor antimicrobial activity and a report suggests that *Pseudomonas putida* was able to utilize limonin as an energy source (Ghosh et al. 2006). This report has also demonstrated that the genes encoding metabolic enzymes are located on a transmissible plasmid, P9 NAH. This transmissible plasmid may spread the gene to other pathogenic bacteria, allowing other bacteria to utilize limonin explaining the poor antibacterial activity of *C. phellodendri* and *R. coptidis*.

By its use in TCM, *R. coptidis* has been found to be an effective antimicrobial drug, and berberine, a major component of *R. coptidis*, has been reported to be strongly effective as an antimicrobial agent (Freile et al. 2003). Research has shown that berberine is effective against *Staphylococcus aureus* and various *Candida species* (Freile. Et. al. 2003). Furthermore, Yu et. al. (2005) demonstrated that 64.0 g/L of berberine could effectively inhibit 90.0 % of MRSA strains. In combination with

oxacillin, berberine was able to restore the effectiveness of the antibiotic against the resistant strains.

The current study has shown that *R. coptidis* was more effective than *C. phellodendri,* but was less effective than *R. sctellariae*, as *R. coptidis* needed a higher concentration than *R. scutellariae* to exhibit the desired effect. This effect may be due to the multi-drug resitance (MDR) pumps which could effectively pump out berberine, rendering it less effective (Tegos et. al. 2002) or other alternative pathways. However, use of an MDR pump inhibitor together with berberine led to a 100 fold increase in effectiveness against *S. aureus* compared to berberine alone (Tegos et. al. 2002). Use of a MDR inhibitor along with *R. coptidis* may improve anti-MRSA and anti-VISA activity. Nevertheless this hypothesis needs to be investigated.

Although, all the herbs were effective in restoring the effectiveness of vancomycin when combined with vancomycin, *R. scutellariae* was the only herb to exhibit synergy when combined with vancomycin. It is evident that *R. scutellariae* was more effective against MRSA and VISA alone and in combination, which may be due to the flavonoids including baicalin and wogonin.

The actual pH of the *R. scutellariae* water extract was 5.2 and change of pH to 5.6 and 7.0 decreased the anti-MRSA and anti-VISA effect suggesting that the lead phytochemical is effective at pH 5.2 and is pH sensitive. The change in pH might have decreased the interaction between *R. scutellariae* and vancomycin through modification of the chemical structure of the active molecule in *R. scutellariae* or the pH may have reduced the structural-activity relationship of lead phytochemical

or the precipitation which occurred during the change in pH could have resulted in reduced concentration of active molecule. Therefore better anti-MRSA and anti-VISA effect observed with *R. scutellariae* hot water extract at actual pH value may be attributed to the presence of active phytochemical and not due to its ability to create a slightly acidic environment as the pH of the agar remained relatively unchanged. Although, some plant extracts are known to exert antimicrobial activity by creating an acidic environment that causes the bacterial cell membrane disruption (Randhir and Shetty 2007), but this was not observed with *R. scutellariae*.

Whilst, *R. coptidis* and *C. phellodendri* was found to be less effective in comparison to *R. scutellariae*, these herbs in TCM are commonly used in combination to treat various disorders including infections (Li et al. 2000, Li et al. 2005). This may be because this combination may exhibit synergistic effect as an anti-infective or due to the nature of certain chemical constituents present in *R. coptidis* and *C. phellodendri* which may block various enzymes involved in bio-synthesis and metabolic pathways and enhance the therapeutic affect of *R. scutellariae* resulting in greater antibacterial power (Oh et. al. 2006). For example Oh et al. (2006) have reported that berberine in addition to its antimicrobial activity could reduce bacterial cell adhesion to host cells, by blocking the enzymes, *sortase A* and *sortase B*, that are responsible for anchoring virulence factors onto the bacterial cell wall and anchoring of the cell to the host. Therefore, blocking the enzyme would render the bacteria less infective. A further investigation is needed to confirm this hypothesis.

In summary, all the three herbs possessed anti-MRSA, anti-VISA and resistance modulatory effects. *R. scutellariae* was the most effective among all the tested herbs as it exhibited synergy with vancomycin and could have significant clinical applications in treatment of vancomycin non-susceptible MRSA infections and in infection control.

The antimicrobial activity of phytochemicals is based on their chemical structures and hence is tailored to target a set of pathogens. The herb *R. scutellariae* contains most effective phytochemicals against vancomycin non-susceptible MRSA strains and may become an effective antibiotic in the coming years as a standalone drug or in a combination with other agents. An unpublished study that investigated cytotoxic effect of *C. phellodendri*, *R. coptidis*, and *R. scutellariae* suggests that *C. phellodendri* and *R. scutellariae* are safe on human cell lines, but further investigation is required to: (i) study the clinical application of RS as a complementary therapy in treatment of MRSA and VISA, (ii) identify the lead phytochemical, (iii) investigate synergistic effect of combination of *R. scutellariae*, *R. coptidis* and *C. phellodendri*, (iv) investigate if an *R. scutellariae* gel / ointment would be safe for human use and (v) investigate gel / ointment formulations of *R. scutellariae* in treatment of SSIs, bed sores, and diabetic ulcers alone or in combination with traditional antibiotics.

CHAPTER 7: MAJOR FINDINGS

7.1 DETECTION OF VANCOMYCIN NON-SUSCEPTIBILITY BY SGE

This work has achieved the aim of developing a rapid, reliable and cost-effective detection method of hVISA/VISA using BHA based SGE. The % CV of the intra-batch reproducibility for MHA, BHA and GBH based SGE ranged between 0 - 22.06, 0 - 9.57 and 0 - 44.9458 respectively. The inter-batch % CVs of the corresponding media ranged between 0 - 31.4, 0 - 8.0464 and 40.4329 – 151.4875 respectively. For inter-observer (intra-batch and inter-batch) reproducibility the % CV ranged between MHA: 0-34.0 and 0-173.20, BHA: 0-10.81 and 0-13.3 and GBH: 0-17.3 and 0-24.790.

Comparison of MICs obtained by SGE showed that 100.0% of the MIC values were within $\pm$1 dilution for BHA based SGE against the refrence method. Notably, 70.0% of the MIC values were within $\pm$1 dilution for MHA based SGE. For GBH based SGE, 56.7% of the MIC values were within $\pm$1 dilution of the reference method. The results obtained by BHA based SGE were comparable to those reported by Pong et. al. (2010) for fastidious organisms.

7.2 INVESTIGATION OF RESISTANCE MECHANISM

This study has achieved its aim to determine the time required for development of non-susceptibility which ranged between 4-22 days for elevation of MIC above 2 mg/L (mean 13 days, median 14 days) and to ≥4 mg/L, ranged from 8-54 days (mean 30 days, median 29 days). All 30 tested isolates developed non-susceptibility within the induction period and, with one exception, lost non-susceptibility upon withdrawal of vancomycin which became a stable phenotype. The proposed molecular mechanism associated with vancomycin non-susceptibility is by means of changes in *vraS* and *graR* during development and loss of non-susceptibility. An additional mutation, D148Q appeared to be required to attain an MIC ≥16 mg/L. The presence of of stop codons may delay non-susceptibility development and absence of stop codon could result in formation of a stable phenotype. The phenotypic changes paralleled genotypic changes with development and loss of resistance. This chapter has demonstrated the need for appropriate AST testing as it was observed that vancomycin non-susceptibility can result in cross resistance to other non-glycopeptide antibiotics and revealed tigecycline as the most effective drug in treatment of vancomycin non-susceptibility. But, the search for consistent genetic marker associated with non-susceptibility development still remains and needs further investigation.

7.3 PREVALENCE OF VANCOMYCIN RESISTANCE AND NEED FOR RESISTANCE DETECTION IN TREATMENT OF MRSA AND VANCOMYCIN NON-SUSCEPTIBLE PHENOTYPES

A total of 14.53% (48/330) of isolates were found to be non-susceptible with vancomycin MIC >2mg/L and all 30 isolates collected from general practitioners were found to be VSSA with MIC ≤ 1mg/L. Although levels of vancomycin non-susceptibility were found to be high in both MRSA and MSSA clinical isolates from hospitalized patients, the former displayed a greater level of resistance. The use of SGE helped in the identification of an additional 2.4% of covert resistant strains. Following incubation with vancomycin (2 mg/L) all presumptive-hVISA strains developed vancomycin non-susceptibility with MICs ranging from 4.12 to 5.29 mg/L by day 14. GRD Etest at 24h and 48h for day 0 strains was 2 mg/L, but resistant sub-populations were observed in only three isolates. The prevalence rate determined by AD method was found to be slightly lower than for SGE, suggesting SGE to be more sensitive in detection of non-susceptibility. Although resistance to various antibiotics were found in isolates from both general practitioner and hospitalized patients, the latter displayed more resistance. A total of 13/200 MRSA strains from hospitalized patients were SCC*mec* type IV showing the spread of typical CA-MRSA strains into the hospital environment.

Although, this study was limited to strains from only one district hospital in Hong Kong, this still represents a large population of Hong Kong. As Hong Kong has a dense urban population concentrated in a small area making variation less likely. The sample of district hospital provides better representation of the Hong Kong

community than the tertiary hospital, as the sample obtained from the tertiary hospital may have been obtained from patients who are severely ill, or may have received therapy at district hospital before being transferred from district hospital. This study has achieved its objective of determining the prevalance of vancomycin non-susceptible isolates in Hong Kong.

7.4 ALTERNATIVE OR COMPLEMENTARY THERAPY

Combination of *C. phellodendri* (3.0 g/L) with vancomycin, *R. coptidis* (2.0 g/L) with vancomycin and combination of *R. scutellariae* (0.25 g/L) with vancomycin resulted in reduction of vancomycin MIC to susceptible level (>2.0 mg/L). *R. scutellariae* in combination exhibited synergistic effect with FIC index of 0.21. The initial pH of *R. scutellariae* extract was 5.2, although the change of pH to pH 5.6 or pH 7.0 resulted in loss of synergistic effect between *R. scutellariae* and vancomycin, these extract retained the desired activity. A further investigation is needed to identify the active phytochemical in order that this agent can be developed and used to improve infection control and clinical outcomes in treatment of MRSA.

CHAPTER 8: CONCLUSIONS AND RECOMMENDATIONS

8.1 INTRODUCTION

The overall purpose of this research was to identify strategies to improve patient management and their outcomes of MRSA infections. This chapter summarizes the conclusions of the results of this research while providing relevant recommendations. In line with the findings from this study, recommendations are identified and further, the potential for utilization and clinical applications are also indicated. In addition, the limitations of this research work are identified and this thesis is concluded with suggestions for further work.

8.2 CONCLUSIONS AND RECOMMENDATIONS

This section revisits the main issues addressed in this research and summarizes the conclusions and recommendations on those issues.

Availability of simple, rapid, reliable, and inexpensive vancomycin non-susceptibility detection tool, especially for the more difficult to detect hVISA phenotypes can go a long way in improving the implementation of effective treatment of MRSA and hVISA/VISA infections, in controlling further development and spread of resistance as well as to for determination of the exact proportion of MRSA with reduced vancomycin susceptibility. Although, PAP-AUC is the gold standard for detection of the resistant sub-population, this method is labor intensive severely restricting its use. Another, recommended method, GRD Etest is a robust, simple and effective tool for detecting vancomycin non-

susceptibility, particularly hVISA phenotypes (Yusof et. al. 2008, Leonard et. al. 2009). But, these specialized Etest strips are relatively expensive restricting their application for routine screening of all MRSA. There is also a need for 48h of incubation to achieve high degree of sensitivity and specificity and this delay for antibiotic change may be critical for a favorable patient outcome.

SGE with a concentration gradient for MIC enables the use of normal media, standard inoculum, and provides greater sensitivity due to the continuous scale of concentrations. It has been shown to be reliable for resistance detection in fastidious organisms (Pong et. al. 2010), but this study was the first report of its use for the detection of hVISA/VISA. The intra-batch, inter-batch and inter-observer reproducibility performed for various vancomycin susceptibility categories produced results superior to AD. The possibility for testing three isolates in a single plate promotes cost-effective detection of vancomycin non-susceptible strains. The concentration gradient across the dilutions provided exact MIC and helped visualize resistant sub-populations after overnight incubation, circumventing some of the limitations associated with other recommended detection methods and supporting the use of SGE for rapid detection of hVISA/VISA. This study also attempted to evaluate SGE as an alternative for detection of vancomycin non-susceptible phenotypes. Comparison of SGE to AD showed that there were no significant differences between the MIC generated by SGE and AD methods. In addition, the use of SGE in Chapter 4, 5 and 6 helped to visualize resistant sub-populations, allowed detection of covert resistance and determined the synergistic effect of TCM herb with vancomycin. Thus, it may be concluded that SGE is a simple, cost-effective, rapid and reliable tool for the

detection of hVISA/VISA and may be used in the examination of combination therapy. This study also recommends SGE for clinical application for effective detection of hVISA/VISA as well as for tracking the change in vancomycin MIC in patients receving vancomycin therapy, in order to provide an appropriate therapy change in a timely manner of necessary for treatment of MRSA infections. It is also inexpensive allowing for surveillance of isolates for epidemiological purposes.

An alternative to phenotypic detection of resistance is molecular detection. Identification of a consistent mutation associated with non-susceptibility development is a pre-requisite for development of a rapid and reliable molecular detection method as well as for better understanding of the molecular mechanism associated with vancomycin non-susceptibility. This study tracked the genotypic and phenotypic changes upon vancomycin exposure in isogenic strains. Variation in mutations observed between strains in both development and loss of vancomycin non-susceptibility between this study and other studies (Kuroda et. al. 2003, Cui et. al. 2005, Howden et. al. 2008, Cui et. al. 2009, Kato et. al. 2010) suggests that changes in *vra*S and *gra*R are important for development and loss of non-susceptibility. But no consistent mutations contributing to non-susceptibility were observed, emphasizing the need of further investigations in other genes. Change in susceptibility was supported by both genotypic and phenotypic changes. This study suggested that MRSA isolates may develop non-susceptibility over 4-22day period (mean 13 days, median 14 days) when exposed to vancomycin and that presence of stop codons could delay development of non-susceptibility, while absence of stop codons could result in formation of stable phenotypes. This study also suggested that change in susceptibility to vancomycin results in altered

susceptibility to other anti-MRSA agents, which may be due to the thickened cell wall hindering the penetration of agents to their site of action. Based on the data obtained in this study, tigecycline appears to be an effective drug against hVISA/VISA, particularly as it continued to be effective even against the laboratory induced VISA-max. However, its use needs further investigation for clinical outcome as its use so far is somewhat limited (Cai et. al. 2011, Saner et. al. 2006).

Moving from laboratory generated strains to clinical isolates, this study demonstrated that determining the prevalence level of hVISA/VISA can help in formulating guidelines for effective identification and treatment, as well as for formulating the infection control guidelines in order to control the development and spread of resistant strains. But, surveillance requires a reliable detection method that is able to detect both hVISA and presumptive-hVISA phenotypes accurately. Currently, there appear to be no reports on reliable detection methods for detection of presumptive-hVISA. This study has revealed a prevalence level of 14.53% of vancomycin non-susceptible phenotypes. Use of SGE helped in identification of additional presumptive-hVISA strains and also demonstrated that these presumptive-hVISA strains were able to develop into hVISA/VISA if exposed to a clinical concentration of vancomycin, for a relatively short period. Although, a resistance sub-populations was observed in eight presumptive-hVISA isolates by SGE, A recent report suggested that resistant sub-populations would rapidly revert to susceptibility after a single passage in drug free medium possibly resulting in vancomycin treatment failure due to undetected hVISA *(Moreillon et. al. 2012)*. However, the *in-situ* organism still retained non-susceptibility and led to

vancomycin treatment failure in animal model. In the absence of a rapid and reliable molecular detection method for such sub-populations, SGE appears to be a suitable method for their detection. It was observed that GRD was effective in detecting only three of the eight presumptive-hVISA in this study, illustrating the superiority of SGE for detecting presumptive-hVISA. The presence of difficult to detect resistant sub-populations emphasizes the need to understand the underlying mechanism in order to find a universal genetic marker and to develop a molecular detection method to help prevent further resistance development and negative clinical outcomes. In the current study all isolates were susceptible to both linezolid and tigecycline as recently reported elsewhere *(Richter et. al. 2011, Namdari et. al. 2012, Saravolatz et. al 2012).* Regardless of the advantages of both linezolid and tigecycline in treatment of MRSA infection, their use is often restricted due to cost, limited experience, and toxicity. Both agents have been approved to treat only certain conditions, linezolid for treatment of SSI and nosocomial pneumonia caused by MRSA; and tigecycline to treat MRSA SSI, and MSSA intra-abdominal infections. The major concern with linezolid is its association with serotonin toxicity and thrombocytopenia (Perry and Jarvis 2001, Lawrence et. al. 2006). Although, toxicity has not been reported with tigecycline treatment, it has been associated with adverse effects such as nausea and vomiting with increase in dose and frequency of administration (Micek 2007); with this the treatment problem associated with vancomycin non-susceptibility remain.

In recent years the antimicrobial efficacy of antibiotics is progressively decreasing, due to increased rates of antibiotic resistance. Very few new anti-MRSA agents have become available and few promising new anti-MRSA agents are in the

pipeline (Arhin et. al. 2012). However, effective anti-MRSA and anti-VISA agents are required for treatment of multi-drug resistant strains and to avoid development of cross-resistance. This study investigated three TCM herbs and found that *R. scutellariae* was the most potent against MRSA and VISA strains. The water extract exhibited synergistic effects with vancomycin and allowing bactericidal effects at attainable vancomycin concentrations. This research demonstrated that further work to investigate clinical application of combination of *Radix scutellariae* with vancomycin to extend the usefulness of vancomycin would be useful.

Overall this research has accomplished its aim of correct detection and appropriate antimicrobial selection in treatment of MRSA and hVISA/VISA (Figure 8.1) through SGE detection method, correlation of genetic changes with exposure time, MIC increase and phenotypic changes. In addition the findings from this study may be useful for the development of novel anti-MRSA and anti-VISA agents.

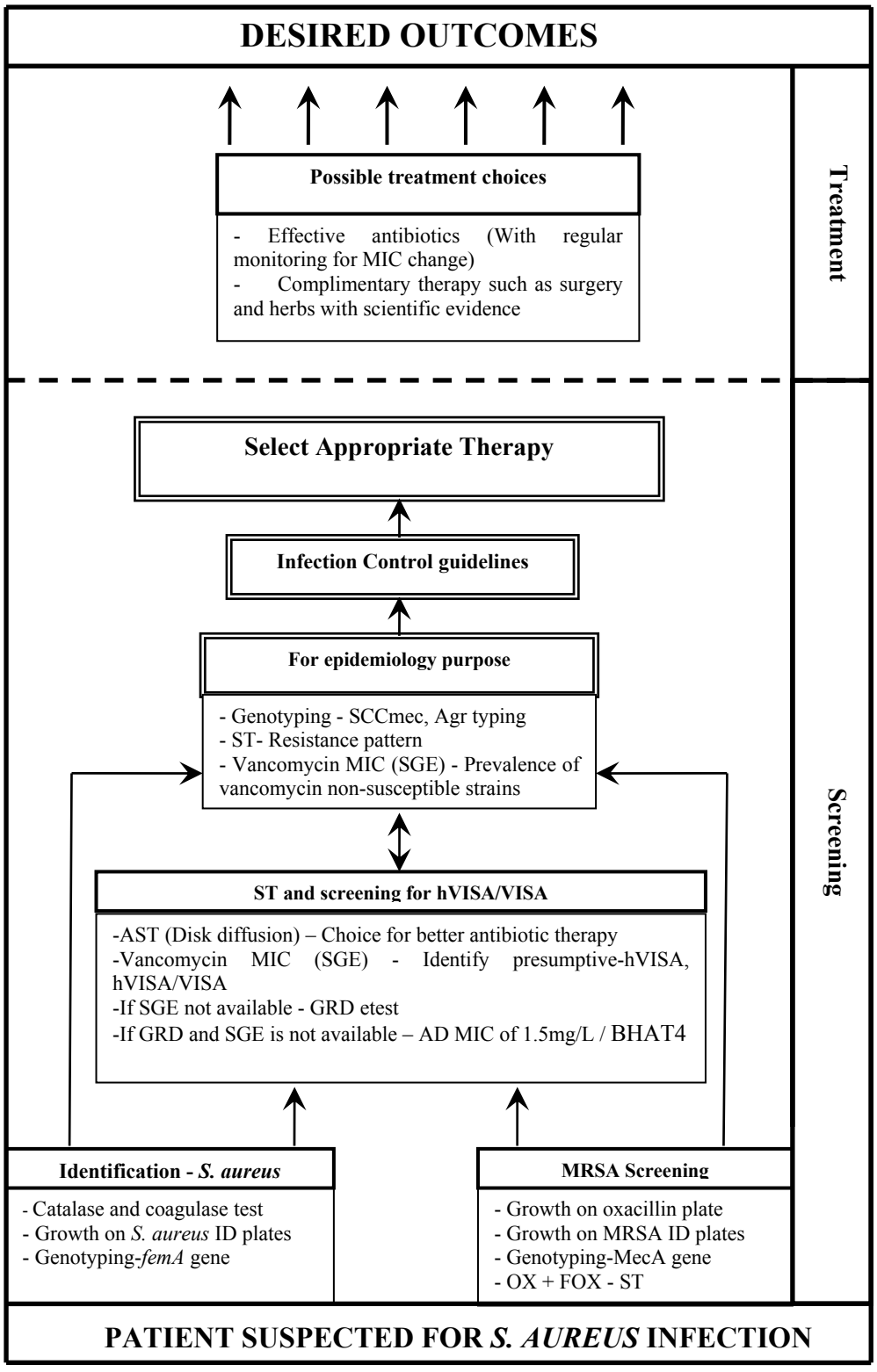


FIGURE 8.1: Framework for desired outcome in treatment and infection control of *S. aureus*

8.3 LIMITATIONS AND SCOPE FOR FURTHER STUDIES

Although, the study successfully completed the objectives described in chapter 2, due to the limited time and resources it does have the following limitations: A multi-center study was not performed and this study recommends further investigation involving several hospitals to investigate intra-laboratory and inter-laboratory precision and accuracy of SGE to determine vancomycin non-susceptibility. The positive predictive value of the SGE for hVISA in comparison to PAP-AUC was not determined in this study and is worthwhile to investigate the same, as a report indicates variable predictive value for vancomycin MIC among AST (Hsu et. al. 2008). It is also worthwhile to investigate the clinical implications of this method in comparison with recommended methods, as a report indicate poor correlation between vancomycin MIC methodologies (van Hal et. al. 2011b) and more recently it has been reported that detection methods needing sub-culturing on drug free media result in poor detection of VISA. Hence these authors have suggested to sub-culture isolates on vancomycin supplemented media to avoid reversion of resistant sub-population to susceptibility (Moreillon et. al. 2012). However, this could result in induction of non-susceptibility due to sub-inhibitory concentrations in the media, possibly use of very low level vancomycin may be useful, but needs further investigation to determine its effectiveness.

This study recommends further work to investigate the accuracy of SGE in determining the effectiveness of combination of antibiotics for combination therapy. A report has suggested that teicoplanin may be more effective in detection of hVISA than vancomycin (Walsh and Howe 2002) and hence it is reasonable to evaluate teicoplanin based SGE in the near feature.

Recently other methods have been suggested for rapid and effective detection of non-susceptibility. The Xpert blood culture assay has been reported to be superior to other automated methods (Kelley et. al. 2011) but needs further evaluation. More recently a delta-hemolysis screening method has also been reported to be effective in detection of hVISA/VISA (Cafiso et. al. 2012b). Thus, further large scale comparative studies between SGE, GRD Xpert assay and delta-hemolysis screening method are needed to to determine the most effective detection method for covert resistant strains and to provide a standardized detection tool for rapid and reliable detection of hVISA/VISA, particularly covert ressitant strains.

Although, this study has investigated *vra, gra* and *rpo*B and has confirmed the association between changes in *vra,* and *gra* genes and vancomycin non-susceptibility as suggested by other studies (Cui et. al. 2009, Gardete et. al. 2012) but, no consistent genetic change was identified. Reports have suggested the importance of changes in other genetic targets including, *mprF, wal*KR, *Yvq*F, *dlt, clp*P and RNAIII for development of non-susceptibility (Dubrac 2007, Kato et. al. 2010, Boyle-Vavra et. al. 2011, Ernst and Peschel 2011, Shoji et. al. 2011, Gardete et. al. 2012, Mehta et. al. 2012, Park et. al. 2012). This study recommends further work on investigating these genes and other genetic loci in the selected set of isogenic strains used in this study to determine if there is a consistent genetic marker and to possibly develop molecular detection method. It is also worthwhile to perform detailed studies on TCS pathways as reports have indicated the importance of TCS in resistance development (West and Stock 2001, Kuroda et. al. 2003, Cui et. al. 2005, Gao and Stock 2009, Galperin 2010) and understanding of these pathways can help understanding determinants associated with different

patterns of non-susceptibility development. In this study no mutations were observed in *rpoB* gene contrary to the findings from other studies (Cui et. al. 2010, Matsuo et. al. 2011, Watanabe et. al. 2011) suggesting further investigation to confirm the role of *rpoB* in vancomycin non-susceptibility. Another limitation of this study is it involved MRSA strains from only one hospital of Hong Kong and only SCC*mec* III. Therefore, it is recommended that other geographically divergent isogenic strains and this should also include other SCC*mec* types, which may help in identification of consistent genetic marker among all vanocmycin non-susceptible MRSA strains. Whether the patterns of development and loss of non-susceptibility is a consequence of specific MRSA clone needs to be determined (Miller et. al. 2012). This study has confirmed the role of stop codons in loss of vancomycin non-susceptibility. This supports a report that indicated inactivation of genes may be associated with loss of non-susceptibility e.g. *sarA* inactivation has been suggested to result in loss of vancomycin non-susceptibility (Lamichhane-Khadka et. al. 2009). In contrast, inactivation of *tcaA* gene has been reported to result in non-susceptibility development (Maki et. al. 2004). A further large scale study is needed to confirm the role of *tcaA* and *sarA* inactivation in development and loss of vancomycin non-susceptibility.

This study determined the prevalence level of vancomycin non-susceptibility among MRSA strains obtained from a district hospital and demonstrated the importance of early detection of presumptive-hVISA. However, this study was limited to strains from only one district hospital of Hong Kong. Hence, it is worthwhile to perform a multi-centre study to determine the prevalence level of hVISA/VISA in Hong Kong. Another limitation of this study was, the

presumptive-hVISA were not confirmed by PAP-AUC. This study has also demonstrated that exposure of presumptive-hVISA to therapeutic levels of vancomycin results in resistance selection supporting the association of clinical failure of vancomycin with increase in MIC and helps explain treatment failure among VSSA strains (Rybak et. al. 2008, Sariono et. al. 2008, Musta et. al. 2009, Holmes et. al. 2011, Takesue et. al. 2011). However, there exists considerable controversy about vancomycin MIC and vancomycin clinical failure. Some reports have associated clinical failure to site of infection including endocarditis and pneumonia rather than the vancomycin MIC (Lubin et. al. 2011, Walraven et. al. 2011). In contrast to the finding of this study i.e role of resistance sub-population in development for further resistance some reports suggests that hVISA is not exclusively associated with tolerance indicating no association between clinical outcome and hVISA and / or vancomycin MIC, and have attributed clinical failure to additional confounding factors such as strain type, age, presence of co-morbidities and severity of the illness as strong predictors for treatment failure (Khatib et. al. 2011, van Hal et. al. 2011, Moore et. al. 2012,). Therefore, this study recommends further study on clinical implications of early and accurate detection of presumptive-hVISA.

Although, this study has demonstrated anti-VISA activity of *R. scutellariae* and its synergistic effect in combination with vancomycin in an *in-vitro* study, further studies on clinical application of *R. scutellariae* as a complementary therapy in treatment of MRSA and VISA are needed. Due to the time limitation the lead phytochemical responsible for the observed activities was not identified, so a further investigation for lead phytochemcial is recommended. It is also worthwhile

to investigate if there is a possibility of synergistic combination of *R. scutellariae*, *R. coptidis* and *C. phellodendri* as these are used in combination in TCM to treat various infections. Further investigations are required for safe human use of *R. scutellariae* topical formulation and to investigate clinical application of these formulations in treatment of SSIs, bed sores, and diabetic ulcer alone or in combination with traditional antibiotics.

In conclusion, this is the first study to demonstrate the application of SGE for rapid detection of vancomycin non-susceptible *S. aureus* including presumptive-hVISA, and anti-VISA activity of *C. phellodendri, R. coptidis* and *R. scutellariae* providing useful insights for development of new antimicrobial agents. Interesting findings in regard to genetic and phenotypic changes during development and loss of vancomycin non-susceptibility in MRSA isolates was role of stopcodons in delaying the development and loss of non-susceptibility and importance of change D148Q in *graR* in acquiring high level of vancomycin resistance. The findings presented here will support further studies in the area of vancomycin non-susceptible *S. aureus,* may have clinical implication in treatment and control of further resistance development.

APPENDIX

Mueller Hinton agar intra-batch data –Day -1

AD	1	1	3		3		5	7	16	18
Sample no / Set	**NRS70**	**B25**	**h20 (EC)**	**h 20 (TEC)**	**h40 (EC)**	**h 40 (TEC)**	**V3**	**NRS1**	**E23**	**E32**
1	1	1	2.494	3.206	3.206	6.002	4.67	5.29	13.17	10.253
2	1	1	2.494	4.67	3.206	5.29	4.67	5.29	13.17	10.253
3	1	1	2.494	3.206	3.206	4.67	4.67	5.29	13.17	10.253
4	1	1	2.494	5.29	3.206	3.67	4.67	5.29	13.17	10.253
5	1	1	2.494	4.67	3.206	5.29	4.67	5.29	13.17	10.253
6	1	1	2.494	3.206	3.206	3.206	4.67	5.29	13.17	10.253
7	1	1	2.494	3.634	3.206	5.29	4.67	5.29	13.17	10.253
8	1	1	2.494	3.206	3.206	4.12	4.67	5.29	13.17	10.253
Mean	1	1	2.494	3.886	3.206	4.69225	4.67	5.29	13.17	10.253
SD	0	0	0	0.8547	0	0.9538	0	0	0	0
CV %	0	0	0	21.994	0	20.3289	0	0	0	0

Mueller Hinton agar intra-batch data –Day -2

AD	1	1	3		3		5	7	17	19
Sample no / Set	**NRS70**	**B25**	**h20 (EC)**	**h 20 (TEC)**	**h40 (EC)**	**h 40 (TEC)**	**V3**	**NRS1**	**E23**	**E32**
1	1	1	2.494	4.67	2.828	3.643	4.12	5.29	14.938	10.253
2	1	1	2.494	3.634	2.828	6.002	4.12	5.29	14.938	10.253
3	1	1	2.494	3.634	2.828	4.12	4.12	5.29	14.938	10.253
4	1	1	2.494	3.206	2.828	4.12	4.12	5.29	14.938	10.253
5	1	1	2.494	3.634	2.828	6.002	4.12	5.29	14.938	10.253
6	1	1	2.494	3.206	2.828	6.002	4.12	5.29	14.938	10.253
7	1	1	2.494	3.634	2.828	4.12	4.12	5.29	14.938	10.253
8	1	1	2.494	4.67	2.828	6.002	4.12	5.29	14.938	10.253
Mean	1	1	2.494	3.786	2.828	5.001375	4.12	5.29	14.938	10.253
SD	0	0	0	0.5767	0	1.0810	0	0	0	0
CV %	0	0	0	15.2325	0	21.6150	0	0	0	0

Mueller Hinton agar intra-batch data –Day -3

AD	1	1	3		3		5	7	17	18
Sample no / Set	**NRS70**	**B25**	**h20 (EC)**	**h 20 (TEC)**	**h40 (EC)**	**h 40 (TEC)**	**V3**	**NRS1**	**E23**	**E32**
1	1	1	2.494	2.828	2.828	4.67	4.12	6.002	14.938	10.253
2	1	1	2.494	3.206	2.828	6.002	4.12	6.002	14.938	10.253
3	1	1	2.494	3.634	2.828	3.206	4.12	6.002	14.938	10.253
4	1	1	2.494	3.206	2.828	5.29	4.12	6.002	14.938	10.253
5	1	1	2.494	3.634	2.828	4.67	4.12	6.002	14.938	10.253
6	1	1	2.494	4.67	2.828	5.29	4.12	6.002	14.938	10.253
7	1	1	2.494	3.634	2.828	3.634	4.12	6.002	14.938	10.253
8	1	1	2.494	3.634	2.828	5.29	4.12	6.002	14.938	10.253
Mean	1	1	2.494	3.5557	2.828	4.7565	4.12	6.002	14.938	10.253
SD	0	0	0	0.5308	0	0.9322	0	0	0	0
CV %	0	0	0	15.1833	0	19.6003	0	0	0	0

Mueller Hinton agar intra-batch data –Day -4

AD	1	1	3		3		5	6	17	19
Sample no / Set	**NRS70**	**B25**	**h20 (EC)**	**h 20 (TEC)**	**h40 (EC)**	**h 40 (TEC)**	**V3**	**NRS1**	**E23**	**E32**
1	1	1	2.828	3.634	3.206	6.002	4.12	5.29	11.623	10.253
2	1	1	2.828	5.29	3.206	6.002	4.12	5.29	11.623	10.253
3	1	1	2.828	3.634	3.206	3.634	4.12	5.29	11.623	10.253
4	1	1	2.828	4.12	3.206	4.02	4.12	5.29	11.623	10.253
5	1	1	2.828	3.634	3.206	6.002	4.12	5.29	11.623	10.253
6	1	1	2.828	4.67	3.206	6.002	4.12	5.29	11.623	10.253
7	1	1	2.828	4.12	3.206	3.634	4.12	5.29	11.623	10.253
8	1	1	2.828	3.206	3.206	4.67	4.12	5.29	11.623	10.253
Mean	1	1	2.828	4.0385	3.206	4.99575	4.12	5.29	11.623	10.253
SD	0	0	0	0.6721	0	1.1223	0	0	0	0
CV %	0	0	0	16.6428	0	22.0653	0	0	0	0

Mueller Hinton agar intra-batch data –Day -5

AD	1	1	3		3		5	7	16	17
Sample no / Set	**NRS70**	**B25**	**h20 (EC)**	**h 20 (TEC)**	**h40 (EC)**	**h 40 (TEC)**	**V3**	**NRS1**	**E23**	**E32**
1	1	1	2.828	3.206	2.828	5.29	4.12	5.29	11.623	14.938
2	1	1	2.828	4.67	2.828	3.206	4.12	5.29	11.623	14.938
3	1	1	2.828	3.634	2.828	3.634	4.12	5.29	11.623	14.938
4	1	1	2.828	3.206	2.828	5.29	4.12	5.29	11.623	14.938
5	1	1	2.828	5.29	2.828	4.12	4.12	5.29	11.623	14.938
6	1	1	2.828	4.12	2.828	5.29	4.12	5.29	11.623	14.938
7	1	1	2.828	5.29	2.828	5.29	4.12	5.29	11.623	14.938
8	1	1	2.828	5.29	2.828	4.12	4.12	5.29	11.623	14.938
Mean	1	1	2.828	4.338	2.828	4.53	4.12	5.29	11.623	14.938
SD	0	0	0	0.9208	0	0.8620	0	0	0	0
CV %	0	0	0	21.2251	0	19.0306	0	0	0	0

Mueller Hinton agar intra-batch data –Day -6

AD	1	1	3		3		5	6	17	17
Sample no / Set	**NRS70**	**B25**	**h20 (EC)**	**h 20 (TEC)**	**h40 (EC)**	**h 40 (TEC)**	**V3**	**NRS1**	**E23**	**E32**
1	1	1	2.494	4.12	3.206	4.67	4.12	4.67	10.253	14.938
2	1	1	2.494	4.12	3.206	4.12	4.12	4.67	10.253	14.938
3	1	1	2.494	5.29	3.206	6.002	4.12	4.67	10.253	14.938
4	1	1	2.494	4.67	3.206	5.29	4.12	4.67	10.253	14.938
5	1	1	2.494	4.67	3.206	6.002	4.12	4.67	10.253	14.938
6	1	1	2.494	6.002	3.206	3.634	4.12	4.67	10.253	14.938
7	1	1	2.494	3.634	3.206	3.634	4.12	4.67	10.253	14.938
8	1	1	2.494	6.002	3.206	4.76	4.12	4.67	10.253	14.938
Mean	1	1	2.494	4.8135	3.206	4.764	4.12	4.67	10.253	14.938
SD	0	0	0	0.8820	0	0.9501	0	0	0	0
CV %	0	0	0	18.3250	0	19.9441	0	0	0	0

Mueller Hinton agar intra-batch data –Day -7

AD	1	1	3		3		5	7	17	18
Sample no / Set	**NRS70**	**B25**	**h20 (EC)**	**h 20 (TEC)**	**h40 (EC)**	**h 40 (TEC)**	**V3**	**NRS1**	**E23**	**E32**
1	1	1	2.828	3.206	3.206	6.002	4.67	6.804	11.623	13.17
2	1	1	2.828	4.67	3.206	5.29	4.67	6.804	11.623	13.17
3	1	1	2.828	6.002	3.206	3.206	4.67	6.804	11.623	13.17
4	1	1	2.828	4.12	3.206	4.67	4.67	6.804	11.623	13.17
5	1	1	2.828	3.643	3.206	4.12	4.67	6.804	11.623	13.17
6	1	1	2.828	5.29	3.206	4.67	4.67	6.804	11.623	13.17
7	1	1	2.828	4.67	3.206	5.29	4.67	6.804	11.623	13.17
8	1	1	2.828	4.12	3.206	4.12	4.67	6.804	11.623	13.17
Mean	1	1	2.828	4.4651	3.206	4.671	4.67	6.804	11.623	13.17
SD	0	0	0	0.8964	0	0.8694	0	0	0	0
CV %	0	0	0	20.0775	0	18.6132	0	0	0	0

Mueller Hinton agar intra-batch data –Day -8

AD	1	1	3		3		5	6	17	16
Sample no / Set	**NRS70**	**B25**	**h20 (EC)**	**h 20 (TEC)**	**h40 (EC)**	**h 40 (TEC)**	**V3**	**NRS1**	**E23**	**E32**
1	1	1	2.494	5.29	2.828	4.67	4.12	4.67	14.938	10.253
2	1	1	2.494	4.12	2.828	6.002	4.12	4.67	14.938	10.253
3	1	1	2.494	6.002	2.828	5.29	4.12	4.67	14.938	10.253
4	1	1	2.494	4.67	2.828	5.29	4.12	4.67	14.938	10.253
5	1	1	2.494	3.206	2.828	3.634	4.12	4.67	14.938	10.253
6	1	1	2.494	4.12	2.828	4.12	4.12	4.67	14.938	10.253
7	1	1	2.494	3.634	2.828	4.12	4.12	4.67	14.938	10.253
8	1	1	2.494	3.634	2.828	4.67	4.12	4.67	14.938	10.253
Mean	1	1	2.494	4.3345	2.828	4.7245	4.12	4.67	14.938	10.253
SD	0	0	0	0.9375	0	0.7743	0	0	0	0
CV %	0	0	0	21.6299	0	16.3905	0	0	0	0

Mueller Hinton agar inter-batch data

S No	N70		B25		h20			h40			V3		NRS1		E23		E32	
Method / Day	**AD**	**SGE**	**AD**	**SGE**	**AD**	**EC**	**TEC**	**AD**	**EC**	**TEC**	**AD**	**SGE**	**AD**	**SGE**	**AD**	**SGE**	**AD**	**SGE**
1	1	1	1	1	3	3.206	6.804	3	2.2	6.002	5	5.29	6	6.002	17	11.623	20	10.253
2	1	1	1	1	3	2.828	6.002	3	2.828	3.634	4	4.12	7	6.804	17	13.17	18	8.288
3	1	1	1	1	3	3.206	4.12	3	3.206	3.634	5	4.67	7	6.804	17	11.623	19	10.253
4	1	1	1	1	3	2.494	4.12	3	2.494	3.634	4	4.12	7	7.744	17	10.253	18	10.253
5	1	1	1	1	3	2.828	4.12	3	2.2	4.12	5	5.29	6	6.002	16	10.253	19	8.288
6	1	1	1	1	3	2.2	2.828	3	2.2	3.206	5	4.67	6	6.002	17	10.253	18	10.253
7	1	1	1	1	3	2.494	2.828	3	2.494	3.206	5	4.12	6	6.002	17	10.253	18	8.288
8	1	1	1	1	3	2.828	4.67	3	2.828	6.002	5	4.12	6	6.804	17	10.253	19	10.253
Mean		1		1		2.760	4.436		2.556	4.179		4.55		6.520		10.960		9.516
SD		0		0		0.3519	1.3931		0.3700	1.1609		0.5159		0.63397		1.0864		1.01698
%CV		0		0		12.7475	31.4012		14.4748	27.7756		11.3400		9.7227		9.9126		10.6869

Brain Heart Infusion agar intra-batch data- Day -1

AD	1	1	3		3		5	7	16	18
Sample no / Set	**NRS70**	**B25**	**h20 (EC)**	**h 20 (TEC)**	**h40 (EC)**	**h 40 (TEC)**	**V3**	**NRS1**	**E23**	**E32**
1	1	1	2.828	5.29	2.828	6.002	4.67	6.804	17	17
2	1	1	2.828	4.12	2.828	6.002	4.67	6.804	17	17
3	1	1	2.828	4.67	2.828	6.002	4.67	6.804	17	17
4	1	1	2.828	4.67	2.828	6.002	4.67	6.804	17	17
5	1	1	2.828	4.12	2.828	6.002	4.67	6.804	17	17
6	1	1	2.828	4.67	2.828	6.002	4.67	6.804	17	17
7	1	1	2.828	4.67	2.828	6.002	4.67	6.804	17	17
8	1	1	2.828	4.67	2.828	6.804	4.67	6.804	17	17
Mean	1	1	2.828	4.61	2.828	6.10225	4.67	6.804	17	17
SD	0	0	0	0.3704	0	0.2835	0	0	0	0
CV %	0	0	0	8.0356	0	4.6466	0	0	0	0

Brain Heart Infusion agar intra-batch data - Day -2

AD	1	1	3		3		5	7	17	19
Sample no / Set	**NRS70**	**B25**	**h20 (EC)**	**h 20 (TEC)**	**h40 (EC)**	**h 40 (TEC)**	**V3**	**NRS1**	**E23**	**E32**
1	1	1	2.828	5.29	2.828	6.804	4.67	6.804	17	17
2	1	1	2.828	4.67	2.828	6.804	4.67	6.804	17	17
3	1	1	2.828	4.67	2.828	6.804	4.67	6.804	17	17
4	1	1	2.828	4.67	2.828	7.714	4.67	6.804	17	17
5	1	1	2.828	4.67	2.828	6.804	4.67	6.804	17	17
6	1	1	2.828	4.67	2.828	6.804	4.67	6.804	17	17
7	1	1	2.828	4.67	2.828	6.804	4.67	6.804	17	17
8	1	1	2.828	4.67	2.828	6.804	4.67	6.804	17	17
Mean	1	1	2.828	4.7475	2.828	6.9177	4.67	6.804	17	17
SD	0	0	0	0.2192	0	0.3217	0	0	0	0
CV %	0	0	0	4.6172	0	4.6508	0	0	0	0

Brain Heart Infusion agar intra-batch data - Day -3

AD	1	1	3		3		5	7	17	18
Sample no / Set	**NRS70**	**B25**	**h20 (EC)**	**h 20 (TEC)**	**h40 (EC)**	**h 40 (TEC)**	**V3**	**NRS1**	**E23**	**E32**
1	1	1	2.828	3.604	2.828	6.002	4.67	6.804	17	17
2	1	1	2.828	4.12	2.828	6.804	4.67	7.774	17	17
3	1	1	2.828	4.12	2.828	6.804	4.67	6.804	17	17
4	1	1	2.828	4.12	2.828	6.002	4.67	6.804	17	17
5	1	1	2.828	4.12	2.828	6.804	4.67	6.804	17	17
6	1	1	2.828	4.12	2.828	6.804	4.67	6.804	17	17
7	1	1	2.828	4.12	2.828	6.804	4.67	6.804	17	17
8	1	1	2.828	4.12	2.828	6.804	4.67	6.804	17	17
Mean	1	1	2.828	4.0555	2.828	6.6035	4.67	6.9252	17	17
SD	0	0	0	0.1824	0	0.3712	0	0.3429	0	0
CV %	0	0	0	4.4984	0	5.6220	0	4.9521	0	0

Brain Heart Infusion agar intra-batch data - Day -4

AD	1	1	3		3		5	6	17	19
Sample no / Set	**NRS70**	**B25**	**h20 (EC)**	**h 20 (TEC)**	**h40 (EC)**	**h 40 (TEC)**	**V3**	**NRS1**	**E23**	**E32**
1	1	1	2.828	4.12	2.828	3.206	4.67	6.002	17	17
2	1	1	2.828	4.12	2.828	3.634	4.67	6.002	17	17
3	1	1	2.828	3.634	2.828	3.634	4.67	6.002	17	17
4	1	1	2.828	3.634	2.828	3.634	4.67	6.002	17	17
5	1	1	2.828	4.12	2.828	4.12	5.295	6.002	17	17
6	1	1	2.828	4.12	2.828	4.12	4.67	6.002	17	17
7	1	1	2.828	4.67	2.828	4.12	4.67	6.002	17	17
8	1	1	2.828	4.12	2.828	4.12	4.67	6.002	17	17
Mean	1	1	2.828	4.0672	2.828	3.8235	4.7481	6.002	17	17
SD	0	0	0	0.3279	0	0.3465	0.2209	0	0	0
CV %	0	0	0	8.0619	0	9.0637	4.6538	0	0	0

Brain Heart Infusion agar intra-batch data - Day -5

AD	1	1	3		3		5	7	16	17
Sample no / Set	**NRS70**	**B25**	**h20 (EC)**	**h 20 (TEC)**	**h40 (EC)**	**h 40 (TEC)**	**V3**	**NRS1**	**E23**	**E32**
1	1	1	2.828	4.12	2.828	4.67	4.67	6.804	17	14.938
2	1	1	2.828	4.12	2.828	3.634	4.67	6.804	17	14.938
3	1	1	2.828	4.12	2.828	3.634	4.67	6.804	17	14.938
4	1	1	2.828	4.12	2.828	3.634	4.67	6.804	17	14.938
5	1	1	2.828	4.12	2.828	3.643	4.67	6.804	17	14.938
6	1	1	2.828	4.12	2.828	4.12	4.67	6.804	17	14.938
7	1	1	2.828	4.67	2.828	4.12	4.67	6.804	17	14.938
8	1	1	2.828	4.12	2.828	4.12	4.67	6.804	17	14.938
Mean	1	1	2.828	4.1887	2.828	3.9468	4.67	6.804	17	14.938
SD	0	0	0	0.1944	0	0.3777	0	0	0	0
CV %	0	0	0	4.6423	0	9.5707	0	0	0	0

Brain Heart Infusion agar intra-batch data - Day -6

AD	1	1	3		3		5	6	17	17
Sample no / Set	**NRS70**	**B25**	**h20 (EC)**	**h 20 (TEC)**	**h40 (EC)**	**h 40 (TEC)**	**V3**	**NRS1**	**E23**	**E32**
1	1	1	2.828	4.67	2.828	5.29	4.67	6.804	17	14.938
2	1	1	2.828	4.67	2.828	4.67	4.67	6.804	17	14.938
3	1	1	2.828	4.67	2.828	4.67	4.67	6.804	17	14.938
4	1	1	2.828	4.67	2.828	4.67	4.67	6.804	17	14.938
5	1	1	2.2	4.67	2.828	4.67	4.67	6.804	17	14.938
6	1	1	2.828	4.67	2.828	4.67	4.67	6.804	17	14.938
7	1	1	2.828	5.29	2.828	4.67	4.67	6.804	17	14.938
8	1	1	2.828	4.12	2.828	4.12	4.67	6.804	17	14.938
Mean	1	1	2.7495	4.6787	2.828	4.6787	4.67	6.804	17	14.938
SD	0	0	0.2220	0.3131	0	0.3131	0	0	0	0
CV %	0	0	8.0753	6.6922	0	6.6922	0	0	0	0

Brain Heart Infusion agar intra-batch data - Day -7

AD	1	1	3		3		5	7	17	18
Sample no / Set	**NRS70**	**B25**	**h20 (EC)**	**h 20 (TEC)**	**h40 (EC)**	**h 40 (TEC)**	**V3**	**NRS1**	**E23**	**E32**
1	1	1	2.828	4.67	2.828	4.12	4.67	6.804	17	17
2	1	1	2.828	4.67	2.828	4.67	4.67	6.804	17	17
3	1	1	2.828	5.29	2.828	4.67	4.67	6.804	17	17
4	1	1	2.828	4.67	2.828	5.29	4.67	6.804	17	17
5	1	1	2.828	4.67	2.828	4.67	4.67	6.804	17	17
6	1	1	2.828	4.67	2.828	4.67	4.67	6.804	17	17
7	1	1	2.828	4.67	2.828	4.67	4.67	6.804	17	17
8	1	1	2.828	4.12	2.828	4.12	4.67	6.804	17	17
Mean	1	1	2.828	4.678	2.828	4.61	4.67	6.804	17	17
SD	0	0	0	0.3131	0	0.3704	0	0	0	0
CV %	0	0	0	6.6922	0	8.0356	0	0	0	0

Brain Heart Infusion agar intra-batch data - Day -8

AD	1	1	3		3		5	6	17	16
Sample no / Set	**NRS70**	**B25**	**h20 (EC)**	**h 20 (TEC)**	**h40 (EC)**	**h 40 (TEC)**	**V3**	**NRS1**	**E23**	**E32**
1	1	1	2.828	5.29	2.828	6.002	4.67	6.002	17	17
2	1	1	2.828	4.67	2.828	6.002	4.67	6.002	17	17
3	1	1	2.828	5.29	2.828	5.29	4.67	6.002	17	17
4	1	1	2.828	4.67	2.828	6.002	4.67	6.002	17	17
5	1	1	2.828	5.29	2.828	6.002	4.67	6.002	17	17
6	1	1	2.828	5.29	2.828	6.002	4.67	6.002	17	17
7	1	1	2.828	5.29	2.828	6.002	4.67	6.002	17	17
8	1	1	2.828	5.29	2.828	5.29	4.67	6.002	17	17
Mean	1	1	2.828	5.135	2.828	5.824	4.67	6.002	17	17
SD	0	0	0	0.2870	0	0.3295	0	0	0	0
CV %	0	0	0	5.5891	0	5.6592	0	0	0	0

Table 3.21: Brain Heart Infusion agar inter-batch data

S No	N70		B25		h20			h40			V3		NRS1		E23		E32	
Method / Day	**AD**	**SGE**	**AD**	**SGE**	**AD**	**EC**	**TEC**	**AD**	**EC**	**TEC**	**AD**	**SGE**	**AD**	**SGE**	**AD**	**SGE**	**AD**	**SGE**
1	1	1	1	1	3	2.828	5.29	3	2.828	8.745	5	5.29	6	6.002	17	17	20	17
2	1	1	1	1	3	2.828	4.67	3	3.206	7.714	4	4.67	7	6.804	17	17	18	17
3	1	1	1	1	3	2.494	5.29	3	2.828	7.714	5	5.29	7	6.002	17	17	19	17
4	1	1	1	1	3	2.494	4.67	3	2.828	6.804	4	4.67	7	6.002	17	17	18	17
5	1	1	1	1	3	2.828	4.67	3	3.206	7.714	5	4.67	6	6.002	16	17	19	17
6	1	1	1	1	3	2.494	4.67	3	3.206	7.714	5	5.29	6	6.002	17	17	18	17
7	1	1	1	1	3	2.494	4.67	3	2.828	7.714	5	5.29	6	5.29	17	17	18	17
8	1	1	1	1	3	2.828	5.29	3	3.206	7.714	5	4.67	6	6.804	17	17	19	17
Mean		1		1		2.661	4.9025		3.017	7.7291		4.98		6.1135		17		17
SD		0		0		0.1785	0.3208		0.2020	0.5295		0.3314		0.49196		0		0
%CV		0		0		6.7091	6.5452		6.6970	6.7214		6.6546		8.0464		0		0

Glucose Brain Heart Infusion agar intra-batch data – Day -1

AD	1	1	3		3		5	7	16	18
Sample no / Set	**NRS70**	**B25**	**h20 (EC)**	**h 20 (TEC)**	**h40 (EC)**	**h 40 (TEC)**	**V3**	**NRS1**	**E23**	**E32**
1	0	1	2.2	2.494	2.2	2.494	3.634	1.941	10.253	14.923
2	1	1	1.712	1.712	1.51	2.2	3.206	2.828	9.044	9.044
3	1	1	2.494	2.494	1.51	1.51	1.712	3.206	9.044	9.044
4	1	1	1.712	1.712	2.494	2.494	3.634	2.828	9.044	8.288
5	1	0	2.494	2.494	1.712	1.941	2.2	1.712	7.038	4.261
6	1	1	1.712	1.712	2.2	2.494	2.828	1.941	6.208	6.208
7	1	1.51	1.51	2.494	2.2	2.494	2.494	2.828	0	9.044
8	1	1	1.941	2.494	1.712	1.941	2.2	1.51	9.004	3.759
Mean	0.875	0.9387	1.9718	2.2007	1.9422	2.196	2.7385	2.3492	7.4543	8.0713
SD	0.3535	0.4191	0.3806	0.4047	0.3748	0.3695	0.7095	0.6397	3.2756	3.5135
CV %	40.4061	44.6548	19.3029	18.3902	19.2983	16.8275	25.9087	27.2341	43.9425	43.5315

Glucose Brain Heart Infusion agar intra-batch data – Day -2

AD	1	1	3		3		5	7	17	19
Sample no / Set	**NRS70**	**B25**	**h20 (EC)**	**h 20 (TEC)**	**h40 (EC)**	**h 40 (TEC)**	**V3**	**NRS1**	**E23**	**E32**
1	1	1	2.492	3.206	1.712	2.494	2.828	2.828	0	9.044
2	1	1	1.941	2.2	2.2	2.2	2.494	4.12	3.795	0
3	1.037	1	2.2	2.494	1.712	1.51	3.634	4.12	3.795	10.253
4	1	1.51	1.332	2.2	1.332	1.51	2.828	4.67	4.261	9.044
5	0	1	2.2	2.494	0	0	2.2	3.634	2.925	9.044
6	1.332	1	2.494	2.492	1.51	1.941	2.828	3.206	4.261	6.208
7	1	1	1.712	3.206	1.712	1.941	2.2	2.828	5.476	9.044
8	1	1.332	2.492	2.494	1.494	1.941	0	8.745	4.261	6.208
Mean	0.9211	1.1052	2.1078	2.5982	1.459	1.6921	2.3765	4.2688	3.5967	7.3556
SD	0.3894	0.2006	0.4221	0.3963	0.6425	0.7574	1.0634	1.9245	1.6178	3.3066
CV %	42.2822	18.1504	20.0292	15.2556	44.0377	44.7647	44.7492	45.0821	44.9804	44.9537

Glucose Brain Heart Infusion agar intra-batch data – Day -3

AD	1	1	3		3		5	7	17	18
Sample no / Set	**NRS70**	**B25**	**h20 (EC)**	**h 20 (TEC)**	**h40 (EC)**	**h 40 (TEC)**	**V3**	**NRS1**	**E23**	**E32**
1	1	1	1.51	1.941	2.2	2.2	1.941	3.206	0	9.044
2	1	1	1.712	1.941	1.51	1.712	2.828	1.941	8.288	8.288
3	1	0	2.2	2.2	2.494	2.494	3.634	4.67	9.044	4.261
4	1	1	1.51	2.494	2.494	2.828	4.12	2.2	8.288	2.925
5	1	0	2.494	2.494	1.332	1.51	4.12	2.494	6.208	5.476
6	1	1	2.828	2.828	1.175	1.51	3.634	1.712	9.044	3.759
7	1	1	1.712	1.941	1.332	1.51	2.2	1.941	8.288	6.208
8	1	0	1.51	1.712	1.941	1.941	2.2	2.828	5.476	10.253
Mean	1	0.625	1.9345	2.1938	1.8097	1.9631	3.0846	2.624	6.8295	6.2767
SD	0	0.5175	0.5101	0.3791	0.5418	0.5022	0.9013	0.9656	3.04841	2.6671
CV %	0	82.8078	26.3690	17.2827	29.9402	25.5819	29.2193	36.8020	44.6360	42.4925

Glucose Brain Heart Infusion agar intra-batch data – Day -4

AD	1	1	3		3		5	6	17	19
Sample no / Set	**NRS70**	**B25**	**h20 (EC)**	**h 20 (TEC)**	**h40 (EC)**	**h 40 (TEC)**	**V3**	**NRS1**	**E23**	**E32**
1	1	1	1.332	1.51	2.494	2.494	2.828	2.494	10.253	11.623
2	1	1	0	0	1.712	2.2	4.12	3.206	9.044	10.253
3	1	1	1.941	2.2	1.712	1.941	3.206	1.941	10.253	13.17
4	1	1	1.941	2.494	0	0	1.941	1.5	9.044	9.044
5	1.037	1	2.2	2.2	1.712	1.712	3.206	2.2	8.288	14.938
6	1	1	1.51	1.941	1.941	1.941	2.494	2.494	9.044	5.476
7	1.941	1	2.2	2.2	1.941	1.941	1.712	1.941	9.044	6.208
8	1.941	1	2.2	2.494	1.941	2.494	1.51	2.494	9.044	9.044
Mean	1.2398	1	1.6655	1.8798	1.6816	1.8403	2.6271	2.2837	9.2517	9.9695
SD	0.4329	0	0.7481	0.8225	0.7262	0.7944	0.8874	0.5126	0.6707	3.2469
CV %	34.9173	0	44.9208	43.756	43.1893	43.1656	33.7807	22.4460	7.2503	32.5684

Glucose Brain Heart Infusion agar intra-batch data – Day -5

AD	1	1	3		3		5	7	16	17
Sample no / Set	**NRS70**	**B25**	**h20 (EC)**	**h 20 (TEC)**	**h40 (EC)**	**h 40 (TEC)**	**V3**	**NRS1**	**E23**	**E32**
1	1	1.037	1.037	1.51	1.175	1.51	1.51	2.828	9.044	8.288
2	1	1.037	1.51	1.712	1.51	1.51	2.2	1.941	6.208	0
3	1	1.037	1.712	2.2	1.332	1.712	2.494	2.2	3.759	9.044
4	1	1.037	2.2	1.941	1.175	1.331	2.828	2.494	6.208	9.044
5	1	1.037	1.712	2.494	1.51	1.941	2.828	2.494	10.253	9.044
6	1	1.037	2.494	2.494	1.712	2.2	1.712	1.941	4.83	6.208
7	1	1.037	2.494	2.494	1.941	2.494	1.1	2.2	5.476	5.476
8	1	1.037	1.51	1.51	2.2	2.2	1.941	2.828	4.261	8.288
Mean	1	1.037	1.8336	2.0443	1.5693	1.8622	2.0766	2.3657	6.2548	6.924
SD	0	0	0.5182	0.4347	0.3646	0.4116	0.6261	0.3537	2.2869	3.1111
CV %	0	0	28.2637	21.2650	23.2329	22.1071	30.1539	14.9533	36.5624	44.9330

Glucose Brain Heart Infusion agar intra-batch data – Day -6

AD	1	1	3		3		5	6	17	17
Sample no / Set	**NRS70**	**B25**	**h20 (EC)**	**h 20 (TEC)**	**h40 (EC)**	**h 40 (TEC)**	**V3**	**NRS1**	**E23**	**E32**
1	1	1	1.51	1.941	1.712	2.2	1.51	2.828	5.476	14.938
2	1	0	1.037	1.51	2.2	2.494	2.828	2.494	5.476	14.938
3	1	1	0	0	1.941	2.2	2.2	3.206	3.316	7.438
4	0	1	1.51	1.51	1.037	1.51	1.941	1.941	5.476	14.938
5	1	1	1.712	1.712	1.037	1.332	3.206	2.828	0	0
6	1.332	1	1.51	1.712	1.712	2.2	2.494	2.494	4.83	14.938
7	1	1	1.037	1.332	2.2	2.494	1.712	1.712	4.83	14.938
8	1	1	1.51	1.712	1.941	2.2	3.634	2.828	5.476	14.938
Mean	0.916	0.875	1.228	1.4286	1.7225	2.078	2.4406	2.5413	4.36	12.1332
SD	0.3881	0.3535	0.5520	0.6055	0.4616	0.4284	0.7432	0.4987	1.9094	5.5608
CV %	42.3477	40.4061	44.9458	42.3881	26.7984	20.6093	30.4548	19.6236	43.7957	45.8314

Glucose Brain Heart Infusion agar intra-batch data – Day -7

AD	1	1	3		3		5	7	17	18
Sample no / Set	**NRS70**	**B25**	**h20 (EC)**	**h 20 (TEC)**	**h40 (EC)**	**h 40 (TEC)**	**V3**	**NRS1**	**E23**	**E32**
1	1	1	1.332	1.51	2.828	2.828	2.494	2.828	11.623	10.253
2	1	1	1.037	1.332	1.712	2.2	3.206	1.712	8.288	5.476
3	1	1	2.494	2.494	1.712	2.941	3.206	2.494	10.253	4.88
4	1	1	2.494	2.494	1.51	1.712	2.828	3.634	9.044	6.208
5	1	1	2.2	2.2	1.332	1.712	3.634	1.941	3.316	9.044
6	1	1	1.51	1.941	1.941	1.941	0	2.828	2.925	2.925
7	1	1	2.2	2.2	2.828	2.828	3.206	3.206	8.288	6.208
8	1	1	2.2	2.494	2.494	2.494	3.206	2.828	4.83	5.476
Mean	1	1	1.9333	2.0831	2.0446	2.332	2.7225	2.6838	7.3208	6.3087
SD	0	0	0.5588	0.4546	0.5922	0.5110	1.1489	0.6290	3.2406	2.3279
CV %	0	0	28.9058	21.8230	28.9641	21.9150	42.2035	23.4389	44.2658	36.9011

Glucose Brain Heart Infusion agar intra-batch data – Day -8

AD	1	1	3		3		5	6	17	16
Sample no / Set	**NRS70**	**B25**	**h20 (EC)**	**h 20 (TEC)**	**h40 (EC)**	**h 40 (TEC)**	**V3**	**NRS1**	**E23**	**E32**
1	1	1	1.51	1.712	1.51	2.2	3.643	2.828	10.253	9.044
2	0	1	1.037	1.51	1.51	1.712	2.2	3.634	9.044	8.288
3	1	1	2.2	2.494	1.037	1.332	2.828	2.2	10.253	9.044
4	1.037	0	1.712	1.712	1.037	1.51	2.494	2.494	4.83	5.476
5	1	1	1.941	1.941	1.51	1.51	2.494	0	10.253	10.253
6	1.037	1	1.51	2.494	1.51	1.712	2.2	3.206	9.044	0
7	1	1	1.712	1.941	1.712	1.712	3.206	3.206	2.276	9.044
8	1	1	1.941	1.941	0	0	3.634	3.206	5.476	10.253
Mean	0.884	0.875	1.695	1.9681	1.228	1.461	2.837	2.596	7.678	7.675
SD	0.3576	0.3535	0.3541	0.3578	0.5520	0.6426	0.5937	1.1435	3.0651	3.4440
CV %	40.4502	40.4061	20.8902	18.1820	44.9458	43.9877	20.9266	44.03786	39.9177	44.8725

Glucose Brain Heart Infusion agar inter-batch data

S No	N70		B25		h20			h40			V3		NRS1		E23		E32	
Method / Day	**AD**	**SGE**	**AD**	**SGE**	**AD**	**EC**	**TEC**	**AD**	**EC**	**TEC**	**AD**	**SGE**	**AD**	**SGE**	**AD**	**SGE**	**AD**	**SGE**
1	1	1	1	1	3	1.037	1.332	3	1.51	1.51	5	2.2	6	3.634	17	11.623	20	11.623
2	1	1	1	0	3	1.037	1.037	3	1.037	1.037	4	0	7	1.332	17	2.508	18	6.208
3	1	0	1	1	3	1.51	1.332	3	1.037	1.037	5	1.037	7	1.037	17	4.88	19	5.476
4	1	1	1	1.037	3	0	0	3	0	0	4	1.332	7	1.037	17	0	18	0
5	1	0	1	1	3	1.332	1.332	3	3.206	3.206	5	1.51	6	4.12	16	2.276	19	5.476
6	1	1	1	1	3	1.712	1.51	3	1.037	1.51	5	1.037	6	1.037	17	0	18	10.253
7	1	0	1	1	3	2.828	3.206	3	1.037	1.037	5	1.332	6	1.51	17	0	18	0
8	1	1	1	1	3	3.634	3.634	3	2.2	3.206	5	3.634	6	1.037	17	0	19	5.476
Mean		0.625		0.8796		1.6362	1.6728		1.383	1.5678		1.5102		1.843		2.6608		5.564
SD		0.5175		0.3556		1.1289	1.1816		0.9560	1.1133		1.0541		1.2740		4.0308		4.1585
%CV		82.8078		40.4329		68.9936	70.6372		69.1302	71.0121		69.7986		69.1307		151.4875		74.7395

The appendix for Chapter three, Chapter four, Chapter five and Chapter six is attached in the enclosed CD-Rom as listed below.

File name	Content	Chapter
Appendix I	SPSS	Chapter Three
Appendix II	Images	Chapter Four
Appendix III	SPSS, Images	Chapter Five
Appendix IV	SPSS, GMP cetrificate	Chapter Six

REFERENCES

AB Biodisk: Etest for MIC determinations of antibiotics. Package insert. *AB Biodisk,* Solna, Sweden. 1992.

Abramson MA, Sexton DJ: Nosocomial methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* primary bacteremia: at what costs? *Infection Control and Hospital Epidemiology.* 1999;20:408-411.

Acar JF, Goldstein FW: Disk susceptibility test. In antibiotics in laboratory medicine *4th Edition. Lorian V (ed.) Williams & Wilkins*, Baltimore. 1996; 1-57.

Ackerman BH, Taylor EH. Olsen M, Abdel-Malak W, Pappas AA: Vancomycin serum protein binding determination by ultrafiltration. *Drug Intelligence & Clinical Pharmacy.* 1988;22:300-303.

Adam HJ, Louie L, Watt C, Gravel D, Bryce E, Loeb M, Matlow A, McGeer A, Mulvey MR, Simor AE: Detection and characterization of heterogeneous vancomycin-intermediate *Staphylococcus aureus* isolates in Canada: results from the Canadian Nosocomial Infection Surveillance Program. *Antimicrobial Agents and Chemotherapy*. 2010;54:945-949.

Adcock PM, Pastor P, Medley F, Patterson JE, Murphy TV. Methicillin-resistant *Staphylococcus aureus* in two child care centers. *Journal of Infectious Diseases.* 1998;178:577–580.

Adhikari RP, Scales GC, Kobayashi K, Smith JM, Berger-Ba¨chi B, Cook GM: Vancomycin-induced deletion of the methicillin resistance gene mecA in *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy.* 2004;54:360–363.

Ahmad NM, Rojtman AD: Successful Treatment of Daptomycin-Nonsusceptible Methicillin-Resistant *Staphylococcus aureus* Bacteremia with the Addition of Rifampin to Daptomycin. *The Annals of Pharmacotherapy.* 2010;44: 918-921.

Aksoy DY, Unal S: New antimicrobial agents for the treatment of gram-positive bacterial infections. *Clinical Microbiology and Infection*. 2008;14:411-420.

Akyama H, Fujii K, Yamasaki O, Oono T, Iwatsuki K: Antimicrobial action of several tannins against *Staphylococcus aureus. Journal of Antimicrobial Chemotherapy.* 2001;48:487-491.

Alanis AJ: Resistance to Antibiotics: Are we in the post-antibiotic Era?. *Archives of Medical Research*. 2005;36: 697–705.

Albrecht LM, Rybak MJ, WarbasseLH, Edwards J: Vancomycin protein binding in patients with infections caused by *Staphylococcus aureus*. *Drug interaction and clinical pharmacy* .1991;25:713-715.

Allen NE, LeTourneau DI, Hobbs JNJ. Molecular interactions of a semisynthetic glycopeptide antibiotic with D-alanyl-D-alanine and D-alanyl-D-lactate residues. *Antimicrobial Agents and Chemotherapy.* 1997;41:66–71

American Thoracic Society, Infectious Diseases Society of America. Guidelines for the management of adults with hospital-acquired, ventilator-associated, and healthcare-associated pneumonia. *American Journal of Respiratory and Critical Care Medicine.* 2005; 171:388–416.

Amsterdam D: Suceptibility testing of antimicrobial in liquid media. *In Antibiotics in laboratory medicine. 4th Edition. Lorian V (ed.), Williams & Wilkins*, Baltimore. 1996; 52-111.

Andreoletti O, Budka H, Buncic S, Colin P, Collins JD, Koeijer AD, Griffin J, Havelaar A, Hope J, Klein G, Kruse H, Magnino S, Antonio, López M, McLauchlin J, Nguyen-The C, Noeckler K, Noerrung B, Maradona MP, Roberts T, Vågsholm I, Vanopdenbosch E: Assessment of the public health significance of meticillin resistant *Staphylococcus aureus* (MRSA) in animals and foods. *European Food Safety Authority Journal.* 2009; 993, 1-73

Andrews JM: Determination of minimal inhibitory concentrations. *Journal of Antimicrobial Chemotherapy.* 2001; 48 Suppl S1: 5-16.

Andrews JM: BSAC standardized disc susceptibility testing method (version 3). *Journal of Antimicrobial Chemotherapy.* 2004;53:713 – 728.

Archer GL, and Niemeyer DM : Origin and evolution of DNA associated with resistance to methicillin in staphylococci. *Trends in Microbiology.* 1994;2:343-347.

Archer G: *Staphylococcus aureus*: a well-armed pathogen. *Clinical Infectious Disease*.1998; 26: 1179-1181.

Arhin FF, Sarmiento I, Parr Jr TR, Moeck G: Activity of oritavancin and comparators in vitro against standard and high inocula of *Staphylococcus aureus*. *International Journal of Antimicrobial Agents.* 2012;39:159-162.

Ariza J, Pujol M, Cabo J, Pena C, Fernandez N, Linares J, Avats J, Gudiol F: Vancomycin in surgical infections due to methicillin-resistant *Staphylococcus aureus* with heterogeneous resistance to vancomycin. *Lancet.* 1999;353:1587-1588.

Arthur M, Brisson-Noel A, Courvalin P: Origin and evolution of genes specifying resistance to macrolide, lincosamide and streptogramin antibiotics: data and hypothesis. *Journal of Antimicrobial Chemotherapy.* 1987;20:783–802.

Arthur M, Reynolds P, Courvalin P: Glycopeptide resistance in enterococci. *Trends in Microbiology.* 1996;4:401-407.

Ashford P and Bew SP: Recent advances in the synthesis of new glycopeptide antibiotics. *Chemical Society Reviews.* 2012; 41:957-978.

Avison MB, Bennett PM, Howe RA, Walsh TR: Preliminary analysis of the genetic basis for vancomycin resistance in *Staphylococcusaureus* strain Mu50. *Journal of Antimicrobial Chemotherapy.* 2002;49:255–260.

Baba T, Takeuchi F, Kuroda M, Yuzawa H, Aoki K, Oguchi A, Nagai Y, Iwama N, Asano K, Naimi T, Kuroda H, Cui L, Yamamoto K, Hiramatsu K: Genome and virulence determinants of high virulence community acquired MRSA. *Lancet.* 2002;359:1819–27.

Backo ME, Gaenger E, Burkart A, Chai YL, Bayer AS: Treatment of experimental staphylococcal endocarditis due to a strain with reduced susceptibility in vitro to vancomycin: efficacy of ampicillin-sulbactam. *Antimicrobial Agents and Chemotherapy*. 1999;43:2565-2568.

Bae IG, Federspiel JJ, Miró JM, Woods CW, Park L, Rybak MJ, Rude TH, Bradley S, Bukovski S, de la Maria CG, Kanj SS, Korman TM, Marco F, Murdoch DR, Plesiat P, Rodriguez-Creixems M, Reinbott P, Steed L, Tattevin P, Tripodi MF, Newton KL, Corey GR, Fowler VG Jr: Heterogeneous vancomycin-intermediate susceptibility phenotype in bloodstream methicillin-resistant Staphylococcus aureus isolates from an international cohort of patients with infective endocarditis: prevalence, genotype, and clinical significance. *Journal of Infectious Diseases.* 2009;200:1355-1366.

Bager F, Madsen M, Christensen J, Aarestrup FM: Avoparcin used as a growth promoter is associated with the occurrence of vancomycin-resistant *Enterococcus faecium* on Danish poultry and pig farms. *Preventive Veterinary Medicine.* 1997;31:95-112.

Baker CN, Stocker SA, Culver DH, Thornsberry C: Comparison of the Etest to agar dilution, broth micro-dilution and agar diffusion susceptibility testing techniques by using a special challenge set of bacteria. *Journal of Clinical Microbiology.* 1991; 29:533-538.

Banerjee T, Anupurba S: Colonization with vancomycin intermediate *Staphylococcus aureus* strains containing *vanA* resistance gene in a tertiary care centre, north India. *Journal of Clinical Microbiology.* Advance access number doi: 10.1128/JCM.06208-11.

Bannerman TL, Hancock GA, Tenover FC, Miller J.M: Pulsed-field gel electrophoresis as a replacement for bacteriophage typing of *Staphylococcus aureus Journal of Cinical Microbilogy*. 1995;33:551-555.

Bannerman TL, Peacock SJ: Staphylococcus, Micrococcus, and other catalase-positive cocci. *In Manual of clinical microbiology, 9th ed.,* Murray PR, Baron EJ, Jorgensen JH, Landry ML, Pfaller MA: *Vol.1. ASM Press,* Washington DC. 2007; 390–411.

Basri DF , Zin NM, Bakar NS, Rahmat F, Mohtar M: Synergistic effects of phytochemcials and oxacillin on laboratory passage-derived Vancomycin-intermediate *Staphylococcus aureus* strain. *International Journal of Medical Sciences*. 2008; 8:131-136.

Bauer AW, Kirby WMM, Sherris JC, Turck M: Antibiotic susceptibility testing by standardized single disk method. *American Journal of Clinical Pathology*. 1966;45:493-496.

Belcheva A, Golemi-Kotra D: A close-up view of the VraSR two-component system. A mediator of *Staphylococcus aureus* response to cell wall damage. *Journal of Biological Chemistry*. 2008; 283:12354-12364

Bennett RW, Yeterian M, Smith W, Coles CM, Sassaman M, McClure FD: *Staphylococcus aureus* identification characteristics and enterotogenicity. *Journal of Food Science.* 1986;51:1337-1339.

Bera A, Biswas R, Herbert S, Kulauzovic E, Weidenmaier C, Peschel A, Gotz F: Influence of wll teichoic acid on lysozyme resistance in *Staphylococcus aureus. Journal of Bacteriology.* 2007;189:280-283

Berg T, Firth N, Apisiridej S, Hettiaratchi A, Leelaporn A, Skurray RA. Complete nucleotide sequence of pSK41: evolution of staphylococcal conjugative plasmids. *Journal of Bacteriology.* 1998;180:4350–4359.

Berger-Bachi, B., Rohrer, S: Factors influencing methicillin resistance in Staphylococci. *Archives of Microbiology.* 2002;178:165-171.

Berglund C, Ito T, Ikeda M, Ma XX, Soderquist B, Hiramatsu K: Novel type of staphylococcal cassette chromosome *mec* in a methicillin-resistant *Staphylococcus aureus* strain isolated in Sweden. *Antimicrobial Agents and Chemotherapy.* 2008;**52**:3512-3516.

Berglund C, Ito T, Ma XX, Ikeda M, Watanabe S, Soderquist B, Hiramatsu K: Genetic diversity of methicillin-resistant *Staphylococcus aureus* carrying type IV SCC*mec* in Örebro County and the western region of Sweden. *Journal of Antimicrobial Chemotherapy.* 2009; 63:32-41.

Berke I, Teeino PM Jr: Comparsion of efficiency and cost-effectiveness of BIOMIC VIDEO and Vitek antimicrobial susceptibility test system for use in the clinical microbiology laboratory. *Journal of Clinical Microbiology.* 1996;34: 1980-1984

Berkley SF, Hightower AW, Broome CV, Reingold AL: The relation- ship of tampon characteristics to menstrual toxic shock syndrome. *Journal of The American Medical Association.* 1987;258:917-920.

Bernal P, Lemaire S, Pinho MG, Mobashery S, Hinds J, Taylor PW: insertion of epicatechin gallate into cytoplasmic membrance of methicillin-resistant *Staphylococcus aureus* disrupts penicillin-binding protein (PBP) 2a-mediated β-lactam resistance by delocalizing PBP2. *Journal of Biological Chemistry.* 2011;31:24055-24065.

Berthoin K, Ampe E, Tulkens PM, Carryn S: Correlation between free and total vancomycin serum concentrations in patients treated for Gram-positive infections. *International Journal of Antimicrobial Agents.* 2009;34:555-560.

Bhagwat SS, McGhee P, Kosowska-Shick K, Patel MV, Appelbaum PC: *In-vitro* activity of the quinolone wck 771 against recent u.s. hospital and community-

acquired *Staphylococcus aureus* pathogens with various resistance types. *Antimicrobial Agents and Chemotherapy.* 2009;53: 811–813.

Bhateja P, Purnapatre K, Dube S, Fatma T, Rattan A: Characterisation of laboratory-generated vancomycin intermediate resistant *Staphylococcus aureus* strains. *International Journal of Antimicrobial agents.* 2006;27:201-211.

Bierbaum G, Fuchs K, Lenz W, Szekat C, Sahl HG: Presence of *Staphylococcus aureus* with reduced susceptibility to vancomycin in Germany. *European Journal of Clinical Microbiology & Infectious Diseases.* 1999;18:691-6

Bischoff M, Berger-Bächi B: Teicoplanin stress-selected mutations increasing σ^B-activity in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*. 2001;45:1714-1720.

Bishop EJ, Howden BP: Treatment of *Staphylococcus aureus* infections: new issues, emerging therapies and future directions. *Expert Opinion on Emerging Drugs*. 2007;12:1-22.

Bishop EJ, Melvin S, Howden BP, Charls PG, Grayson Ml: Good clinical outcomes but high rates of advers reaction during linezolide therapy for serious infections: a proposed protocol for monitering therapy in complex patients. *Antimicrobial Agents and Chemotherapy*. 2006;50:1599-1602.

Blouin RA, Bauer LA, Miller DD, Record KE, Griffen WO Jr: Vancomycin pharmacokinetics in normal and morbidly obese subjects. *Antimicrobial Agents and Chemotherapy.* 1982;21:575–580.

Bonjar GHS, Nik AK, Aghighi S: Antibacterial and antifungal survey in plants used in indigenous herbal-medicine of south east regions of Iran. *Journal of Biological Sciences*. 2004; 4: 405 – 412.

Boyce JM: Vancomycin-resistant enterococci: pervasive and persistent pathogens. *Infection Control & Hospital Epidemiology.* 1995;116:676-679

Boyle-Vavra S, Labischinski H, Ebert CC, Ehlert K, Daum RS: A spectrum of changes occurs in peptidoglycan composition of glycopeptide-intermediate clinical *Staphylococcus aureus* isolates. *Antimicrobial Agents and Chemotherapy.* 2001*;*45*:*280–287.

Boyle-Vavra S, Challapalli M, Daum RS: Resistance to autolysis in vancomycin-selected *Staphylococcus aureus* isolates precedes vancomycin-intermediate resistance. *Antimicrobial Agents and Chemotherapy*. 2003;47:2036–2039

Boyle-Vavra S, Ereshefsky B, Wang C, Daum RS: Successful multiresistant community-associated methicillin-resistant *Staphylococcus aureus* lineage from Taipei, Taiwan, that carries either the novel staphylococcal chromosome cassette mec (SCCmec) Type VT or SCCmec Type IV . *Antimicrobial Agents and Chemotherapy*. 2005;43:4719-4730.

Boyle-Vavra S, Jones M, Gourley BL, Holmes M, Ruf R, Balsam AR, Boulware DR, Kline S, Jawahir S, DeVries A, Peterson SN, Daum RS: Comparative genome sequencing of an isogenic pair of USA800 clinical methicillin-resistant *Staphylococcus aureus* isolates obtained before and after daptomycin treatment failure. *Antimicrobial Agents and Chemotherapy.* 2011*;*55*:*2018–2025.

Brown DJF and Brown L: Evaluation of E test: a novel method for quantifying antimicrobial activity. *Journal of Antimicrobial Chemotherapy*. 1991;27:185-190.

Brown J, Freeman BB: Combining quinupristin/dalfopristin with other agents for resistant infections. *Annals of Pharmacotherapy.* 2004;38: 677-685.

Brunet F, Vedel G, Dreyfus F, Vaxelaire JF, Giraud T, Schremmer B, Monsallier JF: Failure of teicoplanin therapy in two neutropenic patients with staphylococcal septicemia who recovered after administration of vancomycin. *European Journal of Clinical Microbiology & Infectious Diseases.* 1990;9:145-147.

Bugg TDH: Bacterial peptidoglycan biosynthesis and its inhibition. *In Comprehensive natural products chemistry. Pinto M (Ed.), Elsevier,* Oxford. 1999: 241-294.

Burnham CD, Weber CJ, Dunne WM: Novel screening agar for detection of vancomycin-nonsusceptible *Staphylococcus aureus*. *Journal of Clinical Microbiology.* 2010;48:49–951.

Butler MS and Cooper MA: Antibiotics in the clinical pipeline in 2011. *The Journal of Antibiotics.* 2011; 64:413-425.

Cafiso V, Bertuccio T, Spina D, Purrello S, Campanile F, Pietro CD, Purrello M, Stefani S: Modulating activity of vancomycin and daptomycin on the epression of autolysis cell-wall turnover and membrane charge genes in hVISA and VISA strains. *Public Library of Science One.* 2012a;7:1-10.

Cafiso V, Bertuccio T, Spina D, Purrello S, Blandino G, Stefani S: A novel delta-hemolysis screening method to detect hVISA and VISA. *Journal of Clinical Microbiology.* 2012b; access number doi:10.1128/JCM.06307-11.

Cai Y, Wang R, Liang B, Bai N, Liu Y: Systematic review and meta-analysis of the effectiveness and safety of tigecycline for treatment of infectious disease. *Antimicrobial Agents and Chemotherapy.* 2011;55:1162-1172.

Camargo IL, Gilmore MS: *Staphylococcus aureus*-probing for host weakness?. *Journal of Bacteriology.* 2008;190:2253-2256.

Camargo IL, Neoh HM, Cui L, Hiramatsu K. Serial daptomycin selection generates daptomycin-nonsusceptible *Staphylococcus aureus* strains with a heterogeneous vancomycin-intermediate phenotype. *Antimicrobial Agents and Chemotherapy*. 2008;52:4289-4299.

Campanile F, Bongiorno D, Borbone S, Stefani S: Hospital-associated methicillin-resistant *Staphylococcus aureus* (HA-MRSA) in Italy. *Annals of Clinical Microbiology and Antimicrobials.* 2009;24:22-31.

Cartolano GL, Cheron M, Benabid D, Leneveu M, Boisivon A: Methicillin-resistant *Staphylococcus aureus* (MRSA) with reduced susceptibility to glycopeptides (GISA) in 63 French general hospitals. *Clinical Microbiology and Infection.* 2004;10:448–451.

Chen YS, Lee SC, and Kim WJ: Efficacy and tolerability of linezolid in treating severe skin and soft tissue infections caused by Gram-positive pathogens. *The Journal of the Formosan Medical Association.* 2004;103:349-354

CDC. Toxic-shock syndrome United States. Morbidity and Mortality Weekly Report. 1990;39:421-24

CDC Guidelines: http://www.cdc.gov/mrsa/diagnosis/index.html

CDC Guidelines: Siegel JD, Rhinehart E, Jackson M, Chiarello L: Management of multidrug-resistant organisms in healthcare settings. 2006: 1-74

Cepeda1 J, Hayman S, Whitehouse T, Kibbler CC, Livermore D, Singer M, Wilson APR: Teicoplanin resistance in methicillin-resistant *Staphylococcus aureus* in an intensive care unit. *Journal of Antimicrobial Chemotherapy.* 2003;52:533-534.

Cercenado E: Glycopeptide-intermediate *Staphylococcus aureus*: rediscovery of an old problem? *Clinical Microbiology and Infection*. 2000;6:517-518.

Cetinkaya Y, Falk P, Mayhall CG: Vancomycin-Resistant Enterococci. *Clinical Microbiology Reviews.* 2000;13:686–707

Chambers ST: Diagnosis and management of Staphylococcal infections in pacemakers and cardiac defibrillators. *Internal Medicine Journal.* 2005a;35:S63-S71.

Chambers ST: Diagnosis and management of Staphylococcal infections vascular grafts and stens. *Internal Medicine Journal.* 2005b;35:S72-S78.

Chang WN, Lu CH, Wu JJ, Chang HW, Tsai YC, Chen FT, Chien CC: *Staphylococcus aureus* meningitis in adults: A clinical comparison of infections caused by methicillin-resistant and methicillin-sensitive strains. *Infection* 2001;29:245–250

Chang FY, Peacock JE, Musher DM, Triplett P, Mac-Donald BB, Mylotte M, O'Donnell A, Wagener MM, Yu VL: *Staphylococcus aureus* bacteremia: recurrence and the impact of antibiotic treatment in a prospective multicenter study. *Medicine. (Baltimore)* 2003; 82:333-339.

Charlebois ED, Bangsberg DR, Moss NJ, Moore MR, Moss AR, Chambers HF, Perdreau-Remington F: Population-based community prevalence of methicillin-resistant *Staphylococcus aureus* in the urban poor of San Francisco. *Clinical Infectious Diseases*. 2002;34(4):425-433.

Charles PG, Ward BP, Johnson PD, Howden BP, Grayson ML: Clinical features associated with bacteremia due to heterogeneous vancomycin-intermediate *Staphylococcus aureus*. *Clinical Infectious Diseases*. 2004;38:448–451.

Chao S, Young G, Oberg C, Nakaoka K: Inhibition of methicillin-resistant *Staphylococcus aureus* (MRSA) by essential oils. *Flavour and Fragrance Journal.* 2008;23:444–449.

Cho YS, Schiller NL, Oh KH: Antibacterial effects of green tea polyphenols on clinical isolates of methicillin-resistant *Staphylococcus aureus*. *Current Microbiology*. 2008;57:542-546.

Chongtrakool P, Ito T, Ma XX, Kondo Y, Trakulsomboon S, Tiensasitorn C, Jamklang M, Chavalit T, Song JH, Hiramatsu K: Staphylococcal cassette chromosome mec (SCCmec) typing of methicillin-resistant *Staphylococcus aureus* strains isolated in 11 Asian countries: a proposal for a new nomenclature for SCCmec elements. *Antimicrobial Agents and Chemotherapy*. 2006;50:1001-1012.

Chusri S, Voravuthikunchai SP: Damage of staphyloccal cytoplasmic membrane by *Quercus infectoria* G. Oliver and its components. *Letters in Applied Microbiology.* 2011;52:565-572.

Clark NC, Cooksey RC, Hill BC, Swenson JM, Tenover FC: Characterization of glycopeptide-resistant enterococci from US hospitals. *Antimicrobial Agents and Chemotherapy*. 1993;37:2311–2317

Clark NC, Weigel LM, Patel JB, Tenover FC: Comparison of Tn*1546*-like elements in vancomycin-resistant *Staphylococcus aureus* isolates from Michigan and Pennsylvania. *Antimicrobial Agents and Chemotherapy*. 2005;49:470-472.

Clark C, McGhee P, Appelbaum PC, Kosowska-Shick K: Multistep resistance development studies of ceftaroline in gram-positive and –negative bacteria. *Antimicrobial Agents and Chemotherapy*. 2011;55:2344-2351.

http://clinicaltrials.gov, accesed on 02/03/2012.

Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. Nineteenth informational supplement. M100-S20. Wayne, PA: CLSI; 2010.

Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. Nineteenth informational supplement. M100-S20. Wayne, PA: CLSI; 2012.

Coates ARM, Halls G, Hu Y: Novel classes of antibiotics or more of the same?. *British Journal of Pharmacology*. 2011;163:184–194.

Collins LV, Kristian SA, Weidenmaier C, Faigle M, Van Kessel KP, Van Strijp JA, Gotz F, Neumeister B, Peschel A: *Staphylococcus aureus* strains lacking D-alanine modifications of teichoic acids are highly susceptible to human neutrophil killing and are virulence attenuated in mice. *Journal of Infectious Diseases* .2002;186:212-219.

Cosgrove SE, Sakoulas G, Perencevich EN, Schwaber MJ, Karchmer AW, Carmeli Y: Comparison of mortality associated with methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* bacteremia: a meta-analysis. *Clinical Infectious Diseases.* 2003, 36:53-59.

Cosgrove SE, Qi Y, Kaye KS, Harbarth S, Karchmer AW, Carmeli Y: The impact of methicillin resistance in *Staphylococcus aureus* bacteremia on patient outcomes: mortality, length of stay, and hospital charges. *Infection Control and Hospital Epidemiology* . 2005;26:166-174.

Corey GR, Stryjewski ME, Weyenberg W, Yasothan U, Kirkpatrick P: Telavancin. *Nature Reviews Drug Discovery.* 2009;8:929-930.

Corpet F: Multiple sequence alignment with hierarchical clustering". *Nucleic Acids Research*. 1988;16:10881-10890

Cottagnoud P, Cottagnoud M, Tauber MG: Vancomycin acts synergistically with gentamicin aginst penicillin-resistant pneumococci by increasing the intracellular penetration of gentamicin. *Antimicrobial Agents and Chemotherapy*. 2003;47: 144-147.

Couppie P, Cribier B, Prevost G, Grosshans E, Piemont Y: Leukocidin from *Staphylococcus aureus* and cutaneous infections: an epidemiologic study. *Archives of Dermatology.* 1994;130:1208–1209.

Courvalin P: Vancomycin resistance in gram-positive cocci. *Clinical Infectious Diseases.* 2006;42:S25-34.

Craig WA: Pharmacokinetic/pharmacodynamic parameters: rationale for antimicrobial dosing of mice and men. *Clinical Infectious Diseases* . 1998;26:1–10.

Criag WA: Pharmacokinetics of antibiotics with special emphasis on cephalosporins. *Clinical Microbiology and Infection.* 2000; 6 (Supple 3):46-49

Craig WA: Basic pharmacodynamics of antibacterials with clinical applications to the use of beta-lactams, glycopeptides, and linezolid. *Infectious Disease Clinics of North America*. 2003;17:479–501.

Cribier B, Prevost G, Coupie P, Finck-Barbancon V, Grosshans E, Piemont Y: *Staphylococcus aureus* leukocidin: a new virulence factor in cutaneous infections? An epidemiological and experimental study. *Dermatology* 1992;185:175–185.

Crum NF. The emergence of severe, community-acquired methicillin-resistant *Staphylococcus aureus* infections. *Scandinavian Journal of Infectious Diseases.* 2005; 37:651-656.

Cui L, Murakami H, Kuwahara-Arai K, Hanaki H, Hiramatsu K: Contribution of a thickened cell wall and its glutamine nonamidated component to the vancomycin resistance expressed by *Staphylococcus aureus* Mu50. *Antimicrobial Agents and Chemotherapy* . 2000;44:2276–2285.

Cui L, Ma X, Sato K, Okuma K, Tenover FC, Mamizuka EM, Gemmell CG, Kim MN, Ploy MC, N El-Solh, Ferraz V, Hiramatsu K: Cell wall thickening is a common feature of vancomycin resistance in *Staphylococcus aureus*. *Journal of Clinical Microbiology.* 2003;41:5–14.

Cui L, Lian JQ, Neoh HM, Reyes E, Hiramatsu K DNA microarray-based identification of genes associated with glycopeptide resistance in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*. 2005;49: 3404-3413.

Cui L, Iwamoto A, Lian JQ, Neoh HM, Maruyama T, Horikawa Y, Hiramatsu K: Novel mechanism of antibiotic resistance originating in vancomycin-intermediate

Staphylococcus aureus. *Antimicrobial Agents and Chemotherapy*. 2006a;50:428–438.

Cui L, Tominaga E, Neoh H, Hiramatsu K: Correlation between reduced daptomycin susceptibility and vancomycin resistance in vancomycin-intermediate *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*. 2006b;50:1079–1082.

Cui L, Lian JQ, Neoh HM, Shoji M, Hiramatsu K: Contribution of *vraSR* and *graSR* point mutations to vancomycin resistance in vancomycin-intermediate *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy.* 2009;53:1231-1234.

Cui L, Isii T, Fukuda M, Ochiai T, Neoh H, Camargo ILBC, Watanabe Y, Shoji M, Hishinuma T, Hiramatsu K: An RpoB mutation confers dual heteroresistance to daptomycin and vancomycin in *Staphylococcus aureus. Antimicrobial Agents and Chemotherapy*. 2010;54:5222–5233.

D'Agata EMC, Webb GF, Horn MA, Moellering Jr. RC, Ruan S: Modeling the invasion of community- acquired resistant *Staphylococcus aureus* into hospitals. *Clinical Infectious Diseases.*2009; 48:274-284.

Dagan R: Staphylococcus aureus in impetigo. *American Journal of Diseases of Children.* 1991;145:1223.

Daum RS, Ito T, Hiramatsu K, Hussain F, Mongkolrattanothai K, Jamklang M, Boyle-Vavra S, A novel methicillin-resistance cassette in community-acquired methicillin-resistant *Staphylococcus aureus* isolates of diverse genetic backgrounds. *Journal of Infectious Disease* . 2002;186:1344-1347.

David MZ, Daum RS: Community-associated methicillin-resistant *Staphylococcus aureus*: epidemiology and clinical consequences of an emerging epidemic. *Clinical Microbiology Reviews.* 2010;23:616-687.

Davis RL, Smith AL, Koup JR: The “red-man syndrome” and slow infusion of vancomycin. *Annals of Internal Medicine*. 1986;104:285-286.

Davis JS: Management of bone and joint infections due to *Staphylococcus aureus. Internal Medicine Journal.* 2005, 35:S79-S96.

Davis SL, Perri MB, Donabedian SM, Manierski C, Singh A, Vager D, Haque NZ, Speirs K, Muder RR, Robinson-Dunn B, Hayden MK, Zervos MJ: Epidemiology and outcomes of community-associated methicillin-resistant *Staphylococcus aureus* infection. *Journal of Clinical Microbiology*. 2007;45:1705-11.

Denis O, Nonhoff C, Byl B, Knoop C, Bobin-Dubreux S, Struelens MJ: Emergence of vancomycin-intermediate *Staphylococcus aureus* in a Belgian hospital: microbiological and clinical features. *Journal of Antimicrobial Chemotherapy.* 2002;50:383–391.

Deurenberg RH, Stobberingh EE: The Molecular Evolution of Hospital and Community-Associated Methicillin-Resistant *Staphylococcus aureus*. *Current Molecular Medicine.* 2009;9:100-115.

Devabhakthuni S: Antibiotic Pharmacokinetic Monitoring. *American Society of Health-System Pharmacists*. 2011.

Diep BA, Chambers HF, Graber CJ, Szumowsik JD, Miller LG, Han LL, Chen JH, Lin F, LinJ, Phan TH, Carleton HA, McDougal LK, Tenover FC, Cohen DE, Mayer KH, Sensabaugh GH, Perdreau-Remington F: Emergence of multidrug-resistant, community-associated, methicillin-resistant *Staphylococcus aureus* clone USA300 in men who have sex with men. *Annals of Internal Medicine.* 2008;148:249-257.

Donnelly JP, Voss A, Witte W, Murray BE, Hayes PW, Gustafson RH, Lotgering IFK, Mudd AJ: Does the use in animals of antimicrobial agents, including glycopeptide antibiotics, influence the efficacy of antimicrobial therapy in humans? *Journal of Antimicrobial Chemotherapy.* 1996;37:398–392.

Draghi DC, Sheehanb DF, Hoganb P, Sahm DF: Current antimicrobial resistance profiles among methicillin-resistant *Staphylococcus aureus* encountered in the outpatient setting. *Diagnostic Microbiology and Infectious Disease.* 2006;55:129–133.

Draghi DC, Benton BM, Krause KM, Thornsberry C, Pillar C, Sahm DF: Comparative surveillance study of telavancin activity against recently collected gram-positive clinical isolates from across the United States. *Antimicrobial Agents and Chemotherapy*. 2008;52:2383–2388.

Drusano GL: Antimicrobial pharmacodynamics: critical interactions of bug and drug. *Nature Reviews Microbiology*. 2004;2:289–300.

Dubrac S, Bissicchia P, Devine KM Msadek T: A matter of life and death: cell wall homeostasis and the WalKR (YycFG) essential signal transduction pathway. *Molecular Microbiology*. 2008;70:1307-1322.

Dunbar LM, Milata J, McClure T, Wasilewski MM: Comparison of the efficacy and safety of oritavancin front-loaded dosing regimens to daily dosing: an analysis of the SIMPLIFI trial. *Antimicrobial Agents and Chemotherapy*. 2011;55:3476-3484.

Edwards B, Milne K, Lawes T, Cook I, Robb A, Gould IM: Is vancomycin MIC "creep" method dependent? Analysis of methicillin-resistant *Staphylococcus aureus* susceptibility trends in blood isolates from North East Scotland from 2006 to 2010. *Journal of Clinical Microbiology*. 2012;50:318-325.

Eliopoulos GM, Moellering RC: Antimicrobial combinations. In:Lorian V, ed. *Antibiotics in Laboratory Medicine. Baltimore*: Williams and Wilkins, 1996; 330–396.

Ellestad GA, Leese RA, Morton GO, Barbatschi F, Gore WE, McGahren WJ, Armitage IM: Avoparcin and epi-avoparcin. *Journal of the American Chemical Society*. 1982;103: 6522–6524.

El Zoeiby A, Sanachagrin F, Levesque RC: Structure and function of the Mur enzymes: development of novel inhibitors. *Molecular Microbiology.* 2003; 47:1-12.

Engemann JJ, Carmeli Y, Cosgrove SE, Fowler VG, Bronstein MZ, Trivette SL, Briggs JP, Sexton DJ, Kaye KS: Adverse clinical and economic outcomes attributable to methicillin resistance among patients with *Staphylococcus aureus* surgical site infection. *Clinical Infectious Diseases.* 2003, 36:592-598.

Entenza, JM, Hohl P, Heinze-Krauss I, Glauser MP, Moreillon P: BAL9141, a novel extended-spectrum cephalosporin active against methicillin-resistant *Staphylococcus aureus* in treatment of experimental endocarditis. *Antimicrobial Agents and Chemotherapy*. 2002;46:171–177.

Ericsson HM, Sherris JC: Antibiotic susceptibility testing. Report of an international collaborative study. *Acta Pathologica et Microbiologica Scandinavia*. 1971; Section B: Suppl 217.

Ernst CM, Paschel A: Broad-spectrum antimicrobial peptide resistance by MprF-mediated aminoacylation and flipping of phospholipids. *Molecular Microbiology*. 2011;80:290–299.

European Medical Agency. 2010; 1-2.

EUCAST http://www.srga.org/eucastwt/mictab/MICglycopeptides_v2.html. Glycopeptides – EUCAST clinical MIC breakpoints 2009-09-29. Accessed 4 May 2010.

Evers S, Sahm DF, Courvalin P: The *vanB* gene of *Entero coccus faecalis* V583 is structurally related to genes encoding D-Ala, D-Ala ligases and glycopeptide-resistance proteins VanA and VanC. *Gene.* 1993;124:143-144.

Farber BF, Moellering RC Jr: Retrospective study of the toxicity of preparations of vancomycin from 1974 to 1981. *Antimicrobial Agents and Chemotherapy*. 1983;23:138–141.

Faiers M, Geroge R, Jally J, Wheat P: Multipoint methods in the clinical laboratories. 1992. Londan, PHLS.

Farrell DJ, Robbins M, Rhys-Williams W, Love WG: In vitro activity of XF-73, a novel antibacterial agent, against antibiotic-sensitive and -resistant Gram-positive and Gram-negative bacterial species. *International Journal of Antimicrobial Agents.* 2010; 35:531-536.

Ferraro MJ, Jorgensen JH: Suceptability testing instrumentation and computerized expert system for data analysis and interpretation. *In Manual of Clinical Microbiology, 8th ed. Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH (ed.). American Society for Microbiology*. Washington DC. 2004; 208-217.

Ferraz V, Duse AG, Kassel M, Black AD, Ito T, Hiramatsu K: Vancomycin-resistant *Staphylococcus aureus* occurs in South Africa. *South African Medical Journal.* 2000; 90: 1113.

Fergie JE, Purcell K. Community-acquired methicillin-resistant *Staphylococcus aureus* infections in south Texas children. *Pediatric Infectious Disease Journal.* 2001; 20:860-863.

Ferguson MA and Todd JK: Toxic shock syndrome associated with *Staphylococcus aureus* sinusitis in children. *International Journal of Infectious Diseases.* 1990;161:953-955.

Finan JE, Archer GL, Pucci MJ, Climo MW: Role of penicillin-binding protein 4 in expression of vancomycin resistance among clinical isolates of oxacillin-resistant *Staphylococcus aureus. Antimicrobial Agents and Chemotherapy*. 2001;45:3070-3075.

Finck-Barbancon V, Duportail G, Meunier O, Colin DA: Pore formation by a two-component leukocidin from *Staphylococcus aureus* within the membrane of human polymorphonuclear leukocytes. *Biochimica et Biophysica Acta*. 1993.1182:275-282.

Fischer W: Bacterial phosphoglycolipdis and lipoteichoic acids. In M kates (ed), Glycolipids, phosphoglycolipids and sulfoglycolipds. *Plenum press,* New york. 1990 ; 123-234 :

Fishbain JT, Lee JC, Nguyen HD, Mikita JA, Mikita CP, Uyehara CFT, Hospenthal DR: Nosocomial Transmission of Methicillin Resistant *Staphylococcus aureus*: A Blinded Study to Establish Baseline Acquisition Rates. *Infection Control and Hospital Epidemiology* . 2003;24: 415-421.

Fitzgibbon MM, Rossney AS, O'Connell B: Investigation of reduced susceptibility to glycopeptides among methicillin-resistant *Staphylococcus aureus* isolates from patients in Ireland and evaluation of agar screening methods for detection of heterogeneously glycopeptide-intermediate *S. aureus*. *Journal of Clinical Microbiology*. 2007; 45:3263- 3269.

Fowler VG Jr, Olsen MK, Corey GR, Woods CW, Cabell CH, Reller LB, Cheng AC, Dudley T, Oddone EZ: Clinical identifiers of complicated *Staphylococcus aureus* bacteremia. *Archives of Internal Medicine*. 2003;163: 2066-2072.

Fowler VG Jr, Miro JM, Hoen B, Cabell CH, Abrutyn E, Rubinstein E, Corey GR, Spelman D, Bradley SF, Barsic B, Pappas PA, Anstrom KJ, Wray D, Fortes CQ, Anguera I, Athan E, Jones P, van der Meer JTM, Elliott TSJ, Levine DP, Bayer AS: *Staphylococcus aureus* Endocarditis: A Consequence of Medical Progress. *Journal of The American Medical Association,* 2005;293:3012-3021.

Freile ML, Giannini F, Pucci G , Sturnioloc A, Roderoc L, Puccic O, Balzaretia B, Enriz RD. Antimicrobial activity of aqueous extracts and of berberine isolated from *Berberis heterophylla*. *Fitoterapia*. 2003;74: 702–705.

Friedman M: Overview of antibacterial, antitoxin, antiviral, and antifungal activities of tea flavonoids and teas. *Molecular Nutrition & Food Research*. 2007;51:116 – 134.

Fung HB, Kirschenbaum HL, Ojofeitimi BO: Linezolid: An oxazolidinone antimicrobial agent. *Clinical Therapeutics.* 2001; 23:356-391.

Galperin MY: Structural classification of bacterial response regulators: diversity of output domains and domain combinations. *Journal of Bacteriology.* 2006;188:4169-4182.

Gao R, Stock AM: Biological insights from structures of two-component proteins. *Annual Review of Microbiology* . 2009; 63:133-154

Gardete S, Wu SW, Gill S, Tomasz A: Role of *VraSR* in antibiotic resistance and antibiotic-induced stress response in *S. aureus*. *Antimicrobial Agents and Chemotherapy.* 2006; 50: 3424-3434.

Gardete S, Kim C, Hartmann BM, Mwangi M, Roux CM, Dunman PM, Chambers HF, Tomasz A: Genetic Pathway in Acquisition and Loss of Vancomycin Resistance in a Methicillin Resistant *Staphylococcus aureus* (MRSA) Strain of Clonal Type USA300. *PloS Pathogens*. 2012; 8:e1002505.

Garnier F, Chainier D, Walsh T, Karlsson A, Bolmstro¨m A, Grelaud C, Mounier M, Denis F, Ploy M: A 1 year surveillance study of glycopeptide-intermediate *Staphylococcus aureus* strains in a French hospital. *Journal of Antimicrobial Chemotherapy.* 2006;57:146-149.

Garau J, Bouza E, Chaster J, Gudiol F, Harbarth S: Management of methicillin-resistant *Staphylococcus aureus* infections. *Clinical Microbiology and Infection.* 2009;15:125-136.

Geraci J: Vancomycin. *Mayo Clinic Proceedings.* 1977;52:631–634.

Gill SR, Fouts DE, Archer GL, Mongodin EF, DeBoy RT, Ravel J, Paulsen IT, Kolonay JF, Brinkac L, Beanan M, Dodson RJ, Daugherty SC, Madupu R, Angiuoli SV, Durkin AS, Haft DH, Vamathevan J, Khouri H, Utterback T, Lee C, Dimitrov G, Jiang L, Qin H, Weidman J, Tran K, Kang K, Hance IR, Nelson KE,

Fraser CM: Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistant *Staphylococcus epidermidis* strain. *Journal of Bacteriology*. 2005;187:2426-2438.

Gillet Y, Issartel B, Vanhems P, Fournet JC, Lina G, Bes M, Vandenesch F, Piémont Y, Brousse N, Floret D, Etienne J : Association between *Staphylococcus aureus* strains carrying gene for Panton-Valentine leukocidin and highly lethal necrotising pneumonia in young immunocompetent patients. *Lancet.* 2002;359:753–759.

Giuliana B, Loredana S, Pasquale S, Giovanna P, Giorgio C, Laura C, Pellegrino A, Paolo N, Luigi C, Massimo A: Complication of nasal piercing by Staphylococcus aureus endocarditis: a case report and a review of literature. *Cases Journal*. 2010;3:37-40.

Givner LB, Kaplan SL: Meningitis due to *Staphylococcus aureus* in children. *Clinical Infectious Diseases.* 1993;16:766–771.

Goldberg E, Paul M, Talker O, Samra Z, Raskin M, Hazzan R, Leibovici L, Bishara J: Co-trimoxazole versus vancomycin for the treatment of methicillin-resistant *Staphylococcus aureus* bacteraemia: a retrospective cohort study. *The Journal of Antimicrobial Chemotherapy*. 2010;65:1779-1783.

Golper TA, Noonan HM, Elzinga L, Gilbert D, Brummett R, Anderson JL, Bennett WM: Vancomycin pharmacokinetics, renal handling, and nonrenal clearances in normal human subjects. *Clinical Pharmacology & Therapeutics.* 1988;43:565-570.

Gonzalez BE, Martinez-Aguilar G, Hulten KG, et al. Severe Staphylococcal sepsis in adolescents in the era of community-acquired methicillin-resistant *Staphylococcus aureus*. *Pediatrics.* 2005;115: 642-648.

González-Zorn B, Courvalin P: Reflection & Reaction: *van-A* -mediated high level glycopeptide resistance in MRSA. *The Lancet Infectious Diseases*. 2003;63.

Gosbell IB: Diagnosis and management of catheter-related blood-stream infections due to *Staphylococcus aureus. Internal Medicine Journal.* 2005; 35:S45-S62.

Gottlieb GS, Fowler VG, Kong LK, McClelland RS, Gopal AK, Marr KA, Li J, Sexton DJ, Glower D, Corey GR: *Staphylococcus aureus* bacteremia in the surgical patient: a prospective analysis of 73 postoperative patients who developed *staphylococcus aureus* bacteremia at a tertiary care facility. *Journal of the American Chemical Society.* 2000; 190:50-57.

Gould FK, Brindle R, Chadwick PR, Fraise AP, Hill S, Nathwani D, Ridgway GL, Spry MJ, Warren RE: Guidelines (2008) for the prophylaxis and treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) infections in the United Kingdom. *The Journal of Antimicrobial Chemotherapy.* 2009;63:849-861.

Gould IM, Cauda R, Esposito S, Gudiol F, Mazzei T, Garau J: Management of serious meticillin-resistant *Staphylococcus aureus* infections: what are the limits?. *International Journal of Antimicrobial Agents.* 2011;37: 202-209.

Gould IM, David MZ, Esposito S, Garau J, Lina G, Mazzei T, Peters G: New insights into meticillin-resistant *Staphylococcus aureus* (MRSA) pathogenesis, treatment and resistance. *International Journal of Antimicrobial Agents*. 2012;39:96-104.

Graffunder EM, Venezia RA: Risk factors associated with nosocomial methicillin-resistant *Staphylococcus aureus* infection including previous use of antimicrobials. *Journal of Antimicrobial Chemotherapy*. 2002; 49: 999–1005.

Guo QL, Zhao L, You QD, Yang Y, Gu HY, Song GL, Lu N, Xin J: Antihepatitis B virus activity of wogonin in vitro and in vivo. *Antiviral Research*. 2007;74: 16–24.

Gustafson J, Strassle A, Hachler H, Kayser FH, Berger-Bachi B: The *femC* locus of *Staphylococcus aureus* reruired for methicillin resitance includes the glutamine synthestase operon. *Journal of Bacteriology.* 1994;176:1460-1467.

Hanaki H, Labischinski H, Inaba Y, Kondo N, Murakami H, Hiramatsu K: Increase in glutamine-non-amidated muropeptides in the peptidoglycan of vancomycin-resistant *Staphylococcus aureus* strain Mu50. *Journal of Antimicrobial Chemotherapy.* 1998;42:315-320.

Harbarth S, Liassine N, Dharan S, Herrault P, Auckenthaler R, Pittet D: Risk factors for persistent carriage of methicillin-resistant *Staphylococcus aureus. Clinical Infectious Diseases.* 2000;31:1380-1385.

Hartman BJ, Tomasz A: Low-affinity penicillin-binding protein associated with beta-lactam resistance in *Staphylococcus aureus*. *Journal of Bacteriology.* 1984;158:513-516.

Hawkey PM: Pre-Clinical experience with daptomycin. *Journal of Antimicrobial Chemotherapy*. 2008;62: Suppl. 3, 7–14.

Hawser SP, Bouchillon SK, Hoban DJ, Dowzicky M, Babinchak T: Rising incidence of *Staphylococcus aureus* with reduced susceptibility to vancomycin and susceptibility to antibiotics: a global analysis 2004-2009. *International Journal of Antimicrobial Agents*. 2011;37: 219–224.

Health Canada. 2010.

Hebeisen P, Heinze-Krauss I, Angehrn P, Hohl P, Page MG, Then RL: In Vitro and In Vivo Properties of Ro 63-9141, a Novel Broad-Spectrum Cephalosporin with Activity against Methicillin-Resistant Staphylococci. *Antimicrobial Agents and Chemotherapy.* 2001;45:825-836.

Heusser R, Ender M, Berger-Bachi B, McCallum N: Mosaic staphylococcal cassette chromosome mec containing two recombinase loci and a new mec complex, B2. *Antimicrobial Agents and Chemotherapy.* 2007;51:390–393.

Hidayat LK, Hsu DI, Quist R, Shriner KA, Wong-Beringer A: High-dose vancomycin therapy for methicillin-resistant *Staphylococcus aureus* infections: efficacy and toxicity. *Archives of Internal Medicine*. 2006;166:2138-2144.

Hidron AI, Edwards JR, Patel J, Horan TC, Sievert DM, Pollock DA, and Fridkin SK: Antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006–2007. *Infection Control & Hospital Epidemiology.* 2008;29:996–1011.

Hill GB, Schalkousky S: Development and evaluation of the spiral gradient endpoint method for susceptibility testing of anaerobic gram-negative bacilli. *Reviews of Infectious Diseases* 1990; 12(Suppl 2): S200-S209.

Hill EE, Vanderschueren S, Verhaegen J: Risk factors for infective endocarditis and outcome of patients with *Staphylococcus aureus* bacteremia. *Mayo Clinic Proceedings.* 2007;82:1165-1169

Hiramatsu K, Aritaka N, Hanaki H, Kawasaki S,. Hosoda Y, Hori S, Fukuchi Y, Kobayashi I: Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. *Lancet.* 1997a;350:1670-1673.

Hiramatsu K, Hanaki H, Ino T, Yabuta K, Oguri T, Tenover FC: Methicillin-resistant *Staphylococcus aureus* clinical strains with reduced vancomycin susceptibility. *Journal of Antimicrobial Chemotherapy* . 1997b;40:135-136.

Hiramatsu K: Vancomycin-resistant *Staphylococcus aureus*: a new model of antibiotic resistance. *Lancet.* 2001a;1:147-155.

Hiramatsu K, Cui L, Kuroda M, Ito T: The emergence and evolution of methicillin-resistant *Staphylococcus aureus*. *Trends in Microbiology*. 2001b;9:486-493.

Hiramatsu K, Igarashi M, Morimoto Y, Baba T, Umekita M, Akamatsu Y: Curing bacteria of antibiotic resistance: reverse antibiotics, a novel class of antibiotics in nature. *International Journal of Antimicrobial Agents*. 2012;39:478-485.

Hisata K, Kuwahara-Arai K, Yamanoto M, Ito T, Nakatomi Y, Cui L, Baba T, Terasawa M, Sotozono C, Kinoshita S, Yamashiro Y, Hiramatsu K: Dissemination of methicillin-resistant Staphylococci among healthy Japanese children. *Journal of Clinical Microbiology*. 2005; 43:3364-3372

Ho PL, Cheung C, Mak GC, Tse CW, Ng TK, Cheung CH, Que TL, Lam R, Lai RW, Yung RW, Yuen KY: Molecular epidemiology and household transmission of community-associated methicillin-resistant *Staphylococcus aureus* in Hong Kong. *Diagnostic Microbiology and Infectious Disease.* 2007 ;57:145-151.

Ho PL, Lo PY, Chow KH, Lau EH, Lai EL, Cheng VC, Kao RY: Vancomycin MIC creep in MRSA isolates from 1997 to 2008 in a healthcare region in Hong Kong. *Journal of Infection*. 2010;60:140-145.

Hoff AVK and Heck AJR: Interaction of α- and β-avoparcin with bacterial cell wall receptor-mimicking peptides studies by electrospray ionization mass spectroscopy. *Journal of Antimicrobial Chemotherapy* . 1999;44:593-599.

Holmes NE, Turnidge JD, Munckhof WJ, Robinson JO, Korman TM, O'Sullivan MVN, Anderson TL, Roberts SA, Gao W, Christiansen KJ, Coombs GW, Johnson PDR, Howden BP: Antibiotic choice may not explain poorer outcomes in patients with *Staphylococcus aureus* bacteremia and high vancomycin minimum inhibitory concentrations. *Journal of Infectious Disease.* 2011;204:340-347.

Horne KC, Howden BP, Grabsch EA, Graham M, Ward PB, Xie S, Mayall BC, Johnson PDR, Grayson ML: Prospective comparison of the clinical impacts of heterogeneous vancomycin-intermediate methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-susceptible MRSA. *Antimicrobial Agents and Chemotherapy.* 2009; 55:3447-3452.

Howden BP, Ward PB, Charles PG, Korman TM, Fuller A, du Cros P, Grabsch EA, Roberts SA, Robson J, Read K, Bak N, Hurley J, Johnson PD, Morris AJ, Mayall BC, Grayson ML: Treatment outcomes for serious infections caused by methicillin-resistant *Staphylococcus aureus* with reduced vancomycin susceptibility. *Clinical Infectious Diseases*. 2004; 38:521–528.

Howden BP. Recognition and management of infections caused by vancomycin intermediate *Staphylococcus aureus* (VISA) and heterogenous VISA (hVISA). *Internal Medicine Journal* . 2005;35:S136-S140.

Howden BP, Johnson PDR, Ward PB, Stinear TP, Davies JK: Isolates with Low-level vancomycin resistance associated with persistent methicillin-resistant *Staphylococcus aureus* bacteremia. *Antimicrobial Agents and Chemotherapy.* 2006;50:3039-3047.

Howden BP, Stinear TP, Allen DL, Johnson PD, Ward PB, Davies JK: Genomic analysis reveals a point mutation in the two-component sensor gene graS that leads to intermediate vancomycin resistance in clinical *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy.* 2008;52:3755-3762.

Howden BP, Davies JK, Johnson PDR, Stinear TP, Grayson ML: Reduced Vancomycin susceptibility in *Staphylococcus aureus*, including vancomycin-intermediate and heterogeneous vancomycin- intermediate strains: resistance mechanisms, laboratory detection, and clinical implications. *Clinical Microbiology Reviews.* 2010;23:99–139.

Howden BP, McEvoy CR, Allen DL, Chua K, Gao W, Harrison PF, Bell J, Coombs G, Bennett-Wood V, Porter JL, Robins-Browne R, Davies JK, Seemann T, Stinear TP: Evolution of multidrug resistance during *Staphylococcus aureus* infection involves mutation of the essential two component regulator WalKR. *PloS Pathogens.* 2011;7: e1002359.

Hsu DI, Hidayat LK, Quist R, Hindler J, Karlsson A, Yusof A, Wong-Beringer A: Comparison of method-specific vancomycin minimum inhibitory concentration

values and their predictability for treatment outcome of meticillin-resistant *Staphylococcus aureus* (MRSA) infections. *International Journal of Antimicrobial Agents*. 2008;32:378-385.

Hu ZQ, Zhao WH, Hara Y, Shimamura T: Epigallocatechin gallate synergy with ampicillin/salbactam against 28 clinical isolates of methicillin-resistant *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy*. 2001;48:361-364.

Hu Z, Zhao W, Asano N, Yoda Y, Hara Y, Shimamura T: Epigallocatechin gallate synergistically enhances the activity of carbapenems against methicillin-resistant *Staphylococcus aureus. Antimicrobial Agents and Chemotherapy.* 2002;46:558–560.

Huang YT, Liao CH, Teng LJ, Hsueh PR: Comparative bactericidal activities of daptomycin, glycopeptides, linezolid and tigecycline against blood isolates of Gram-positive bacteria in Taiwan. *Clinical Microbiology and Infection*. 2008;14:124–129.

Hubert SK, Mohammed JM, Fridkin SK, Gaynes RP, McGowan JE,. Tenover FC: Glycopeptide-intermediate *Staphylococcus aureus*: evaluation of a novel screening method and results of a survey of selected U.S. hospitals. *Journal of Clinical Microbiology.* 1999;37:3590–3593.

Hussain A, Alleyne S, Jenkins D: A survey of attitudes towards methicillin-resistant Staphylococcus aureus bacteraemias amongst United Kingdom microbiologists. *Journal of Antimicrobial Chemothererapy*. 2010;65:347-349.

Infectious Diseases Society of America: The 10 × 20 initiative: pursuing a global commitment to develop 10 new antibacterial drugs by 2020. *Clinical Infectious Disease*. 2010;50:1081-1083.

International Organization for Standardization. Clinical laboratory testing and in vitro diagnostic test systems usceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices. Part 2:

evaluation of performance of antimicrobial susceptibility test devices. ISO 20776-2. *Geneva: International Organization for Standardization*; 2007.

International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC). Classification of staphylococcal cassette chromosome *mec* (SCC*mec*): guidelines for reporting novel SCC*mec* elements. *Antimicrobial Agents and Chemotherapy* . 2009;53: 4961-4967.

Ip M, Lyon DJ, Chio F, Cheng AF: A longitudinal analysis of methicillin resistant *Staphylococcus aureus* in a Hong Kong teaching hospital. *Infection Control and Hospital Epidemiology*. 2004;25:126-129.

Ip M, Chau SS, Lui SL, Leung E, Ling T: Vancomycin minimum inhibitory concentrations (MICs) for meticillin-resistant *Staphylococcus aureus* (MRSA) in Hong Kong. *International Journal of Antimicrobial Agents*. 2010; 36:386-387.

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

Ito T, Katayama Y, Asada K, Mori N, Tsutsumimoto K, Tiensasitorn C and Hiramatsu K: Structural comparison of three types of staphylococcal cassette chromosome *mec* integrated in the chromosome in methicillin-resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*. 2001;45:1323-1336.

Ito T, Okuma K, Ma XX, Yuzawa H, Hiramatsu K: Insights on antibiotic resistance of *Staphylococcus aureus* from its whole genome: genomic island SCC. *Drug Resistance Updates* 2003;6:41-52.

Ito T, Ma XX, Takeuchi F, Okuma K, Yuzawa H, Hiramatsu K: Novel type V staphylococcal cassette chromosome mec driven by a novel cassette chromosome recombinase, ccrC. *Antimicrobial Agents and Chemotherapy*. 2004;48: 2637-2651.

James PA: Comparison of four methods for determination of MIC and MBC of penicillin for viridian streptococci and the implication for penicillin tolerance. *Journal of Antimicrobial Chemotherapy* . 1990;25:209-216.

James PA, Young SEJ, White DG: Incidence of penicillin tolerance among blood cultures of *Streptococcus sangius*. *Journal of Clinical Pathology.* 1991;44: 160-163.

Jaksic B, Martinelli G, Perez-Oteyza J, Hartman CS, Leonard LB, Tack K J: Efficacy and safety of linezolid compared with vancomycin in a randomized, double-blind study of febrile neutropenic patients with cancer. *Clinical Infectious Diseases* . 2006;42:597–607.

Jariyasethpong T, Tribuddharat C, Dejsirilert S, Kerdsin A, Tishyadhigama P, Rahule S, Sawanpanyalert P, Yosapol P, Aswapokee N: MRSA carriage in a tertiary governmental hospital in Thailand: emphasis on prevalence and molecular epidemiology. *European Journal of Clinical Microbiology & Infectious Diseases. Dis.* 2010;29:977–985.

Jauregui LE, Babazadeh S, Seltzer E, Goldberg L, Krievins D, Frederick M, Krause D, Satilovs I, Endzinas Z, Breaux J, O'Riordan W: Randomized, double-blind comparison of once-weekly dalbavancin versus twice-daily linezolid therapy for the treatment of complicated skin and skin structure infections. *Clinical Infectious Diseases.* 2005; 41:1407–1415

Jansen WT, Beitsma MM, Koeman CJ, van Wamel WJ, Verhoef J, Fluit AC: Novel mobile variants of staphylococcal cassette chromosome mec in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy.* 2006;50: 2072-2078.

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

Jarraud S,1 Cozon GG, Vandenesch FO,1 Bes ML, Etienne J, Lina G: Involvement of enterotoxins G and I in staphylococcal toxic shock syndrome and staphylococcal scarlet fever. *Journal of Clinical Microbiology*. 1999;37:2446–2449.

Jernigan JA, Pullen AL, Flowers L, Bell M, Jarvis WR: Prevalence of and risk factors for colonization with methicillin-resistant *Staphylococcus aureus* in an outpatient clinic population. *Infection Control and Hospital Epidemiology*. 2003;24:445-450.

Jevitt LA., Smith AJ, Williams PP, Raney PM, McGowan JE, Tenover FC: In vitro activities of daptomycin, linezolid, and quinupristin-dalfopristin against a challenge panel of staphylococci and enterococci, including vancomycin-intermediate *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium*. *Microbial Drug Resistance*. 2003;9:389–393.

Jevons MP. Celbenin-resistant staphylococci. *British Medical Journal*. 1961;124–125.

Jiang W: Therapeutic wisdom in traditional Chinese medicine: a perspective from modern science. *Trends in Pharmacological Sciences*.2005;26:558-563.

Jones RN: Microbiological features of vancomycin in the 21st century: minimum inhibitory concentration creep, bactericidal/static activity, and applied breakpoints to predict clinical outcomes or detect resistant strains. *Clinical Infectious Disease*. 2006;42 (Suppl 1):S13-S24.

Jones RN, Mendes RE, Sader HS: Ceftaroline activity against pathogens associated with complicated skin and skin structure infections: results from an international surveillance study. *Journal of Antimicrobial Chemotherapy*. 2010; 65 Suppl 4: 17–31

Jones RN, Farrell DJ, Mendes RE, Sader HS: Comparative ceftaroline activity tested against pathogens associated with community-acquired pneumonia: results

from an international surveillance study. *Journal of Antimicrobial Chemotherapy*. 2011;66 Suppl 3: 69–80.

Jorgensen JH, Ferraro MJ: Antimicrobial susceptibility testing: a review of general principles and contemporary practices. *Clinical Infectious Diseases.* 2009;49:1749-1755.

Ju O, Woolley M, Gordon D: Emergence and spread of rifampicin-resistant, methicillin-resistant *Staphylococcus aureus* during vancomycin-rifampicin combination therapy in an intensive care unit. *European Journal of Clinical Microbiology & Infectious Diseases.* 2006; 25:61–62.

Julian K, Kosowska-Shick K, Whitener C, Roos M, Labischinski H, Rubio A, Parent L, Ednie L, Koeth L, Bogdanovich T, Appelbaum PC: Characterization of a daptomycin nonsusceptible vancomycin-intermediate *Staphylococcus aureus* strain in a patient with endocarditis. *Antimicrobial Agents and Chemotherapy.* 2007;51:3445-3448.

Jung SI, Kiem S, Lee NY, Kim YS, Oh WS, Cho HL, Peck KR, Song JH: One-point population analysis and effect of osmolarity on detection of hetero-vancomycin-resistant *Staphylococcus aureus*. *Journal of Clinical Microbiology*. 2002;40:1493–1495.

Jung YJ, Koh Y, Hong S, Chung JW, Choi SH, Kim NJ, Kim M, Choi IS, Han SY, Kim W, Yun S, Lim C: Effect of vancomycin plus rifampicin in the treatment of nosocomial methicillin-resistant *Staphylococcus aureus* pneumonia. *Critical Care Medicine.* 2010;38:175-180.

Kaplan SL, Hulten KG, Gonzalez BE, Hammerman WA, Lamberth L, Versalovic J, Mason EO Jr: Three-year surveillance of community-acquired *Staphylococcus aureus* infections in children. *Clinical Infectious Diseases.* 2005;40:1785-1791.

Katayama Y, Ito T and Hiramatsu K: A new class of genetic element, staphylococcal cassette chromosome *mec,* encodes methicillin resistance in

Staphylococcus aureus. Antimicrobial Agents and Chemotherapy. 2000;44:1549-1555.

Katayama Y, Ito T, Hiramatsu K: Genetic organization of the chromosome region surrounding mecA in clinical staphylococcal strains: role of IS431-mediated mecI deletion in expression of resistance in mecA-carrying, low-level methicillin-resistant *Staphylococcus haemolyticus*. *Antimicrobial Agents and Chemotherapy.* 2001;45:1955-1963.

Katayama Y, Takeuchi F, Ito T, Ma XX, Ui-Mizutani, Kobayashi I and Hiramatsu K: Identification in methicillin-susceptible *Staphylococcus hominis* of an active primordial mobile genetic element, staphylococcal cassette chromosome *mec* of methicillin resistant *Staphylococcus aureus. Journal of Bacteriology.* 2003;185: 2711-2722.

Katayama Y, Murakami-Kuroda H, Cui L, Hiramatsu K: Selection of heterogeneous vancomycin-intermediate *Staphylococcus aureus* by imipenem. *Antimicrobial Agents and Chemotherapy*. 2009;53:3190-3196.

Kato Y, Suzuki T, Ida T, Maebashi K: Genetic changes associated with glycopeptide resistance in *Staphylococcus aureus*: predominance of amino acid substitutions in YvqF/VraSR. *Journal of Antimicrobial Chemotherapy*. 2010;65: 37–45

Kazakova SV, Hageman JC, Matava M, Srinivasan A, Phelan L, Garfinkel B, Boo T, McAllister S, Anderson J, Jensen B, Dodson D, Lonsway D, McDougal LK, Arduino M, Fraser VJ, Killgore G, Tenover FC, Cody S, Jernigan DB: A clone of methicillin-resistant *Staphylococcus aureus* among professional football players. *New England Journal of Medicine* . 2005; 352:468-475.

Kehrmann J, Kaase M, Szabados F, Gatermann SG, Buer J, Rath PM, Steinmann J: Vancomycin MIC creep in MRSA blood culture isolates from Germany: a regional problem? *European Journal of Clinical Microbiology and Infectious Disease.* 2011; 30:677–683.

Kelley PG, Grabsch EA, Farrell J, Xie S, Montgomery J, Mayall B, Howden BP: Evaluation of the Xpert™ MRSA/SA blood culture assay for the detection of *Staphylococcus aureus* including strains with reduced vancomycin susceptibility from blood culture specimens. *Diagonistic Microbiology and Infectious Diseases.* 2011;70: 404-407.

Khan SA: Plasmid rolling-circle replication: highlights of two decades of research. *Plasmid*. 2005;53:126–136.

Khatib R, Johnson LB, Fakih MG, Riederer K, Khosrovaneh A, Shamse Tabriz M, Sharma M, Saeed S: Persistence in *Staphylococcus aureus* bacteremia: incidence, characteristics of patients and outcome. *Scandinavian Journal of Infectious Diseases.* 2006;38:7–14.

Khatib R, Jose J, Musta A, Sharma M, Fakih MG, Johnson LB, Riederer K, Shemes S: Relevence of vancomycin-intermediate *Staphylococcus aureus* bacteraemia. *Journal of Antimicrobial and Chemotherapy.* 2011;66:1594-1599.

Kim T, Oh PI, Simor AE: The economic impact of methicillin-resistant Staphylococcus aureus in Canadian hospitals. *Infection Control and Hospital Epidemiology.* 2001;22:99-104.

Kim MN, Hwang SH, Pyo Y, Mun H, Pai CH: Clonal spread of *Staphylococcus aureus* heterogeneously resistant to vancomycin in a university hospital in Korea. *Journal of Clinical Microbiology.* 2002; 40:1376- 1380.

Kim A, Sakoulas G, Kuti JL, Nicolau DP: Prevalence of methicillin-resistant *Staphylococcus aureus* strains with vancomycin minimum inhibitory concentration 2 mg/l in New York city area hospitals and implications on pharmacodynamic target attainment. *Infectious Diseases in Clinical Practice.* 2009;17: 95-98

Kloucek P, Svobodova B, Polesny Z, Langrova I, Smrcek S, Kokoska L: Antimicrobial avticity of some medicinal barks used in Peruvian Amazon. *Journal of Enthopharmacy.* 2007;111:427–429.

Klugman KP, Friedland IR, Bradley JS: Bactericidal activity against cephalosporin *Streptococcus pneumoniae* in cerebrospinal fluid of children with acute meningitis. *Antimicrobial Agents and Chemotherapy* . 1995;39: 1988-1992.

Kluytmans J, van Belkum A, Verbrugh H: Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks. *Clinical Microbiology Reviews* . 1997;10:505-520.

Köck R, Becker K, Cookson B, van Gemert-Pijnen JE, Harbarth S, Kluytmans J, Mielke M, Peters G, Skov RL, Struelens MJ, Tacconelli E, Torné1 AN, Witte W, Friedrich AW: Methicillin-resistant *Staphylococcus aureus* (MRSA): burden of disease and control challenges in Europe. *Euro Surveillance.* 2010;15: 1-7.

Koehl JL, Muthaiyan A, Jayaswal RK, Ehlert E, Labischinski H, Wilkinson BJ: Cell wall composition and decreased autolytic activity and lysostaphin susceptibility of glycopeptide-intermediate *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy.* 2004; 48:3749-3757.

Kollef MH: New antimicrobial agents for methicillin-resistant *Staphylococcus aureus*". *Critical Care & Resuscitation.* 2009;11: 282–6.

Kola A, Kirschner P, Gohrbandt B, Chaberny IF, Mattner F, Struber M, Gastmeier P, Suerbaum S: An infection with linezolid-resistant *S. aureus* in a patient with left ventricular assist system. *Scandinavian Journal of Infectious Diseases.* 2007; 39:463–465.

Kondo Y, Ito T, Ma XX, Watanabe S, Kreiswirth B N, Etienne J, Hiramatsu K: Combination of multiplex PCRs for staphylococcal cassette chromosome mec type assignment: rapid identification system for mec, ccr, and major differences in junkyard regions *Antimicrobial Agents and Chemotherapy.* 2007;51: 264–274.

Kosowska-Shick K, Ednie LM, McGhee P, Smith K, Todd CD, Wehler A, Appelbaum PC: Incidence and characteristics of vancomycin nonsusceptible

strains of methicillin-resistant *Staphylococcus aureus* at Hershey Medical Center. *Antimicrobial Agents and Chemotherapy.* 2008;52:4510–4513.

Korakaki E, Aligizakis A, Manoura A, Hatzidaki E, Saitakis E, Anatoliotaki M, Velivasakis E, Maraki S and Giannakopoulou C: Methicillin-resistant *Staphylococcus aureus* osteomyelitis and septic arthritis in neonates: diagnosis and management. *Japan Journal of Infectious Diseases.* 2007; 60:129-131.

Kuehnert MJ, Hill HA, Kupronis BA, Tokars JI, Solomon SL, and Jernigan DB: Methicillin-resistant *Staphylococcus aureus* hospitalizations, United States. *Emerging Infectious Disease.* 2005; 11:868–872.

Kumar V, Abbas AK, Fausto N, Mitchell RN: *Robbins Basic Pathology. 8th Edition. Saunders Elsevier*, Oxford. 2007:810-811.

Kuroda M, Kuroda H, Oshima T, Takeuchi F, Mori H, Hiramatsu K: Two-component system *VraSR* positively modulates the regulation of cell-wall biosynthesis pathway in *Staphylococcus aureus*. *Molecular Microbiology.* 2003;49: 807–821.

Kuroda M, Kuwahara-Arai K, Hiramatsu K: Identification of the up- and down-regulated genes in vancomycin-resistant *Staphylococcus aureus* strains Mu3 and Mu50 by cDNA differential hybridization method. *Biochemical and Biophysical Research Communications.* 2000;269:485–490.

Kuzma L, Rozalski M, Walencka E, Rozalska B, Wysokinska H: Antimicrobial activity of diterpenoids from hairy roots of *Salvia sclarea* L.: Salvipisone as a pontial anti-biofilm agent active against antibiotic resistant *Staphylococci. Phytomedicine*. 2007;14:31-35.

Kwon NH, Park KT, Moon JS, Jung WK, Kim SH, Kim JM, Hong SK, Koo HC, Joo YS, Park YH: Staphylococcal cassette chromosome mec (SCCmec) characterization and molecular analysis for methicillin-resistant *Staphylococcus*

aureus and novel SCCmec subtype Ivg isolated from bovine milk in Korea. *Journal of Antimicrobial Chemotherapy.* 2005;56: 624-632.

Labandeira-Rey M, Couzon F, Boisset S, Brown EL, Bes M, Benito Y, Barbu EM, Vazquez V, Höök M, Etienne J, Vandenesch F, Bowden MG: *Staphylococcus aureus* Panton-Valentine leukocidin causes necrotizing pneumonia. *Science.* 2007;315:1130-1133.

Lamichhane-Khadka R, Cantore SA, Riordan JT, Delgado A, Norman AEA, Duenas S, Zaman S, Horan S, Wilkinson BJ, Gustafson JE,: sarA inactivation reduces vancomycin-intermediate and ciprofloxacin resistance expression by *Staphylococcus aureus*. *International Journal of Antimicrobial Agents.* 2009;34:136-141.

Leclercq RE, Duval DJ, Courvalin P: Plasmid-mediated resistance to vancomycin and teicoplanin in *Enterococcus faecium*. *New England Journal of Medicine* . 1988;319:157–160.

Leclercq RE: Mechanisms of resistance to macrolides and lincosamides: nature of the resistance elements and their clinical implications. *Clinical Infectious Diseases* . 2002;34: 482-492.

Levine DP, Fromm BS, Reddy BR: Slow response to vancomycin or vancomycin plus rifampin in methicillin-resistant *Staphylococcus aureus* endocarditis. *Annals of Internal Medicine* ., 1991;115:674–80.

Leonard SN, Rossi KL, Newton KL, Rybak MJ: Evaluation of the Etest GRD for the detection of *Staphylococcus aureus* with reduced susceptibility to glycopeptides. *Journal of Antimicrobial Chemotherapy.* 2009;63: 489–492.

Leonard SN, Rybak MJ: Evaluation of vancomycin and daptomycin against methicillin-resistant *Staphylococcus aureus* and heterogeneously vancomycin-intermediate *S. aureus* in an in vitro pharmacokinetic/pharmacodynamic model

with simulated endocardial vegetations. *Journal of Antimicrobial Chemotherapy.* 2009;63:155–160.

Lew DP, Waldvogel F: Osteomyelitis. *Lancet.* 2004;364:369-379.

Li BQ, Fu T, Dongyan Y, Mikovits JA, Ruscetti FW, Wang JM: Flavonoid baicalin inhibits HIV-1 infection at the level of viral entry. *Biochemical and Biophysical Research Communications*. 2000;276:534–538.

Li CR, Li Y, Li GQ, Yang XY, Zhang WX, Lou RH, Liu JF, Yuan M, Huang P, Cen S, Yu LY, Zhao LX, Jiang JD, You XF: In vivo antibacterial activity of nemonoxacin, a novel non-fluorinated quinolone. *Journal of Antimicrobial Chemotherrapy*. 2010;65:2411-2415.

Li JJ, Ji BP, Li B, Zhou F, Zhao L: *In-vitro* antimicrobial activity study on 10 extracts. *Food Science*. 2006; 27: 147-150.

Li S, Skov RL, Han X, Larsen AR, Larsen J, Sørum M, Wulf M, Voss A, Hiramatsu K, Ito T: Novel types of staphylococcal cassette chromosome*mec* elements identified in clonal complex 398 methicillin-resistant *Staphylococcus aureus* strains. *Antimicrobial Agents and Chemotherapy.* 2011;55:3046-3050.

Lina G, Piemont Y, Godail-Gamot F, Bes M, Marie-Odile P, Gauduchon V, Vandenesch F, Etienne J: Involvement of panton-valentine leukocidin-producing *Staphylococcus aureus* in primary skin infections and pneumonia. *Clinical Infectious Diseases.* 1999;29:1128-1132.

Lindsay JA, Holden MTG: *Staphylococcus aureus*: Superbug, super genome?. *Trends in Microbiology*. 2004;12:378-385.

Lindsay LA, Moore CE, Day NP, Peacock SJ, Witney AA, Stabler RA, Husain SE, Butcher PD, Hinds J: Microarrays reveal that each of the ten dominant lineages of

Staphylococcus aureus has a unique combination of surface-associated and regulatory genes. *Journal of Bacteriology.* 2006;188:669-676.

Liu GW. Clincal essentials of contemporary series Chinese herbal medicine. 2001; Ed. Liu GW, Trans. Cao L. Hua Xia Publishing House: Beijing.

Liu C, Chambers HF. *Staphylococcus aureus* with heterogeneous resistance to vancomycin: epidemiology, clinical significance, and critical assessment of diagnostic methods. *Antimicrobial Agents and Chemotherapy.* 2003;47:3040–3045.

Liu C, Bayer A, Cosgrove SE, Daum RS, Fridkin SK, Gorwitz RJ, Kaplan SL, Karchmer AW, Levine DP, Murray BE, Rybak MJ, Talan DA, Chambers HF: Clinical practice guidelines by the Infectious Diseases Society of America for the treatment of methicillin-resistant *Staphylococcus aureus* infections in adults and children: executive summary. *Clinical Infectious Disease*. 2011; 52: 285–92.

Lodise TP, Graves J, Evans A, Graffunder E, Helmecke M, Lomaestro BM, Stellrecht K: Relationship between vancomycin mic and failure among patients with methicillin-resistant *Staphylococcus aureus* bacteremia treated with vancomycin. *Antimicrobial Agents and Chemotherapy.* 2008;52:3315–3320.

Lodise TP, Patel N, Lomaestro BM, Rodvold KA, Drusano GL: Relationship between initial vancomycin concentration-time profile and nephrotoxicity among hospitalized patients. *Clinical Infectious Diseases*. 2009;49:507–514.

Löffler B, Hussain M, Grundmeier M, Brück M, Holzinger D, Varga G, Roth J, Kahl BC, Proctor RA, Peters G: *Staphylococcus aureus* panton-valentine leukocidin is a very potent cytotoxic factor for human neutrophils. *PLoS Pathogens.* 2010;6:e1000715.

Logman JF, Stephens J, Heeg B, Haider S, Cappelleri J, Nathwani D[d], Tice A, van Hout BA: Comparative effectiveness of antibiotics for the treatment of MRSA complicated skin and soft tissue infections. *Current Medical Research and Opinion*. 2010;26:1565-1578.

Lowy FD: *Staphylococcus aureus* infections. *New England Journal of Medicine.* 1998, 339:520-532.

Lubin AS, Snydman DR, Ruthazer R, Bide P, Golan Y: Predicitng high vancomycin minimum inhibitory concnetration in methicillin-resistant *Staphylococcus aureus* bloodstream infections. *Clinical Infectious Diseases*. 2011;52:997-1002.

Lucet J, Chevret S, Durand-Zaleski I, Chastang C, Re´gnier B: Prevalence and risk factors for carriage of methicillin-resistant *Staphylococcus aureus* at admission to the intensive care unit. *Archives of Internal Medicine*. 2003;163:181-188.

Lulitanond A, Engchanil C, Chaimanee P, Vorachit M, Ito T, Hiramatsu K: The first vancomycin-intermediate *Staphylococcus aureus* strains isolated from patients in Thailand. *Journal of Clinical Microbiology.* 2009;47: 2311–2316.

Ma XX, Ito T, Tiensasitorn C, Jamklang M, Chongtrakool P, Boyle-Vavra S, Daum RS, Hiramatsu K: Novel type of staphylococcal cassette chromosome *mec* identified in community-acquired methicillin-resistant *Staphylococcus aureus* strains. *Antimicrobial Agents and Chemotherapy.* 2002;46:1147-1152.

Ma XX, Ito T, Chongtrakool P, Hiramatsu K: Predominance of clones carrying Panton-Valentine leukocidin genes among methicillin-resistant *Staphylococcus aureus* strains isolated in Japanese hospitals from 1979 to 1985. *Journal of Clinical Microbiology.* 2006;44:4515–4527.

McCallum N, Karauzum H, Getzmann R, Bischoff M, Majcherczyk P, Berger-Bächi B, Landmann R: In vivo survival of teicoplanin-resistant *Staphylococcus aureus* and fitness cost of teicoplanin resistance. *Antimicrobial Agents and Chemotherapy* 2006;50:2352–2360.

MacDonald KL, Osterholm MT, Hedberg CW, Schrock CG, Peterson GF, Jentzen JM, Leonard SA, Schlievert PM: Toxic shock syndrome: a newly recognized

complication of influenza and influenza like illness. *Journal of The American Medical Association* 1987;257:1053-1058.

Macias EA, Mason EO Jr, Ocera HY, Lorococco MT: Comparison of Etest with standard broth microdilution for determining antibiotic susceptibility of penicillin-resistant strains of *Streptococcus pneumoniae. Journal of Clinical Microbiology*. 1994; 32:430-432.

Mainardi JL, Shlaes DM, Goering RV, Shlaes JH, Acar JF, Goldstein FW: Decreased teicoplanin susceptibility of methicillin-resistant strains of *Staphylococcus aureus*. *Journal of Infectious Diseases.* 1995;171:1646–1650.

Maki H, McCallum N, Bischoff M, Wada A, Berger-Bachi B: tcaA inactivation increases glycopeptide resistance in *Staphylococcus aureus. Antimicrobial Agents and Chemotherapy.* 2004; 48:1953–9.

Mangili A, Bica I, Snydman DR, Hamera DH: Daptomycin-resistant, methicillin-resistant *Staphylococcus aureus* bacteremia. *Clinical Infectious Diseases*. 2005; 40:1058–1060.

Maor Y, Rahav G, Belausov N, Ben-David D, Smollan G, Keller N: Prevalence and characteristics of heteroresistant vancomycin-intermediate *Staphylococcus aureus* bacteremia in a tertiary care center. *Journal of Clinical Microbiology.* 2007;45:1511–1514.

Maor Y, Hagin M, Belausov N, Keller N, Ben-David D, Rahav G: Clinical features of heteroresistant vancomycin-intermediate *Staphylococcus aureus* bacteremia versus those of methicillin-resistant S. aureus bacteremia. *The Journal of Infectious Disease*. 2009;199:619-624.

Marchese A, Balistreri G, Tonoli E, Debbia EA, Schito GC: Heterogeneous vancomycin resistance in methicillin-resistant *Staphylococcus aureus* strains isolated in a large Italian hospital. *Journal of Clinical Microbiology.* 2000; 38: 866–869.

Marschall J, Mu¨hlemann K: Duration of methicillin-resistant *Staphylococcus aureus* carriage, according to risk factors for acquisition. *Infection Control and Hospital Epidemiology.* 2006;27: 1206-1212.

Matsuda Y, Kato H, Yamada R, Okano H, Ohta H, Imanishi K, Kikuchi K, Totsuka K, Uchiyama T: Early and definitive diagnosis of toxic shock syndrome by detection of marked expansion of t-cell-receptor vβ2-positive t cells. *Emerging Infectious Diseases.* 2003;9:387-389.

Matsuo M, Hishinuma T, Katayama Y, Cui L, Kapi M, Hiramatsu K: Mutation of RNA polymerase subunit (*rpob*) promotes hVISA-to-VISA phenotypic conversion of strain Mu3. *Antimicrobial Agents and Chemotherapy*. 2011;55: 4188–4195.

Mattana CM, Satorres SE, Sosa A, Fusco M, Alcaráz LE: Antibacterial activity of extracts of *acacia aroma* against methicillin-resistant and methicillin-sensitive *Staphylococcus. Brazilian Journal of Microbiology.* 2010; 41:581-587.

Matzke GR, McGory RW, Halstenson CE, Keane WF: Pharmacokinetics of vancomycin in patients with various degrees of renal function. *Antimicrobial Agents and Chemotherapy.* 1984;25:433–437.

McDougal LK, Steward CD, Killgore GE, Chaitram JM, McAllister SK, Tenover FC. Pulsed-field gel electrophoresis typing of oxacillin-resistant *Staphylococcus aureus* isolates from the United States: establishing a national database. *Journal of Clinical Microbiology.* 2003; 41:5113-5120.

Meehl M, Herbert S, Gotz F, Cheung, A: Interaction of theGraRS two-component system with the VraFG ABCtransporter to support vancomycin-intermediate resistance in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*. 2007;51:2679–2689.

Meng Z, Sop CJ, You XF, Xing D, Wang W, Du LJ. Characteristics of Baicalin Synergy with Penicillin or Notopterygium Ethanol Extracts Against *Staphylococcus aureus*. Tsinghua Science and Technology. 2006; 11: 459-461.

Mehta S, Cuirolo AX, Plata KX, RioSA S, Silverman JA, Rubio A, Rosato RR, Rosato AE: VraSR two-component regulatory system contributes to *mprf*-mediated decreased susceptibility to daptomycin in *in vivo*-selected clinical strains of methicillin-resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*. 2012;56:92:102.

Meka VG, Gold HS: Antimicrobial resistance to linezolid. *Clinical Infectious Diseases*. 2004;39:1010-1015.

Mergenhagen KA, Pasko MT: Daptomycin use after vancomycin-induced neutropenia in a patient with left-sided endocarditis. *The Annals of Pharmacotherapy*. 2007;41:1531-1535.

Miller LG, Perdreau-Remington F, Rieg G, Mehdi S, Periroth J, Bayer AS, Tang AW, Phung TO, Spellberg B: Necrotizing fasciitis caused by community-associated methicillin-resistant *Staphylococcus aureus* in Los Angeles. *New England Journal of Medicine* .2005;352:1445-1453.

Miller CE, Batra R, Cooper BS, Patel AK, Klein J, Otter JA, Kypraios T, French GL, Tosas O, Edgeworth JD: An association between bacterial genotype combined with a high-vancomycin minimum inhibitory concentration and risk of endocarditis in methicillin-resistant *Staphylococcus aureus* bloodstream infection. *Clinical Infectious Disease*. 2012;54:591-600.

Milheirico C, Oliveira DC, de Lencastre H: Multiplex PCR strategy for subtyping the staphylococcal cassette chromosome mec type IV in methicillin-resistant *Staphylococcus aureus*: 'SCCmec IV multiplex'. *Journal of Antimicrobial Chemotherapy*. 2007a;60:42–48.

Milheirico C, Oliveira DC, de Lencastre H:. Update to the multiplex PCR strategy for assignment of mec element types in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* . 2007b;51:3374-3377.

Mitchell DH, Howden BP: Diagnosis and management of *Staphylococcus aureus* bacteraemia. *Internal Medicine Journal* 2005, 35(2):S17-S24.

Mody L, Maheshwari S, Galecki A, Kauffman CA, Bradley SF: Indwelling device use and antibiotic resistance in nursing homes: identifying a high-risk group. *Journal of American Geriatric Society*. 2007; 55:1921–1926.

Moellering RC Jr.: Pharmacokinetics of vancomycin. *Journal of Antimicrobial Chemotherapy.* 1984;14: 43-52.

Moellering RC Jr. Vancomycin: a 50-year reassessment. *Clinical Infectious Diseases*. 2006; 42:S3–4

Mohiyiddeen G, Brett I, Jude E: Infective endocarditis caused by *Staphylococcus aureus* in a patient with atopic dermatitis: a case report. *Journal of Medical Case Reports.* 2008;2:143-145.

Moise PA, Schentag JJ: Vancomycin treatment failures in *Staphyloccus aureus* lower respiratory tract infections. *International Journal of Antimicrobial Agents.* 2000, 16(Suppl 1):S31-34.

Moise-Broder PA, Forrest A, Birmingham MC, Schentag JJ: Pharmacodynamics of vancomycin and other antimicrobials in patients with *Staphylococcus aureus* lower respiratory tract infections. *Clinical. Pharmacokinetics*. 2004, 43:925–942.

Moise-Border PA, Sakoulas G, Forrest A, Schentag JJ: Vancomycin *in-vitro* bactericidal activity and its relationship to efficacy in clearance of methicillin-resistant *Staphyloccus aureus* bacteremia. *Antimicrobial Agents and Chemotherapy.* 2007;51:2582-2586.

Moise PA, Smyth DS, El-Fawal N, Robinson DA, Holden PN, Forrest A, Sakoulas G: Microbiological effects of prior vancomycin use in patients with methicillin-resistant *Staphylococcus aureus* bacteraemia. *Journal of Antimicrobial Chemotherapy*. 2008;61:85-90.

Moise PA, Hershberger E, Amodio-Groton MI, Lamp KC: Safety and clinical outcomes when utilizing high-dose (> or =8 mg/kg) daptomycin therapy. *The Annals of Pharmacotherapy.* 2009;43:1211-1219.

Morales G, Picazo JJ, Baos E, Candel FJ, Arribi A, Peláez B, Andrade R, de la Torre MA, Fereres J, Sánchez-García M: Resistance to linezolid is mediated by the cfr gene in the first report of an outbreak of linezolid-resistant *Staphylococcus aureus. Clinical Infectious Diseases.* 2010;50:821-825.

Moreillon P, Que YA, Glauser MP: *Staphylococcus aureus* – Principles and practice of infectious diseases. *In*: Mandell GL, Bennett JE, Dolin R (eds) 6th edn. Elsevier Churchill Livingstone, Philadelphia, 2005;2321–2351.

Moreillon P, Bizzini A, Giddey M, Vouillamoz J, Entenza JM: Vancomycin-intermediate *Staphylococcus aureus* selected during vancomycin therapy of experimental endocarditis are not detected by culture-based diagnostic procedures and persist after treatment arrest. *Journal of Antimicrobial Chemotherapy.* 2012; 67: 652-660.

Moore CL, Lu M, Cheema F, Osaki-Kiyan P, Perri MB, Donabedian S, Haque NZ, Zervos MJ: Predection of failure in vancomycin-treated methicillin-resistant *Staphylococcus aureus* bloodstream infection: a clinically useful risk stratification tool. *Clinical Infectious Diseases.* 2012;54:51-58

Morbidity and Mortality Weekly Report: Outbreaks of community-associated methicillin-resistant *Staphylococcus aureus* skin infections-Los Angeles county, California, 2002-2003. *Morbidity and Mortality Weekly Report.* 2003;52:88-88.

Moreira B, Boyle-Vavra S, deJonge BL, Daum RS. Increased production of penicillin-binding protein 2, increased detection of other penicillin-binding proteins, and decreased coagulase activity associated with glycopeptide resistance in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*. 1997;41:1788–1793.

Moreira B, Boyle-Vavra S, Dejonge BLM, Daum RS: Increased production of penicillin-binding protein 2, increased detection of other penicillin-binding proteins, and decreased coagulase activity associated with glycopeptide resistance in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*. 1997; 41:1788–1793.

Mortin LI, Li T, Van Praagh AG, Zhang S, Zhang XX, Alder JD: Rapid bactericidal activity of daptomycin against methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* peritonitis in mice as measured with bioluminescent bacteria. *Antimicrobial Agents and Chemotherapy* . 2007; 51:1787–1794.

Murray BE: The life and times of the enterococcus. *Clinical Microbiology Reviews.* 1990;3:45-65.

Murray RJ: *Staphylococcus aureus* infective endocarditis: diagnosis and management guideline. *Internal Medicine Journal.* 2005; 35:S25-S44.

Musta AC, Riederer K, Shemes S, Chase P, Jose J, Jhonson LB, Khatib R: Vancomycin MIC plus heteroresistance and the outcome of methicillin-resistant *Staphylococcus aureus* bacteremia: trends over 11 years. *Journal of Clinical Microbiology*. 2009; 47: 1640–1644.

Muthaiyan A, Jayaswal RK, Wilkinson BJ: Intact *mutS* in laboratory-derived and clinical glycopeptide-intermediate *Staphylococcus aureus* strains. *Antimicrobial Agents and Chemotherapy.* 2004;48:623-625.

Mwangi MM., Wu SW, Zhou Y, Sieradzki K, de Lencastre H, Richardson P, Bruce D, Rubin E, Myers E, Siggia ED, Tomasz A.: Tracking the in vivo evolution of multidrug resistance in *Staphylococcus aureus* by whole-genome sequencing. *Proceedings of the National Academy of Sciences.* 2007;104:9451–9456.

Naimi TS, Ledell KH, Como-Sabetti K, Borchardt SM, Boxrud DJ, Etienne J, Johnson SK, Vandenesch F, Fridkin S, O'Boyle C, Danila RN, Lynfield R:

Comparison of community and health care-associated methicillin-resistant *Staphylococcus aureus* infection. *Journal of The American Medical Association.* 2003;290:2976-2984.

Namdari H, Tan TH, Dowzicky MJ: Activity of tigecycline and comparators against skin and skin structure pathogens: global results of the tigecycline evaluation and surveillance trial, 2004-2009. *International Journal of Antimicrobial Agents.* 2012;16:e60-e66.

Neuhaus FC, Baddiley J. A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in gram positive bacteria. *Microbiology and Molecular Biology Reviews*. 2003; 64: 686-723.

Nelson JL, Rice KC, Slater SR, Fox M, Archer GL, Bayles KW, Fey PD, Kreiswirth BN, Somerville GA: Vancomycin-intermediate *Staphylococcus aureus* strains have impaired acetate catabolism: implications for polysaccharide intercellular adhesion synthesis and autolysis. *Antimicrobial Agents and Chemotherapy.* 2007;51:616–622.

Neoh HM, Cui L, Yuzawa H, Takeuchi F, Matsuo M, Hiramatsu K: Mutated response regulator *graR* is responsible for phenotypic conversion of *Staphylococcus aureus* from heterogeneous vancomycin-intermediate resistance to vancomycin-intermediate resistance. *Antimicrobial Agents and Chemotherapy.* 2008;52:45–53.

Ngui-Yen JH, Bryce EA, Poter C, Smith JA: Evaluation of the E test by using selected gram-positive bacteria. *Journal of Clinical Microbiology*. 1992;30:2150-2152.

Nishi H, Komatsuzawa H, Fujiwara T, McCallum N, Sugai M: Reduced content of lysyl-phosphatidylglycerol in the cytoplasmic membrane affects susceptibility to moenomycin, as well as vancomycin, gentamicin, and antimicrobial peptides, in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy.* 2004; 48: 4800–4807.

Nolte FS, Krisher KK, Beltran LA, Christian NP, Sheridan GE: Rapid and overnight microdilution antibiotic susceptibility testing with the sensitive autoreader system. *Journal of Clinical Microbiology.* 1998;26:1079-1084.

Noskin GA: Tigecycline: A new glycylcycline for treatment of serious infections. *Clinical Infectious Diseases*. 2005; 41:S303–314

Odds FC: Synergy, antagonism, and what the chequerboard puts between them. *Journal of Antimicrobial Chemotherapy*. 2003;52:1.

O'Donoghue MM, Boost MV: The prevalence and source of methicillin-resistant *Staphylococcus aureus* (MRSA) in the community in Hong Kong. *Epidemiology and Infection.* 2004;132:1091–1097.

Oh K, Oh M, Kim J, Shin D, Shin J: Inhibition of sortase-mediated Staphylococcus aureus adhesion to fibronectin via fibronectin-binding protein by sortase inhibitors. *Applied Microbiology and Biotechnology.* 2006;70:102-106.

Ohta T, Hirakawa H, Morikawa K, Maruyama A, Inose Y, Yamashita A, Oshima K, Kuroda M, Hattori M, Hiramatsu K, Kuhara S, Hayashi H: Nucleotide substitutions in *Staphylococcus aureus* strains, Mu50, Mu3, and N315. *DNA Research.* 2004;11:51–56.

Ogston A: "On abscesses". Classics in infectious diseases". *Reviews of Infectious Diseases.* 1984*;*6:122-28.

Oliveira DC, Tomasz A, de Lencastre H: The evolution of pandemic clones of methicillin-resistant *Staphylococcus aureus:* identification of two ancestral genetic backgrounds and the associated mec elements. *Microbial Drug Resistance.* 2001;7:349-361.

Oliveira DC, Milheirico C, de Lencastre H: Redefining a structural variant of staphylococcal cassette chromosome *mec*, SCCmec yype VI. *Antimicrobial Agents and Chemotherapy*. 2006a; 50:3457-3459.

Oliveira DC, Milheirico C, Vinga S, de Lencastre H: Assessment of allelic variation in the ccrAB locus in methicillin-resistant *Staphylococcus aureus* clones. *Journal of Antimicrobial Chemotherapy.* 2006b;58:23-30.

Olson MW, Ruzin A, Feyfant E, Rush TS, O'Connell J, Bradford PA.. Functional, biophysical, and structural bases for antibacterial activity of tigecycline. *Antimicrobial Agents and Chemotherapy.* 2006;50:2156–2166.

O'Neill AJ, Larsen AR, Skov R, Henriksen AS, Chopra I: Insertional inactivation of mutS in *Staphylococcus aureus* reveals potential for elevated mutation frequencies, although the prevalence of mutators in clinical isolates is low. *Journal of Antimicrobial Chemotherapy.* 2002;50:161-169.

O'Neill AJ, Chopra I: Chracterization of the epidemic European fusidic acid-resistant impetigo clone of *Staphylococcus aureus. Journal of Clinical Microbiology.* 2007;45:1505-1510.

Ornelas-Soares A, de Lencastre H, de Jonge B, Gage D, Chang YC, Tomasz A: The peptidoglycan composition of a *Staphylococcus aureus* mutant selected for reduced methicillin resistance. *Journal of Biological Chemistry.* 1993;268:26268-26272.

Pallares R, Viladrich PF, Linares J, Cubellos L, Gudiol F: Impact of antibiotic resistance on chemotherapy for pneumococcal infections. *Microbial Drug Resistance*. 1998;4:339-347.

Park C, Shin NY, Byun JH, Shin HH, Kwon EY, Choi SM, Kim SH, Kwon JC, Park SH, Choi JH, Yoo JH, Yoo JI, Chung GT, Lee DG: Downregulation of RNAIII in vancomycin-intermediate *Staphylococcus aureus* strains regardless of the presence of agr mutation. *Journal of Medical Microbiology*. 2012;61:345-352.

Parkinson JS: Signal transduction schemes of bacteria. *Cell*. 1993;73:857-871.

Patel M, Waites KB, Hoesley CJ, Stamm AM, Canupp KC, Moser SA: Emergence of USA300 MRSA in a tertiary medical centre: implications for epidemiological studies. *Journal of Hospital Infection*. 2008; 68:208-213.

Paton JH, Holt HA, Bywater MJ: Measurement of MICs of antibacterial agents by spiral gradient endpoint method compared with convential dilution method. *International Journal of Experimental and Clinical Chemotherapy* . 1990;3:31-38.

Pereira PM, Filipe SR, Tomasz A, Pinho MG: Fluorescence ratio imaging microscopy shows decreased access of vancomycin to cell wall synthetic sites in vancomycin-resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy.* 2007;51:3627-33

Perichon B, Courvalin P: VanA -Type Vancomycin-Resistant *Staphylococcus aureus*. . *Antimicrobial Agents and Chemotherapy*. 2009; 53: 4580-4587.

Peschel A, Otto M, Jack RW, Kalbacher H, Jung G, Gotz F: Inactivation of the dlt operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides. *Journal of Biological Chemistry*. 1999;274: 8405–8410.

Peschel A, Vuong C, Otto M, Gotz F: The D-alanine residues of *Staphylococcus aureus* teichoic acids alter the susceptibility to vancomycin and the activity of autolytic enzymes. *Antimicrobial Agents and Chemotherapy*. 2000;44: 2845–2847.

Petersen PJ, Bradford PA, Weiss WJ, Murphy TM, Sum PE, Projan SJ: *In-vitro* and *In-vivo* activities of tigecycline (gar-936), daptomycin, and comparative antimicrobial agents against glycopeptide-intermediate *Staphylococcus aureus* and other resistant gram-positive pathogens. *Antimicrobial Agents and Chemotherapy*. 2002;46:2595–2601.

Pinho MG, de Lencastre H, Tomasz A: An acquired and a native penicillin-binding protein cooperate in building the cell wall of drug-resistant staphylococci. *Proceedings of the National Academy of Sciences.* 2001; 98:10886–10891.

Pinho MG, Errington J: Recruitment of penicillin binding protein PBP2 to the division site of *Staphylococcus aureus* is dependent on its transpeptidation substrates. *Molecular Microbiology*. 2005; 55:799-807.

Pintado V, Meseguer MA, Fortun J, Cobo J, Navas E, Quereda C, Corral I, Moreno S: Clinical study of 44 cases of *Staphylococcus aureus* meningitis. *European Journal of Clinical Microbiology & Infectious Diseases.* 2002;21:864–868.

Ploy MC, Grelaud C, Martin CC, de Lumley L, Dennis F: First clinical isolate of vancomycin-intermediate *Staphylococcus aureus* in a French hospital. *Lancet.* 1998;351:1212.

Pong R, Boost MV, O'Donoghue MM Appelbaum PC: Spiral gradient endpoint susceptibility testing: a fresh look at a neglected technique. *Journal of Antimicrobial Chemotherapy.* 2010; 65: 1959–1963.

Popovich KJ, Weinstein RA, Hota B: Are community-associated methicillin-resistant *Staphylococcus aureus* (MRSA) strains replacing traditional nosocomial MRSA strains? *Clinical Infectious Diseases* .2008;46:795-798.

Pootoolal J, Neu J, Wright GD: Glycopeptide antibiotic resistance. *Annual Review of Pharmacology and Toxicology.* 2002; 42:381-408.

Poupard JA, Rittenhouse SF, Walsh LR: The evolution of antimicrobial susceptibility testing methods. *In antimicrobial susceptibility testing*. Poupard JA et al (ed.) 1994. New York. Plenum Press

Prakash V, Lewis JS II, Jorgensen JH: Vancomycin MICs for methicillin-resistant *Staphylococcus aureus* isolates differ based upon the susceptibility test method used. *Antimicrobial Agents and Chemotherapy*. 2008; 52:4528.

Prevost G, Couppie P, Prevost P, Gayet S, Petiau P, Cribier B Monteil H, Piemont Y: Epidemiological data on *Staphylococcus aureus* strains producing synergohymenotropic toxins. *Journal of Medical Microbiology.* 1995;42:237–245.

Price J, Atkinson S, Llewelyn M, Paul J. Paradoxical relationship between the clinical outcome of *Staphylococcus aureus* bacteremia and the minimum inhibitory concentration of vancomycin. *Clinical Infectious Diseases*. 2009;48:997-998.

Quintiliani R: Using pharmacodynamics and pharmacokinetics concepts to optimize treatment of infectious diseases. *Infections in Medicine.* 2004; 21: 219-232.

Randhir A, Shetty K: Improved α-amylase and *Helicobacter pylori* inhibition by fenugreek extracts derived via solid-state bioconversion using *Rhizopus oligosporus. Asia Pacific Journal of Clinical Nutrition.* 2007;16 :382-392.

Reithinger R, Aadil K, Kolaczinski J, Mohsen M, Hami S: Methicillin-resistant *Staphylococcus aureus* toxic shock syndrome. *Emerging Infectious Diseases.* 2005;2: 637-636.

Renz L, Thurn JD, Finn HA, Lynch JP, Moss J: Oral antihistamines reduce the side effects from rapid vancomycin *Infusion. Anesthesia and Analgesia.* 1998;87:681-685.

Reipert A, Ehlert K, Kast T, Bierbaum G: Morphological and genetic differences in two isogenic Staphylococcus aureus strains with decreased susceptibilities to vancomycin. *Antimicrobial Agents and Chemotherapy.* 2003;47:568-576.

Renzoni A, Francois P, Li D, Kelley WL, Lew DP, Vaudaux P, Schrenzel J: Modulation of fibronectin adhesions and other virulence factors in a teicoplanin-resistant derivative of methicillin-resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy.* 2004; 48:2958–2965.

Renzoni A, Barras C, Francois P, Charbonnier Y, Huggler E, Garzoni C, Kelley WL, Majcherczyk P, Schrenzel J, Lew DP, Vaudaux P: Transcriptomic and functional analysis of an autolysis-deficient, teicoplanin-resistant derivative of methicillin-resistant Staphylococcus aureus. *Antimicrobial Agents and Chemotherapy*. 2006;50:3048–3061.

Renzoni A Kelley WL, Barras C, Monod A, Huggler E, François P, Schrenzel J, Studer R, Vaudaux P, Lew DP: Identification by genomic and genetic analysis of two new genes playing a key role in intermediate glycopeptide resistance in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*. 2009;53:903–911.

Reverdy ME, Jarraud S, Bobin-Dubreux S, Bure E, Girardo P, Lina G, Vandenesch F, Etienne J: Incidence of *Staphylococcus aureus* with reduced susceptibility to glycopeptides in two French hospitals. *Clinical Microbiology and Infection.* 2001;7:267-272.

Reynolds PE, Brown DF: Penicillin-binding proteins of beta-lactam-resistant strains of *Staphylococcus aureus.* Effect of growth conditions. *Federation of European Biochemical Societies letter.* 1985;192:28-32.

Reynolds PE: Structure, biochemistry and mechanism of action of glycopeptide antibiotics. *European Journal of Clinical Microbiology and Infectious Diseases.* 1989; 8:943–950.

Rice LB: Emergence of Vancomycin-Resistant Enterococci. *Emerging Infectious Disease.* 2001;7:183-187.

Richter SS, Satola SW, Crispell EK, Heilmann KP, Dohrn CL, Riahi F, Costello AJ, Diekema DJ, Doern GV: Detection of *Staphylococcus aureus* isolates with heterogeneous intermediate-level resistance to vancomycin in the United States. *Journal of Clinical Microbiology.* 2011;49:4203-4207.

Robetrs S, Chambers S: Diagnosis and management of *Staphylococcus aureus* infections of the skin and soft tissue. *Internal Medicine Journal* 2005, 35:S97-S105.

Robert J, Bismuth R, Jarlier V: Decreased susceptibility to glycopeptides in methicillin-resistant *Staphylococcus aureus*: a 20 year study in a large French teaching hospital, 1983–2002. *Journal of Antimicrobial Chemotherapy.* 2006;57:506–510.

Rodvold KA, Blum RA, Fischer JH, Zokufa HZ, Rotschafer JC, Crossley KB, Riff LJ: Vancomycin pharmacokinetics in patients with various degrees of renal function. *Antimicrobial Agents and Chemotherapy.* 1988; 32:848-852.

Rohrer SK, Ehlert K, Tschierske M, Labischinski H, Berger-Bächi B.. The essential *Staphylococcus aureus* gene fmhB is involved in the first step of peptidoglycan pentaglycine interpeptide formation. *Proceedings of the National Academy of Sciences USA*. 1999;96:9351-9356.

Rohrer SK, Berger-Bächi B: FemABX peptidyl transferases: A link between branched-chain cell wall peptide formation and β-lactam resistance in Gram-positive cocci. *Antimicrobial Agents and Chemotherapy.* 2003; 47: 837–846.

Rose WE, Leonard SN, Sakoulas G, Kaatz GW, Zervos MJ, Sheth A, Carpenter CF, Rybak MJ: Daptomycin activity against *Staphylococcus aureus* following vancomycin exposure in an in vitro pharmacodynamic model with simulated endocardial vegetations. *Antimicrobial Agents and Chemotherapy*. 2008;52:831–836.

Rose WE, Leonard SN, Rossi KL, Kaatz GW, Rybak MJ: Impact of inoculum size and heterogeneous vancomycin-intermediate *Staphylococcus aureus* (hVISA) on vancomycin activity and emergence of VISA in an in vitro pharmacodynamic model. *Antimicrobial Agents and Chemotherapy.* 2009; 53:805-807.

Rotschafer JC, Crossley K, Zaske DE, Mead K, Sawchuk RJ, Solem LD: Pharmacokinetics of vancomycin: observations in 28 patients and dosage recommendations. *Antimicrobial Agents and Chemotherapy.* 1982;22:391–394.

Rotun SS, McMath V, Schoonmaker DJ, Maupin PS, Tenover FC, Hill BC, Ackman DM:. *Staphylococcus aureus* with reduced susceptibility to vancomycin isolated from a patient with fatal bacteremia. *Emerging Infectious Disease.* 1999;5:147-149.

Rowland J, Dyke KGH: Characterization of the staphylococcal β-lactamase transposon Tn552. *European Molecular Biology Organization Journal.* 1989; 8:2761–2773.

Rubin RJ, Harrington CA, Poon A, Dietrich K, Greene JA, and Moiduddin A: The economic impact of *Staphylococcus aureus* infection in New York City hospitals. *Emerging Infectious Disease.* 1999;5:9–17.

Rubinstein E, Lalani T, Corey GR, Kanafani ZA, Nannini EC, Rocha MG, Rahav G, Niederman MS, Kollef MH, Shorr AF, Lee PC, Lentnek AL, Luna CM, Fagon JY, Torres A, Kitt MM, Genter FC, Barriere SL, Friedland HD, Stryjewski ME: Telavancin versus vancomycin for hospital-acquired pneumonia due to gram-positive pathogens. *Clinical Infectious Diseases*. 2011;52:31-40.

Ruzin A, Severin A, Moghazeh SL, Etienne J, Bradford PA, Projan SJ, Shlaes DM: Inactivation of mprF affects vancomycin susceptibility in Staphylococcus aureus. *Biochimica et Biophysica Acta*. 2003.1621:117-121.

Ryan KJ: Staphylococci. *In: Ryan KJ, Ray CG, editors. Sherris medical microbiology an introduction to infectious diseases. 4th ed., McGraw Hill,* New York:; 2004: 261-271.

Rybak MJ, Cha R, Cheung CM, Meka VG, Kaatz GW: Clinical isolates of *Staphylococcus aureus* from 1987 and 1989 demonstrating heterogeneous resistance to vancomycin and teicoplanin. *Diagnostic Microbiology and Infectious Disease.* 2005; 51:119–125.

Rybak MJ. The pharmacokinetic and pharmacodynamic properties of vancomycin. *Clinical Infectious Diseases* . 2006a;42:S35–9.

Rybak MJ. Pharmacodynamics: Relation to antimicrobial resistance. *American Journal of Medicine* . 2006b;119:S37–44.

Rybak MJ, Leonard SN, Rossi KL, Cheung CM, Sadar HS, Jones RN: Characterization of vancomycin heteroresistant *Staphylococcus aureus* (hVISA) from the Detroit Metropolitan area over a 22-year period (1986-2007). *Journal of Clinical Microbiology.* 2008; 46:2950-2954.

Rybak M, Lomaestro B, Rotschafer JC, Moellering R, Jr, Craig W, Billeter M, Dalovisio JR, Levine DP:. Therapeutic monitoring of vancomycin in adult patients: a consensus review of the American Society of Health-System Pharmacists, the Infectious Diseases Society of America, and the Society of Infectious Diseases Pharmacists. *American Journal of Health-System Pharmacy.* 2009a;66:82–98.

Rybak MJ, Lomaestro BM, Rotschafer JC, Moellering RC, Craig WA, Billeter M, Dalovisio JR, Levine DP: Vancomycin therapeutic guidelines: a summary of consensus recommendations from the infectious diseases Society of America, the American Society of Health-System Pharmacists, and the Society of Infectious Diseases Pharmacists. *Clinical Infectious Diseases*. 2009b;49:325-327.

Sader HS, Fritsche TR, Kaniga K, Ge Y, Jones RN: Antimicrobial activity and spectrum of PPI-0903M (T-91825), a novel cephalosporin, tested against a worldwide collection of clinical strains. *Antimicrobial Agents and Chemotherapy.* 2005;49:3501–3512.

Sader HS, Fey PD, Limaye AP, Madinger N, Pankey G, Rahal J, Rybak MJ, Snydman DR, Steed LL, Waites K, Jones RN: Evaluation of vancomycin and daptomycin potency trends (MIC creep) against methicillin-resistant *Staphylococcus aureus* isolates collected in nine U.S. medical centers from 2002 to 2006. *Antimicrobial Agents and Chemotherapy*.2009;53:4127-4132.

Sakoulas G, Gold HS, Venkataraman L, DeGirolami P, Elipoulos GM, Qian Q: Methicillin-Resistant *Staphylococcus aureus*: Comparison of Susceptibility Testing Methods and Analysis of *mecA*-Positive Susceptible Strains. *Journal of Cinical Microbilogy.* 2001;39:3946-3951.

Sakoulas G, Eliopoulos GM, Moellering RC Jr, Novick RP, Venkataraman L, Wennersten C, DeGirolami PC, Schwaber MJ, Gold HS: Staphylococcus aureus accessory gene regulator (agr) group II: is there a relationship to the development of intermediate-level glycopeptide resistance?. *Journal of Infectious Disease.* 2003;187:929-938.

Sakoulas G, Moise-Border PA, Schentag JJ, Forrest A, Moellering RC Jr, Eliopoulos GM: Relationship of MIC and bactericidal activity to efficacy of vancomycin for treatment of methicillin-resistant *Staphylococcus aureus* bacteraemia. *Journal of Clinical Microbiology*. 2004;42:2398-2402.

Sakoulas G, Eliopoulos GM, Flower VG Jr, Moellering RC Jr, Novick RP, Lucindo N, Yeaman MR, Baver AS: Reduced susceptibility of *Staphylococcus aureus* to vancomycin and platelet microbicidal protein correlates with defective autolysis and loss of accessory gene regulator (*agr*) function. *Antimicrobial Agents and Chemotherapy*. 2005;49:2687–2692.

Sakoulas G, Gold HS, Cohen RA, Venkataraman L, Moellering RC, Eliopoulos GM: Effects of prolonged vancomycin administration on methicillin-resistant *Staphylococcus aureus* (MRSA) in a patient with recurrent bacteraemia. *Journal of Antimicrobial Chemotherapy*. 2006;57:699–704.

Sakoulas G, and Moellering Jr. RC: Increasing Antibiotic Resistance among Methicillin-Resistant *Staphylococcus aureus* Strains. *Clinical Infectious Diseases.* 2008;46:S360–367.

Sancak B, Ercis S, Menemenlioglu D, Colakoglu S, Hascelik G: Methicillin-resistant *Staphylococcus aureus* heterogeneously resistant to vancomycin in a Turkish university hospital. *Journal of Antimicrobial Chemotherapy.* 2005;56:519-523.

Saner FH, Heuer M, Rath P, Gensicke J, Radtke A, Dru¨he A, Ru¨ngeler E, Nadalin S, Malago M, Broelsch CE: Successful salvage therapy with tigecycline

after linezolid failure in a liver transplant recipient with mrsa pneumonia. Liver Transplantation. 2006; 12:1689-1692.

Saravolatz L, Pawlak J, Johnson L: In vitro activity of ceftaroline against community-associated methicillin-resistant, vancomycin-intermediate, vancomycin-resistant, and daptomycin-nonsusceptible *Staphylococcus aureus* isolates. *Antimicrobial Agents and Chemotherapy.* 2010;54:3027-3030.

Saravolatz LD, Pawlak J, Johnson LB: *In-Vitro* Susceptibilities and Molecular Analysis of Vancomycin-Intermediate and Vancomycin-Resistant *Staphylococcus aureus* Isolates. *Clinical Infectious Diseases*. 2012a. Advance Access number: 10.1093/cid/cis492.

Saravolatz LD, Pawlak J, Johnson L, Bonilla H, Saravolatz LD 2nd, Fakih MG, Fugelli A, Olsen WM: In-vitro Activity of LTX-109 a Synthetic Antimicrobial Peptide against MRSA, VISA, VRSA, Daptomycin-non-susceptible Staphylococcus aureus (DNSSA) and Linezolid-non-susceptible SA (LNSSA). *Antimicrobial Agents and Chemotherapy*. 2012b. Advance Access number:10.1128/AAC.00194-12.

Schaaff F, Reipert A, Bierbaum G: An elevated mutation frequency favors development of vancomycin resistance in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* . 2002;46:3540-3548.

Schalkowsky S: Preliminary user guide: Determination of antimicrobial susceptibility test by the Spiral Gradient Endpoint test. 1985; Spiral system Instrument Inc. Bethesda, MD.

Schriever CA, Fernández CA, Rodvold KA, Danziger LH: Daptomycin: A novel cyclic lipopeptide antimicrobial. *American Journal of Health-System Pharmacy*. 2005;62:1145-1158.

Schulthess B, Meier S, Homerova D, Goerke C, Wolz C, Kormanec J, Berger-Bachi B, Bischoff M: Functional characterization of the sigmaB-dependent yabJ-

spoVG operon in *Staphylococcus aureus*: role in methicillin and glycopeptide resistance. *Antimicrobial Agents and Chemotherapy*. 2009;53:1832-1839.

Scott WJ: Water relations of *Staphylococcus aureus* at 30 degrees C. *Australian Journal of Biological Sciences*. 1953;6:549-564.

Seidl K, Stucki M, Ruegg M, Goerke C, Wolz C, Harris L, Berger-Bachi B, Bischoff M: *Staphylococcus aureus* CcpA affects virulence determinant production and antibiotic resistance. *Antimicrobial Agents and Chemotherapy*. 2006;50:1183-1194.

Seybold U, Kourbatova EV, Johnson JG, Halvosa SJ, Wang YF, King MD, Ray SM, Blumberg HM: Emergence of community-associated methicilin-resistant *Staphylococcus aureus* USA300 genotype as a major cause of health care-associated blood stream infections. *Clinical Infectious Diseases.* 2006;42: 647-656.

Sheagren JN: *Staphylococcus aureus* infections of skin and skin structure. *Cutis.* 1985, 36:2-6.

Sheagren JN: *Staphylococcus aureus* infections in truma patients. *Critical Care Medicine* . 1999;27:692-693.

Sherman-Weber S, Axelrod P, Suh B, Rubin S, Beltramo D, Manacchio J, Furukawa S, Weber T, Eisen H, Samuel R. Infective endocarditis following orthotopic heart transplantation: 10 cases and a review of the literature. *Transplant Infectious Disease.* 2004; 6:165–170.

Shimada M, Kamakura T, Itasaka H, Matsumata T, Hashizume M, Sugimachi K: The significance of methicillin-resistant *Staphylococcus Aureus* infection in general surgery: A multivariate analysis of risk factors and preventive approaches. *Surgery Today*. 1999; 23:880-884.

Shoji M, Cui L, Iizuka R, Komoto A, Neoh H, Watanabe Y, Hishimuma, Hiramatsu K: walK and clpP mutations confer reduced vancomycin susceptibility

in *staphylococcus aureus. Antimicrobial Agents and Chemotherapy.* 2011;55: 8 3870-3881.

Shopsin B, Mathema B, Alcabes P, Said-Salim B, Lina G, Matsuka A, Martinez J, Kreiswirth BN: Prevalence of *agr* specificity groups among *Staphylococcus aureus* strains colonizing children and their guardians. *Journal of Clinical Microbilogy*. 2003;41:456-459.

Shore A, Rossney AS, Keane CT, Enright MC, Coleman DC: Seven novel variants of the staphylococcal chromosomal cassette mec in methicillin-resistant *Staphylococcus aureus* isolates from Ireland. *Antimicrobial Agents and Chemotherapy.* 2005;49: 2070-2083.

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

Sieradzki K., Pinho MG, Tomasz A: Inactivated *pbp4* in highly glycopeptide-resistant laboratory mutants of *Staphylococcus aureus*. *Journal of Biological Chemistry.* 1999a; 274:18942-18946.

Sieradzki K., Tomasz A: Gradual alterations in cell wall structure and metabolism in vancomycin-resistant mutants of *Staphylococcus aureus*. *Journal of Bacteriology.* 1999b; 181:7566-7570.

Sieradzki K., Tomasz A: Alterations of cell wall structure and metabolism accompany reduced susceptibility to vancomycin in an isogenic series of clinical isolates of *Staphylococcus aureus*. *Journal of Bacteriology.* 2003;185:7103-7110.

Sievert DM, Boulton ML, Stoltman G: *Staphylococcus aureus* resistant to vancomycin –United States, 2002. *Morbidity and Mortality Weekly Report*. 2002; 51:565-567.

Singh N, Paterson DL, Chang FY, Gayowski T, Squier C, Wagener MM, Marino IR: Methicillin-resistant *Staphylococcus aureus*: the other emerging resistant gram-positive coccus among liver transplant recipients. *Clinical Infectious Diseases.* 2000;30:322-327.

Singh VK, Schmidt JL, Jayaswal RK, Wilkinson BJ: Impact of sigB mutation on Staphylococcus aureus oxacillin and vancomycin resistance varies with parental background and method of assessment. *International Journal of Antimicrobial Agents.* 2003; 21:256–261.

Skhirtladze K, Hutschala D, Fleck T, Thalhammer F, EhrlichM, Vukovich T, Muller M Tschernko EM: Impaired target site penetration of vancomycin in diabetic patients following cardiac surgery. *Antimicrobial Agents and Chemotherapy* . 2006; 50:1372–1375

Skulnick M, Small GW, Lo P, Patel MP, Poter CR, Low DE, Matsumura S, Mazzulli T: Evaluation of accuracy and reproducibility of E test for susceptibility testing of *Streptococcus pneumoniae* to penicillin, ceftriaxone. *Journal of Clinical Microbiology*. 1995; 33: 2334-2337.

Smith TL, Pearson ML, Wilcox KR, Cruz C, Lancaster MV, Robinson-Dunn B, Tenover FC, Zervos MJ, Band JD, White E, Jarvis WR: Emergence of vancomycin resistance in *Staphylococcus aureus*. *New England Journal of Medicine*. 1999; 340: 493-501.

Sobral RG Ludovice Am, de Lencastre H, Tomasz A: Role of *mur*F in cell wall biosynthesis: Isolation and characterization of a *mur*F conditional mutant of *Staphylococcus aureus*. *Journal of Bacteriology.* 2006;188:2543-72523.

Sola C Lamberghini R, Ciarlantini M, Egea AL, Gonzalez P, Diaz EG, Huerta V, Gonzalez J, Corso A, Vilaro M, Petiti JP, Torres A, Vindel A, Bocco JL: Heterogeneous vancomycin-intermediate susceptibility in a community-associated methicillin-resistant *Staphylococcus aureus* epidemic clone, in a case of Infective

Endocarditis in Argentina. *Annals of Clinical Microbiology and Antimicrobials*. 2011;10:15-20.

Soriano A, Marco F, Martinez JA, Pisos E, Almela M, Dimova P, Alamo D, Ortega M, Lopez J, Mensa J: Influence of vancomycin minimum inhibitory concentration on the treatment of methicillin-resistant *Staphylococcus aureus* bacteremia. *Clinical Infectious Diseases.* 2008; 46:193-200.

Steinkraus G, White R, Friedrich L: Vancomycin MIC creep in non-vancomycin-intermediate *Staphylococcus aureus* (VISA), vancomycin-susceptible clinical methicillin-resistant S. aureus (MRSA) blood isolates from 2001-05. *Journal of Antimicrobial Chemotherapy*. 2007;60:788–794.

Stevens DL, Wallace RJ, Hamilton SM, Bryant AE: Successful treatment of staphylococcal toxic shock syndrome with linezolid: a case report and in vitro evaluation of the production of toxic shock syndrome toxin type 1 in the presence of antibiotics. *Clinical Infectious Diseases*. 2006;42:507–514.

Streit JM, Sader HS, Fritsche TR, Jones RN: Dalbavancin activity against selected populations of antimicrobial-resistant Gram-positive pathogens. *Diagnostic Microbiology and Infectious Disease* . 2005;53:307–310.

Stryjewski ME, Graham DR, Wilson SE, O'Riordan W, Young D, Lentnek A, Ross DP, Fowler VG, Hopkins A, Friedland HD, Barriere SL, Kitt MM, Corey GR: Telavancin versus vancomycin for the treatment of complicated skin and skin-structure infections caused by gram-positive organisms. *Clinical Infectious Diseases*. 2008;46:1683-1693.

Stokes EJ, Ridgway GL, Wren MW: Clinical Microbiology, *7th ed., Edward Arnold,* London. 1993.

Sun W, Chen H, Liu Y, Zhao C, Nichols WW, Chen M, Zhang J, Ma Y, Wang H: Prevalence and characterization of heterogeneous vancomycin-intermediate

Staphylococcus aureus isolates from 14 cities in China. *Antimicrobial Agents and Chemotherapy.* 2009;53:3642-3649.

Supersac G, Prevost G, Piemont Y: Sequencing of leucocidin R from *Staphylococcus aureus* P83 suggests that staphylococcal leucocidins and gamma-hemolysin are members of a single, two-component family of toxins. *Infection and Immunity.* 1993; 61:580-587

Swenson JM, Anderson KF, Lonsway DR, Thompson A, McAllister SK, Limbago BM, Carey RB, Tenover FC, Patel JB: Accuracy of commercial and reference susceptibility testing methods for detecting vancomycin-intermediate *Staphylococcus aureus*. *Journal of Clinical Microbiology.* 2009;47:2013-2017

Sztramko R, Katz K, Antoniou T, Mulvey MR, Brunetta J, Crouzat F, Kovacs C, Merkley B, Tilley D, and Loutfy MR: Community-associated methicillin-resistant *Staphylococcus aureus* infections in men who have sex with men: A case series. *Canadian Journal of Infectious Diseases & Medical Microbiology* 2007; 18: 257–261.

Taneja C, Haque N, Oster G, Shorr AF, Zilber S, Kyan PO, Reyes KC, Moore C, Spalding J, Kothari S, Zervos M: Clinical and economic outcomes in patients with community-acquired *Staphylococcus aureus* pneumonia. *Journal of Hospital Medicne* 2010;5:528–534.

Tang HJ, Chen CC, Cheng KC, Toh HS, Su BA, Chiang SR, Ko WC, Chuang YC: In vitro efficacy of fosfomycin-containing regimens against methicillin-resistant *Staphylococcus aureus* in biofilms. *The Journal of Antimicrobial Chemotherapy*. 2012;67:944-950.

Takano T, Higuchi W, Otsuka T, Baranovich T, Enany S, Saito K, Isobe H, Dohmae S, Ozaki K, Takano M, Iwao Y, Shibuya M, Okubo T, Yabe S, Shi D, Reva I, Teng LJ, Yamamoto T: Novel characteristics of community-acquired methicillin-resistant *Staphylococcus aureus* belonging to multilocus sequence type 59 in Taiwan. *Antimicrobial Agents and Chemotherapy.* 2008;52:837-845.

Takesue Y, Nakajima K, Takahashi Y, Ichiki K, Ishihara M, Tsuchida T, Uchino M, Ikeuchi H: Clinical characteristics of vancomycin minimum inhibitory concentration of 2 mg/L methicillin-resistant *Staphylococcus aureus* strains isolated from patients with bacteremia. *Journal of Infection and Chemotherapy*. 2011;17:52-57.

Tegos G, Stermitz FR, Lomovskaya O, Lewis K: Multidrug pump inhibitors uncover remarkable activity of plant antimicrobials. *Antimicrobial Agents and Chemotherapy*. 2002;46: 3133-3141.

Tenover FC, Lancaster MV, Hill BC, Steward CD, Stocker SA, Hancock GA, O'Hara CM, McAllister SK, Clark NC, Hiramatsu K: Characterization of staphylococci with reduced susceptibilities to vancomycin and other glycopeptides. *Journal of Clinical Microbiology*. 1998;36:1020-1027.

Tenover FC, Biddle JW, Lancaster MV: Increasing resistance to vancomycin and other glycopeptides in *Staphylococcus aureus*. *Emerging Infectious Disease.* 2001;7:327-332.

Thati V, Shivannavar CT, Gaddad SM: Vancomycin resistance among methicillin resistant *Staphylococcus aureus* isolates from intensive care units of tertiary care hospitals in Hyderabad. *Indian Journal of Medical Research*. 2011;134:704-708.

Trakulsomboon S, Danchaivijitr S, Rongrungruang Y, Dhiraputra C, Susaemgrat W, Ito T, Hiramatsu K: First report of methicillin-resistant *Staphylococcus aureus* with reduced susceptibility to vancomycin in Thailand. *Journal of Clinical Microbiology.* 2001;39:591–595.

Trenholme GM, Kaplan Rl, Karakusis PH, Stine T, Fuhrer J, Landau W, Levin S: Clinical impact of rapid identification and susceptibility testing of bacterial blood culture isolates. *Journal of Clinical Microbiology.* 1989; 27: 1342-1345.

Todd JK, Fishaut M, Kapral F, Welch T. Toxic shock syndrome associated with phage-group-I staphylococci. *Lancet.* 1978;2:1116-1118

Todd JK. Toxic shock syndrome. *Clinical Microbiology Reviews.* 1988;1:432-446.

Tomasz A: The staphylococcal cell wall – Gram-positive pathogens, V. A. Fischetti, R. P. Novick, J. J. Ferretti, D. A. Portnoy, and J. I. Rood (ed.), 2nd ed. ASM Press, Washington, DC. 2006;443-455

Trotonda MP, Xiong YQ, Memmi G, Bayer AS, Cheung AL: Role of mgrA and sarA in methicillin-resistant *Staphylococcus aureus* autolysis and resistance to cell wall-active antibiotics. *Journal of Infectious Diseases*.2009;199:209–218.

Turnidge JD, Nimmo GR, Francis G. Evolution of resistance in *Staphylococcus aureus* in Australian teaching hospitals. Australian Group on Antimicrobial Resistance (AGAR). *Medical Journal of Australia.* 1996; 164:68–71.

Turnidge JD, Jorgensen JH: Antimicrobial susceptibility testing: General considerations. *In Manual of clinical microbiology (7th Edition),* Murray PR, Baron EJ, Pfaller MA, Tenover, Yolken RH (ed). American Society for Microbiology, Washington DC 1999;1469-1473.

Turnidge JD, Paterson DL: Setting and revising antibacterial susceptibility breakpoints. *Clinical Microbiology Reviews* . 2007; 20: 391–408.

Udo EE, Pearman JW, Grubb WB: Genetic analysis of community isolates of methicillin-resistant *Staphylococcus aureus* in Western Australia. *Journal of Hospital Infection.* 1993;22:97-108.

Utaida S, Dunman PM, Macapagal D, Murphy E, Projan SJ, Singh VK, Jayaswal RK, Wilkinson BJ: Genome-wide transcriptional profiling of the response of *Staphylococcus aureus* to cell-wall-active antibiotics reveals a cell-wall-stress stimulon. *Microbiology*. 2003; 149:2719-2732.

US Food and Drug and Administration . Class II special controls guidance document: antimicrobial susceptibility test (AST) systems; guidance for industry and FDA. Rockville, MD*: US Food and Drug Administration; 2003*

Uttley AH, Collins CH, Naidoo J, George RC: Vancomycin-Resistant Enterococci. *Lancet.* 1988;1:57-8.

Van Belkum, Verkaik NJ, de Vogel CP, Boelens HA, Verveer J, Nouwen JL, Verbrugh HA, Wertheim1 HFL: Reclassification of *Staphylococcus aureus* Nasal Carriage Types. *Journal of Infectious Diseases*. 2009;199:1820–1826.

Van Cleef BAGL, Broens EM, Voss A, Huijsdens XW, Zuchner L, Van Benthem BHB, Kluytmans JAJW, Mulders MN, Van De Giessen AW: High prevalence of nasal MRSA carriage in slaughterhouse workers in contact with live pigs in The Netherlands. *Epidemiology and Infection.* 2010;138: 756–763.

Van Den Bogaard AE, Bruinsma N, Stobberingh EE: The effect of banning avoparcin on VRE carriage in the Netherlands. *Journal of Antimicrobial Chemotherapy.* 2000; 46: 146–148.

Van Den Broek IVF, Van Cleef BAGL, Broens EM, Van Der Wolf PJ, Van Den Broek MJM, Huijsdens XW, Kluytmans JAJW, Van De Giessen AW, Tiemersma EW: Methicillin-resistant *Staphylococcus* aureus in people living and working in pig farms. *Epidemiology and Infection.* 2008;137: 700–708.

Vandenesch F, Naimi T, Enright MC, Lina G, Nimmo GR, Heffernan H, Liassine N, Bes M, Greenland T, Reverdy ME, Etienne J: Community-acquired methicillin-resistant *Staphylococcus aureus carrying* panton-valentine leukocidin genes: Worldwide emergence. *Emerging Infectious Disease.* 2003: 5:9-17.

van Hal1 SJ, Jones M, Gosbell IB, Paterson DL: Vancomycin heteroresistance is associated with reduced mortality in st239 methicillin-resistant *Staphylococcus aureus* blood stream infections. *Public Library of Science ONE*. 2011a; 6: e21217.

van Hal1 SJ, Wehrhahn MC, Barbagiannakos, Mercer J, Chen D, Jones M, Paterson DL, Gosbell IB: Perfomrance of various testing methodologies for detection of heteroresistant vancomycin-intermediate *Staphylococcus Aureus* blood stream infections. *Journal of Clinical Microbiology.* 2011b; 49: 1489-1494.

Van Griethuysen A, Van't Veen A, Buiting A, Walsh T, Kluytmans J: High percentage of methicillin-resistant *Staphylococcus aureus* isolates with reduced susceptibility to glycopeptides in The Netherlands. *Journal of Clinical Microbiology*. 2003; 41:2487–2491.

Voss A, Mouton JW, van Elzakker EP, Hendrix RG, Goessens W, Kluytmans JA, Krabbe PF, de Neeling HJ, Sloos JH, Oztoprak N Howe RA Walsh TR: A multi-center blinded study on the efficiency of phenotypic screening methods to detect glycopeptide intermediately susceptible *Staphylococcus aureus* (GISA) and heterogeneous GISA (h-GISA). *Annals of Clinical Microbiology and Antimicrobials.* 2007;6:1-5.

Wagner, H., Bauer, R., Peigen, X., Jianming, C., Grieblinger, E., and Fischer, M: Chinese drug monographs and analysis *Rhizoma coptidis* huanglian. Vol. 5, No. 28. Verlag für Ganzheitliche Medizin: Wald. 2004.

Wald ER. Epidemiology, pathophysiology, and etiology of sinusitis. *Pediatric Infectious Disease Journal.* 1985;4(Suppl):S51-S54.

Walsh TR, Bolmstrom A, Qwarnstrom A, Ho P, Wootton M, Howe RA, MacGowan AP, Diekema D: Evaluation of current methods for detection of staphylococci with reduced susceptibility to glycopeptides. *Journal of Clinical Microbiology*. 2001;39:2439–2444.

Walsh TR, Howe R A: The prevalence and mechanisms of vancomycin resistance in *Staphylococcus aureus*. *Annual Review of Microbiology* . 2002; 56:657–675.

Walsh TR, Bolmstrom A, Qwarnstrom A, Ho P, Wootton M, Howe RE, Macgowan AP, Diekema D: Evaluation of current methods for detection of staphylococci with reduced susceptibility to glycopeptides. *Journal of Clinical Microbiology.* 2001;39:2439–2444.

Walraven CJ, North MS, Marr-Lyon L, Deming P, Sakoulas G, Mercier R: Site of infection rather than vancomycin MIC predicts vancomycin treatment failure in

methicillin-resistant *Staphylococcus aureus* bacteraemia. *Journal of Antimicrobial Chemotherapy.* 2011;66:2386–2392.

Wang G, Hindler JF, Ward KW, Bruckner DA: Increased vancomycin MICs for *Staphylococcus aureus* clinical isolates from a university hospital during a 5-year period. *Journal of Clinical Microbiology*. 2006;44:3883–3886.

Ward PD, Turner WH: Identification of staphylococcal Panton-Valentine leukocidin as a potent dermonecrotic toxin. *Infection and Immunity*. 1980;28:393–397

Wardenburg BJ, Patel RJ, and Schneewind O: Surface proteins and exotoxins are required for the pathogenesis of *Staphylococcus aureus* pneumonia. *Infection and Immunity*. 2007,75:1040–1044.

Warnock DW: Methods with antifungal drugs. *In Medical mycology: a practical approach*. *Evans EGV (ed.), IRL Press,* New York. 1989;235-251.

Watanabe Y, Cui L, Katayama Y, Kozue K, Hirakawa H: Impact of *rpoB* mutations on reduced vancomycin susceptibility in *Staphylococcus aureus*. *Journal of Clinical Microbiology.* 2011;49:2680–2684.

Weber JT. Community-associated methicillin-resistant *Staphylococcus aureus*. *Clinical Infectious Diseases* . 2005; 41:S269-S272.

Weinbern M and Struthers K: Emergence of MRSA with reduced susceptibility to teicoplanin during therapy. *Journal of Antimicrobial Chemotherapy.* 2002;50:293-307.

Wenzel RP, Perl TM: The significance of nasal carriage of *Staphylococcus aureus* and the incidence of postoperative wound infection. *Journal of Hospital Infection.* 1995, 31:13-24

Werner G, Cuny C, Schmitz FJ, Witte W: Methicillin-resistant, quinupristin-dalfopristin-resistant *Staphylococcus aureus* with reduced sensitivity to glycopeptides. *Journal of Clinical Microbiology*. 2001;39:3586–3590

Wertheim HF, van Kleef M, Vos MC, Ott A, Verbrugh HA, Fokkens W: Nose picking and nasal carriage of *Staphylococcus aureus*. *Infection Control and Hospital Epidemiology*. 2006;27:863-867.

West AH, Stock AM. Histidine kinases and response regulator proteins in two-component signaling system. *Trends in Biochemical Sciences.* 2001;26:369-376.

Wexler HM, Lavin PT, Molitoris E, Finegold SM: Statistical analysis of the effect of trial, reading and replicates on MIC determination for cefoxitin. *Journal of Antimicrobial Chemotherapy.* 1990;19:143-146.

Wexler HM, Molitoris E, Jashnian F, Finegold SM: Comparison of spiral gradient and conventional agar dilution for susceptibility testing of anaerobic bacteria. *Antimicrobial Agents and Chemotherapy.* 1991;35:1196-1202.

Wilson P, Andrews JA, Charlesworth R, Walesby R, Singer M, Farre DJ, Robbins M: Linezolid resistance in clinical isolates of *Staphylococcus aureus. Journal of Antimicrobial Chemotherapy.* 2003;51:186-188.

Wong SSY, Ho PL, Woo PCY, Yuen KY: Bacteremia caused by staphylococci with inducible vancomycin heteroresistance. *Clinical Infectious Diseases.* 1999;29:760–767.

Wood GL, Washington JA: Antibacterial susceptibility tests: dilution and disk diffusion methods; *In manual of clinical microbiology*. *Murray. P.R (ed.), ASM Press,* Washington DC: 1995; 1327-1341.

Wootton M, Howe RA, Hillman R, Walsh TR, Bennett PM, MacGowan AP: A modified population analysis profile (PAP) method to detect hetero-resistance to

vancomycin in *Staphylococcus aureus* in a UK hospital. *Journal of Antimicrobial Chemotherapy*. 2001;47:399–403.

Wootton M, MacGowan AP, Walsh TR, Howe RA: A multicenter study evaluating the current strategies for isolating *Staphylococcus aureus* strains with reduced susceptibility to glycopeptides. *Journal of Clinical Microbiology.* 2007;45:329–332.

Xu G, Dou J, Zhang L, Guo QL, Zhou CL: Inhibitory effects of baicalein on the influenza virus *in-vivo* is determined by baicalin in the serum. *Biological & Pharmaceutical Bulletin.* 2010;33:238-243.

Yamakawa J, Aminaka M, Okuzumi K, Kobayashi H, Katayama Y, Kondo S, Nakamura A, Oguri T, Hori S, Cui L, Ito T, Jin J, Kurosawa H, Kaneko K, Hiramatsu K: Heterogeneously vancomycin-intermediate *Staphylococcus aureus* (hVISA) emerged before the clinical introduction of vancomycin in Japan: a retrospective study. *Journal of Infection and Chemotherapy.* 2012;8:406-409.

Yang, Q, Zhang F, Gao SH, Sun LN, Chen WS: Determination of bioactive compounds in *Cortex phellodendri* by high-performance liquid chromatography. *Journal of AOAC International.* 2010;93:855-861.

Yang S, Bayer AS, Mishra N, Meehl M, Ledala N, Yeaman MR, Xiong YQ, Cheung AL: The *Staphylococcus aureus* two-component regulatory system, grars, senses and confers resistance to selected cationic antimicrobial peptides. *Infection and Immunity.* 2012:80;74-81.

Yu HH, Kim KJ, Cha JD, Kim HK, Lee YE, Choi NY, You YO: Antimicrobial activity of berberine alone and in combination with ampicillin or oxacillin against methicillin-resistant *Staphylococcus aureus*. *Journal of Medicinal Food*. 2005; 8: 454-461.

Yusof A, Engelhardt A, Karlsson A, Bylund L, Vidh P, Mills K, Wootton M, Walsh TR: Evaluation of a new Etest vancomycin-teicoplanin strip for detection of

glycopeptide-intermediate *Staphylococcus aureus* (GISA), in particular, heterogeneous GISA. *Journal of Clinical Microbiology.* 2008;46:3042–3047.

Zhanel GG, Rossnagel1 E, Nichol1 K, Cox L, Karlowsky JA, Zelenitsky S, Noreddin AM, Hoban1 DA: Ceftaroline pharmacodynamic activity versus community-associated and healthcare-associated methicillin-resistant *Staphylococcus aureus*, heteroresistant vancomycin-intermediate S. aureus, vancomycin-intermediate S. aureus and vancomycin-resistant S. aureus using an in vitro model. *Journal of Antimicrobial Chemotherapy*. 2011;doi: 10.1093/jac/dkr110.

Zhang K, McClure JA, Elsayed S, Louie T, Conly JM: Novel multiplex PCR assay for characterization and concomitant subtyping of staphylococcal cassette chromosome *mec* types I to V in methicillin-resistant *Staphylococcus aureus. Journal of Cinical Microbilogy.* 2005;43:5026–5033.

Zhao W, Hu1 Z, Okubo1 S, Hara Y, Shimamura1 T: Mechanism of synergy between epigallocatechin gallate and β-lactams against methicillin-resistant *Staphylococcus aureus. Antimicrobial Agents and Chemotherapy.* 2001;45:1737-1742.

Zhao C, Sun H, Wang H, Liu Y, Hu B, Yu Y, Sun Z, Chu Y, Cao B, Liao K, Lei J, Hu Z, Zhang L, Zhang X, Xu Y, Wang Z, Chen M: Antimicrobial resistance trends among 5608 clinical Gram-positive isolates in China: results from the Gram-positive cocci resistance surveillance program (2005-2010). *Diagnostic Microbiology and Infectious Disease.* 2012;73:174-181

Zinderman CE, Conner B, Malakotti MA, LaMar JE, Armstrong A, Bohnker BK: Community-acquired methicillin-resistant *Staphylococcus aureus* among military recruits. *Emerging Infectious Disease* . 2004;10:941-944

Zinn CS, Westh H, Rosdahl VT: An international multicenter study of antimicrobial resistance and typing of hospital *Staphylococcus aureus* isolates from

21 laboratories in 19 countries or states. *Microbial Drug Resistance*. 2004;10:160–168.

Zucca M, Savoia D: The post-antibiotic era: Promising developments in the therapy of infectious disease. *International Journal of Biomedical Science*. 2010;6:77–86.