LINEZOLID SUSCEPTIBILITY AND POTENTIAL RESISTANCE MECHANISMS AMONG MRSA ISOLATED FROM TWO MAJOR PUBLIC HOSPITALS IN MALAYSIA

THIRUCHELVI PULINGAM

UNIVERSITI SAINS MALAYSIA

2014

LINEZOLID SUSCEPTIBILITY AND POTENTIAL RESISTANCE MECHANISMS AMONG MRSA ISOLATED FROM TWO MAJOR PUBLIC HOSPITALS IN MALAYSIA

by

THIRUCHELVI PULINGAM

Thesis submitted in fulfilment of the

requirements for the degree of

Master of Science

August 2014

ACKNOWLEDGEMENTS

I would like to express my deepest appreciation to Dr. Toh Seok Ming, my project supervisor. Her continuous motivation, persistent support and boundless encouragement have enabled this thesis to see the light of day. Without her limitless guidance, countless obstacles that I have stumbled upon along the way would not have been removed so effortlessly. I am also grateful for all the innumerable ways she have assisted me during my years as a research student.

I would also like to thank Assoc. Prof. Dr. Pazilah Ibrahim for granting me a space in their laboratory to conduct the initial part of this study and also for her kind contribution of clinical MRSA isolates from Hospital Universiti Sains Malaysia (HUSM) in Kubang Kerian, Kelantan. Not forgetting the TB lab members: - Mr Thaigarajan Parumasivam (mononymously known as Tiger) for his assistance in microbial work and thesis coordination and Miss Adila Ibrahim for sharing her vast knowledge in handling bacteria.

I owe a debt of gratitude to my family members for their continuous love and support throughout my studies. My parents' constant motivation has been my pillar of strength. I am also grateful to Mr Jimmy Nelson, a special person, for his never ending enthusiasm and optimism for making this thesis a reality. Lastly, this work would not have been possible without the funding from Universiti Sains Malaysia's Research University Grant (1001/PFARMASI/815074) and MyMaster scholarship from the Malaysian Ministry of Education.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

µg/ml	Micro gram per milliliter
A	Adenine
C	Cytosine
CA-MRSA	Community-acquired methicillin-resistant *Staphylococcus aureus*
CLSI	Clinical and Laboratory Standards Institute
cSSSI	Complicated skin and skin structure infection
DEPC	Diethylpyrocarbonate
DFI	Diabetic foot infection
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FDA	Food and Drug Administration
G	Guanine
HA-MRSA	Hospital-acquired methicillin-resistant *Staphylococcus aureus*
HPP	Hospital Pulau Pinang
HUSM	Hospital Universiti Sains Malaysia
ICU	Intensive care unit
IDSA	Infectious Disease Society of America
MHA	Mueller-Hinton Agar
MHB	Mueller-Hinton Broth
MIC	Minimal inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSA	Mannitol Salt Agar
PBP	Penicillin-binding proteins
PBP2A	Penicillin-binding proteins 2A
PCR	Polymerase chain reaction
PTC	Peptidyl transferase center
rDNA	Ribosomal deoxyribonucleic acid

rRNA	Ribosomal ribonucleic acid
SCC	Staphylococcal cassette chromosome
SSTI	Skin and soft tissue infection
T	Thymine
TMP-SMX	Trimethoprim-sulfamethoxazole
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
U	Uridine
VISA	Intermediately vancomycin-resistant *Staphylococcus aureus*
VRE	Vancomycin-resistant enterococci
VRSA	Vancomycin-resistant *Staphylococcus aureus*

KERENTANAN DAN MEKANISME KERINTANGAN YANG MUNGKIN BAGI LINEZOLID DALAM KALANGAN MRSA YANG DIISOLAT DARI DUA HOSPITAL AWAM UTAMA DI MALAYSIA

ABSTRAK

Linezolid merupakan antibiotik pertama dari kelas struktur baru, oksazolidinon, yang telah diluluskan untuk kegunaan klinikal setelah 35 tahun. Ejen anti-mikrob ini berkesan secara meluas terhadap patogen gram-positif rintang-antibiotik yang sering menjadi punca jangkitan bakteria. Walau bagaimanapun, kewujudan ubat ini di hospital-hospital kerajaan Malaysia adalah agak rendah dan maklumat mengenai keberkesanan linezolid dalam rawatan MRSA adalah kurang memadai. Objektif kajian ini adalah untuk menyiasat sensitiviti terhadap linezolid dalam kalangan *Staphylococcus aureus* rintang-metisilin (MRSA) di Malaysia. Berasaskan 100 isolat-isolat yang dikumpulkan dari dua hospital kerajaan iaitu Hospital Universiti Sains Malaysia (HUSM) dan Hospital Pulau Pinang (HPP), didapati bahawa semua isolat MRSA klinikal adalah sensitif terhadap linezolid dengan sepenuhnya. Sensitiviti terhadap 5 antibiotik lain yang turut dikaji untuk 100 isolat-isolat tersebut. Tiga isolat dari HPP menunjukkan kadar rintangan perantaraan untuk vankomisin dengan nilai MIC 3 - 8μg/ml, 4% daripada isolat-isolat MRSA klinikal adalah rintang terhadap kloramfenikol, 20% adalah rintang terhadap klindamisin, 33% adalah rintang terhadap eritromisin dan kesemua isolat adalah rintang terhadap oksasilin (menurut definisi mikrobiologi MRSA). Akhirnya, kewujudan mekanisme molekul yang boleh menyumbang kepada kerintangan terhadap linezolid juga telah dikaji. Kaedah yang paling biasa MRSA memperoleh kerintangan terhadap linezolid iaitu penggantian bes G2576U dalam RNA 23S ribosom, adalah ternyata tidak ditemui dalam

mana-mana isolat. Pemerolehan gen kerintangan, *cfr*, yang mengkodkan enzim Cfr metiltransferase, boleh menyebabkan kerintangan terhadap linezolid. Walau bagaimanapun, kehadiran gen ini dalam komposisi genetik MRSA klinikal tidak dapat dikesan dalam analisis gel agaros dari kaedah *cfr*-PCR yang dijalankan dalam kajian ini. Kaedah penjujukan DNA telah mengenal pasti sejumlah 26 jenis penggantian dan sejenis penghapusan bes yang terdapat dalam domain V 23S rRNA yang diasingkan dari 11 isolat-isolat MRSA klinikal. Berdasarkan pada data-data yang dikumpulkan dalam kajian ini, linezolid disyorkan sebagai alternatif yang sesuai kepada vankomisin untuk rawatan jangkitan MRSA di dalam persekitaran hospital di Malaysia.

LINEZOLID SUSCEPTIBILITY AND POTENTIAL RESISTANCE MECHANISMS AMONG MRSA ISOLATED FROM TWO MAJOR PUBLIC HOSPITALS IN MALAYSIA

ABSTRACT

Linezolid is the first antibiotic of a new structural class, the oxazolidinones, to be approved for clinical use in 35 years. This antimicrobial agent is broadly effective against drug-resistant gram-positive pathogens which commonly cause infections. However, the availability of this drug in Malaysian government hospitals is relatively low and the knowledge on linezolid's efficacy in MRSA treatment is lacking. The objective of this research was to determine the susceptibility towards linezolid among methicillin-resistant *Staphylococcus aureus* (MRSA) in Malaysia. From the 100 strains collected from two government hospitals, namely Hospital Universiti Sains Malaysia (HUSM) and Hospital Pulau Pinang (HPP), it was found that all clinical MRSA isolates were fully susceptible to linezolid. The susceptibilities of 100 isolates against 5 other antibiotics were also studied. Three strains from HPP showed intermediate vancomycin resistance with MICs of 3-8µg/ml, 4% of clinical MRSA were resistant to chloramphenicol, 20% were resistant to clindamycin, 33% were resistant to erythromycin and all were resistant to oxacillin (as per microbiological definition of MRSA). Finally, the presence of molecular mechanisms conferring linezolid resistance was investigated. The most common way through which MRSA acquires resistance to linezolid, the G2576U base substitution at 23S ribosomal RNA, was noticeably absent in all isolates. Acquisition of a natural resistance gene, *cfr*, which encodes for the Cfr methyltransferase enzyme, could render

resistance to linezolid. However, the presence of this gene in the genetic makeup of clinical MRSA was not detected according to agarose gel analysis of *cfr*-PCR conducted in this study. DNA sequencing revealed a total of 26 types of base substitutions and one type of base deletion within domain V of 23S rRNA of 11 clinical MRSA isolates. Based on data accumulated in this study, linezolid is recommended as an acceptable alternative to vancomycin for MRSA infections treatment in Malaysian healthcare settings.

CHAPTER 1

INTRODUCTION

1.1 PROBLEM STATEMENT

Methicillin-resistant *Staphylococcus aureus* (MRSA) infections constitute an important and still evolving global health challenge. The highly pathogenic MRSA readily acquires resistance against most classes of antibiotics through gene conversion (mutation) or horizontal transfer of resistance genes from other bacteria. Methicillin resistance is clinically important since it renders MRSA resistant against many members of drugs within the commonly prescribed ß-lactam family of antibiotics (Grundmann, Aires-de-Sousa, Boyce, & Tiemersma, 2006). Colonized or infected MRSA-positive patients are major reservoirs for this microorganism while transitory carriage of this pathogen on the hands of healthcare workers is the most common mechanism of transmission from patient-to-patient in hospital settings (Bertrand et al., 2012).

According to a 2009 health report published by the Malaysian Ministry of Health, the prevalence of MRSA infections in Malaysia was the highest in Kuala Lumpur General Hospital (KLGH) at 28.5% and it was followed closely by Penang General Hospital (PGH) at 28% (Institute for Medical Research, 2010). It is a worrisome situation in hospitals to record a high number of MRSA incidence. MRSA are often resistant to a number of antibiotics which leads to increased morbidity and mortality in nosocomial infections (Wunderink et al., 2012).

Vancomycin has been used for MRSA treatment for the past 40 years since 1958 due to its efficacy in eradicating this 'superbug' (Wilhelm & Estes, 1999). However, in 1997, the very first vancomycin-intermediate-resistant *S. aureus* (VISA) was reported in Japan (Hiramatsu et al., 1997). The emergence of vancomycin-resistant *S. aureus* (VRSA) due to horizontal gene transfer of *vanA* which confers resistance towards high concentrations of vancomycin in vancomycin-resistant enterococci (VRE) demonstrates complete vancomycin resistance (Chang et al., 2003).

The American Food and Drug Administration (FDA) approved the first oxazolidinone antibiotic, linezolid, in April 2000 for the treatment of MRSA. Since then, this antibiotic has been used to treat a multitude of serious infections caused by MRSA with an optimum amount of success (Watkins, Lemonovich, & File, 2012). The use of linezolid to treat MRSA will eventually reduce the pressure on excessive vancomycin usage (Dennis L. Stevens et al., 2002). However, resistance towards linezolid was reported as soon as it was deployed for use in the clinical setting. The first resistant strain was isolated from the peritoneal fluid of an 85-year-old man undergoing linezolid therapy for peritonitis (Tsiodras et al., 2001). Since then, linezolid-resistant MRSA strains have been reported worldwide even though the emergence of resistance towards linezolid remains very rare (Ikeda-Dantsuji, Hanaki, Nakae, et al., 2011).

This is the first study that will report and characterise linezolid sensitivity among nosocomial MRSA strains isolated from Penang General Hospital in Pulau Pinang (PGH) and Hospital Universiti Sains Malaysia (HUSM) in Kelantan, Malaysia. Three methods to investigate linezolid resistance were

carried out. These surveillance methods will be further discussed in detail in upcoming chapters.

1.2 GENUS *STAPHYLOCOCCUS*

Staphylococcus literally means "a bunch of grapes" in the Greek language and this name was first introduced by a 19th century surgeon, Sir Alexander Ogston in 1883 when a group of micrococci was studied for causing inflammation and pus formation. The name was derived as such due to the bacteria's cocci and grape-like cluster appearance when viewed under the microscope. The genus was formally described in 1884 by Friedrich Julius Rosenbach and he further classified the genus into two separate species *Staphylococcus aureus* and *Staphylococcus albus* (now known as *Staphylococcus epidermidis*) (Jones & Niven, 1964).

As of 2009, there are more than 50 species and subspecies of this genus which have been greatly described and characterised in length. However, the most prominent member of this genus is *Staphylococcus aureus* which is a notorious human-infecting pathogen (Ng et al., 2009). Members of this genus are Gram-positive, a classification due to its significantly thick peptidoglycan layer which is a trademark of all Gram-positive prokaryotes (Cummins & Harris, 1956). Their unusual 'bunch of grapes' formation is due to incomplete binary fission which enables them to multiply in more than one axis. The approximate size of this coccus is 0.5 – 1.5µm in diameter. They are all non-motile, non-sporulate and have limited capsule forming ability or are non-encapsulated altogether. All members of this genus are catalase-positive, where they are able to convert harmful reactive oxygen species (ROS) to water and oxygen to

prevent oxidative damage. They are also facultative anaerobic microorganisms (able to conduct aerobic respiration in the presence of oxygen and also capable of fermentation using glucose), able to ferment mannitol to produce acidic by-products, have a G + C DNA composition of 33 – 39 mol% and genome size in the range of 2 to 3Mb (Baird-Parker, 1963; Evans, 1947; Götz, Bannerman, & Schleifer, 2006).

Some of the well-known members of this genus include *S. aureus, S. epidermidis, S. saprophyticus, S. haemolyticus* and *S. hominis*. These species are human pathogens however the first two species are more commonly isolated amongst clinical samples. Stapylococcal infections often result from transmission of this bacterium from an infected person to a susceptible individual who may remain asymptomatic. Both of the mentioned species recurrently exist as normal flora of the upper respiratory tract and often reside on the skin without causing any harm. Nevertheless, staphylococci may cause a variety of skin infections including boils, acne and impetigo as well as pneumonia, meningitis and osteomyelitis. Many staphylococcal diseases cause pus formation in patients and thus staphylococci are referred to as pyogenic. Even though the majority of staphylococcal carriers remain asymptomatic for most of their lifetime, serious infections may surface when immunological status of human host fluctuates due to underlying disease or aging processes (Madigan, Martinko, Paul, & Clark, 2009).

1.3 *STAPHYLOCOCCUS AUREUS*

1.3.1 Characteristics

Staphylococcus aureus is perhaps the most common causative agent of skin infections because humans serve as a natural reservoir of this bacterium. It has been reported that almost 30 - 50% of healthy adults are carriers of *S. aureus* with 10 - 20% being persistent carriers (Lowy, 1998). Colonisation with *S. aureus* increases one's risk of developing nosocomial infections and up to 30% of nosocomial infections have been reported to be due to colonisation with this microorganism (Bloemendaal et al., 2009).

S. aureus often express various cell surface-associated and extra-cellular proteins which may function as potential virulence factors. These factors enable adherence to the host cell while other factors allow the bacterial invasion through evasion of the host immunological response. Fibronectin-binding proteins, collagen-binding proteins, staphylococcal protein A and clumping-factors target components of the human extracellular matrix such as collagen, fibronectin and fibrinogen to initiate staphylococcal infections (Foster, 1996). Exoproteins are secreted by *S. aureus* to alter host tissues into nutrients that are essential for bacterial growth thus causing disease in mammalian hosts, mainly humans. This includes proteases, lipases, nucleases, hyaluronidases and collagenases which are secreted by almost all strains of *S. aureus* (Justyna Bien, Olga Sokolova, & Przemyslaw Bozko, 2011). Exotoxins such as α-hemolysin, β-hemolysin, γ-hemolysin are known to possess cytolytic activities where the secreted toxins form pores in the plasma membrane and cause cell lysis. Alpha-hemolysin has been reported to be particularly cytolytic towards human platelets and monocytes (Dinges, Orwin, & Schlievert, 2000). Pathogenesis of

staphylococcal disease is multifactorial and the disease manifests due to the simultaneous production of several virulence factors and therefore the precise role of exotoxins excreted by *S. aureus* is difficult to be determined (Bhakdi & Tranum-Jensen, 1991). However, the correlations found between strains that have been isolated from a particular disease and the increased expression of certain proteins within these strains aid in unveiling their importance in pathogenesis (Foster, 1996).

1.4 METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

1.4.1 Epidemiology

The first emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) was reported in United Kingdom hospitals in 1961, where a large sample pool derived from surrounding hospitals was tested for any occurrence of resistance towards the then new penicillinase-resistant penicillin called 'celbenin' (Jevons, 1961). 'Celbenin' was another name for methicillin produced by Beecham Research Laboratories in 1959 for battling against staphylococcal penicillinase which compromised the use of penicillin for treating infections caused by staphylococci (Çetin & Ang, 1962; Montgomery, 1962). MRSA belongs to the species of *Staphylococcus aureus* and it is the antibiotic-resistant form of the mentioned species but more pathogenic (Gordon & Lowy, 2008).

Up to date, two groups of MRSA have been identified. They comprise hospital-acquired MRSA (HA-MRSA) and community-acquired MRSA (CA-MRSA) (Enright, 2006). This study is associated with HA-MRSA since our isolates are derived from two hospitals in Malaysia. HA-MRSA is globally distributed and it is the leading cause of nosocomial infections reported

throughout the world (Maple, Hamilton-Miller, & Brumfitt, 1989). After the first emergence of HA-MRSA in 1961, this pathogen spread rapidly and successively and now creates tremendous problems in hospital settings which are associated with increased treatment cost and prolonged hospital stay (Mulligan et al., 1993).

This opportunistic pathogen usually infects individuals who have compromised immune response or chronic diseases like diabetes mellitus or breached epithelial surfaces of the body such as broken skin. MRSA infections in humans cause a great number of illnesses such as septicaemia, skin and soft tissue infections (SSTIs), pneumonia, toxic shock syndrome and endocarditis (Moran et al., 2006; Sandlin, 2008). Ailments such as necrotising fasciitis and necrotising pneumonia also evolve from SSTIs (Morgan, 2011).

Not all MRSA infections cause illness or most importantly show symptoms. It may colonize areas in the human body such as the respiratory tract, nasal cavity and urinary tract but the patient remains asymptomatic. These infected individuals are known as carriers and they are one of the reasons for MRSA dispersal in hospital settings through nursing contact (Wenzel, Nettleman, Jones, & Pfaller, 1991). Patients admitted to the intensive care unit are often at high risk of MRSA exposure due to invasive procedures carried out in the unit. Medical apparatus including intravenous catheters, open surgical wounds, excessive use of antibiotics and prolonged hospital stay clearly multiply the chances of MRSA colonization and infection (Coello, Glynn, Gaspar, Picazo, & Fereres, 1997). In some cases, enteral feedings too pose an increased risk of infection as it may serve as a route of entry for the pathogen. It may be due to the tainted nutrition solution or contaminated feed tube caused by a greater number of handlings during the administration or assembly of the food duct (Graffunder

& Venezia, 2002). This certainly emphasizes the use of aseptic intravenous tubes, hand sanitization and early catheter removal to reduce MRSA epidemics worldwide. An improvement of 12% in hand hygiene routine was predicted to have compensated for staff shortage in intensive care units and prevented MRSA transmission during the times of high workload and patient overcrowding (Grundmann, Hori, Winter, Tami, & Austin, 2002).

Previous antibiotic therapy is associated with colonisation or infection with MRSA. It was documented that 80% of nosocomial bacteraemia will be resistant towards methicillin if any one type of antibiotic was used for treatment more than once in the patient's past medical history and regardless of the antimicrobial agent, patients will be predisposed to MRSA (Lodise, Peggy S. McKinnon, & Rybak, 2003). Due to excessive usage of unnecessary antibiotics in the United States, the Society for Healthcare Epidemiology of America (SHEA) reported that this situation creates a competitive advantage for the bacteria through development of resistance. They further suggested a reduction in the use of antibiotics especially fluoroquinolones to decrease the persistent carriage of MRSA (Muto et al., 2003). It was also noted that the higher the proportion of colonised patients in an intensive care unit, the higher are the chances of contracting MRSA regardless of the unit size (Tacconelli, De Angelis, Cataldo, Pozzi, & Cauda, 2008).

Prior hospitalisation has been determined as a risk factor in acquiring MRSA infections or colonisation. All patients who were included in a prospective study at the time of admission for being MRSA positive had been previously admitted to a hospital in the previous year (Lodise et al., 2003). In another report, residents of a nursing care facility were found to be colonised with this pathogen

due to hospital admission and this factor was found to be the most important marker for positive infection (Warshawsky et al., 2000).

Patients with diabetic foot ulcers are susceptible to MRSA infection due to peripheral arterial disease and have poor penetration of antibiotics to lower limb tissues. This situation presents an excellent niche for MRSA to breach the broken skin barrier of the patient thus causing a full-fledged infection (Raymakers et al., 2001). It was noted that MRSA infection in diabetic foot ulcers was reported to occur in 18% of total patients with this disease and previous hospitalisation for the same condition and cross transmission from the hands of care-givers may have contributed to this circumstance (Hartemann-Heurtier et al., 2004).

It has been reported that MRSA transmission from colonized patients or healthcare workers to their household contacts are as high as 47% and it can be said that the spread of the infection happens in nearly half of all cases studied. One more intriguing fact is that once the infection happens in a household, nearly two-thirds of the house population will be MRSA positive carriers (Mollema et al., 2010). Besides direct contact transmission, there are cases which demonstrate that the environment of the MRSA positive person such as door knobs, bathroom sinks, light switches and remote controls may serve as the source of spreading for the household members to be positively colonized with this pathogen (Uhlemann et al., 2011).

Simple daily acts of respiratory secretions including sneezing, coughing and even kissing would definitely play a contributory action of disseminating MRSA into the environment. It is not only important to swab bacterial samples from

anterior nares alone but the throat too must be scrutinised for colony establishment (Snyder et al., 2008). The proximity between the household contact and the MRSA positive person too determines the risk of contraction, the risk being higher if linens are shared and frequent body contact is displayed (Hall, Bixler, & Haddy, 2009).

The increasing transmission of MRSA in health care settings has prompted the use of infection control gowns and gloves which are required to be worn by healthcare workers. A related study demonstrated that MRSA strains were frequently isolated from these gowns and gloves and the detection frequency was 18% but it is important to note that even after removal of these protective barriers, it was found that MRSA was acquired by these healthcare workers especially on their hands (Snyder et al., 2008).

1.4.2 ß-lactam antibiotic resistance mechanism

Wild type *S. aureus* strains have 4 penicillin-binding proteins (PBPs) which are anchored on the cytoplasmic membrane that take part in the cross-linking of peptidoglycan layer which constitutes the bacterial cell wall. These PBPs have high affinity towards ß-lactam agents which bind and halts the assembly of the bacterial cell wall leading to cell death (Palavecino, 2007). All MRSA strains which are examined so far are known to contain the *mecA* gene, the causative agent of methicillin resistance in this pathogen. This gene has been identified to encode a 78 kDa protein called penicillin-binding protein 2A (PBP2A). PBP2A has a decreased affinity towards ß-lactam antibiotics and the protein only gets activated when staphylococcal PBPs are bound to the ß-lactam antibiotics in the medium and are unable to synthesise peptidoglycan. PBP2A has been proven to

take over the synthesis of peptidoglycan when the antibiotic threshold level reaches 5µg/ml (de Jonge & Tomasz, 1993).

However, the *mecA* gene is not exclusively found in *Staphylococcus aureus* but also in another species known as *Staphylococcus sciuri* (Wu, de Lencastre, & Tomasz, 2001). This bacterium is often found as a commensal on the skin of rodents and primitive mammals. It is also described as a relatively rare microorganism to be found in humans (Couto, Wu, Tomasz, & de Lencastre, 2003). The *mecA* gene is located on a mobile genetic element called staphylococcal cassette chromosome (SCC) which is widely dispersed among staphylococci and primarily causes methicillin-resistance when acquired by a susceptible strain (Katayama, Zhang, Hong, & Chambers, 2003). This element incorporates into the *S. aureus* chromosome at a specific location called *attB scc* which can be found near the origin of replication (de Lencastre, Oliveira, & Tomasz, 2007). It is further classified into types and subtypes and it is now a customary practice to identify MRSA strains with their SCC*mec* type (Elements, 2009). Currently there are 6 types of SCC*mec* elements that have been categorised and typing of MRSA is principally done by PCR fragment analysis (Bartels et al., 2013).

1.5 MRSA TREATMENTS (Disinfectant and antibiotics)

1.5.1 Chlorhexidine

'Prevention is better than cure' is always practiced in hospitals when it comes to MRSA infection. In this case, decolonization of MRSA is entirely necessary in preventing successive infections especially to reduce the rate of MRSA infection dispersal in health care and community settings (Buehlmann et

al., 2008). A chemical agent called chlorhexidine is largely used as surface antiseptic in hospitals and mainly in intensive care units (ICU) to prevent MRSA colonization. It has been reported that in a stable 20% prevalence of MRSA in ICU wards, the usage of chlorhexidine maintained a reduction in MRSA dispersal. There is also evidence that usage of this antiseptic on MRSA carriers and all ICU patients is useful in governing MRSA based colonization which could have led to subsequent infection (Batra et al., 2010). Another report states that daily chlorhexidine-bath for ICU patients have reduced MRSA acquisition by 32%. This skin disinfection method is practiced as an improvement of barrier protection for the prevention of MRSA transmission from a carrier to a non-carrier in ICU wards (Climo et al., 2009).

1.5.2 Mupirocin

One decolonizing agent widely used in hospitals throughout the world is mupirocin, a topical antibiotic originally isolated from *Pseudomonas fluorescens* NCIMB 10586 when bacterial inhibition activity was observed towards *S. aureus* (NCTC 6571) (Fuller et al., 1971) . This antimicrobial agent has been in use since the 1980s to eradicate *S. aureus* in the nasal cavity based on the notion that *S. aureus* carriers create a higher risk of contracting MRSA infections and have higher chances of transmitting it to other patients or healthcare workers through contact (Parras et al., 1995; Rode, Hanslo, de Wet, Millar, & Cywes, 1989). Completion of mupirocin treatment has been shown to reach almost 81.5% to 100% of MRSA colony eradication and only in rarer conditions the success level was 6% due to poor patient compliance (Coates, Bax, & Coates, 2009).

1.5.3 Trimethoprim-Sulfamethoxazole (TMP-SMX)

Oral antibiotics are typically used to treat skin and soft tissue infections (SSTI) in the hospitals and where *S. aureus* is the causative agent of SSTIs including boils, carbuncles, abscesses and surgical site infections. A 33% death rate is associated with MRSA-caused SSTIs and 16% for MSSA-caused SSTIs (Wolk et al., 2009). Trimethoprim-sulfamethoxazole (TMP-SMX) is one of the common oral antibiotics often prescribed along with other antimicrobial drugs to combat MRSA causing SSTI (Cadena et al., 2011). This drug is well tolerated, offers better penetration into tissues and most importantly it is an economical and effective treatment against SSTIs caused by MRSA (Goldstein & Proctor, 2008).

1.5.4 Daptomycin (Cubicin)

Daptomycin is a cyclic lipopeptide antibiotic reported as a promising treatment against MRSA infections particularly complicated skin and skin structure infections (cSSSIs) which was approved for use in the United States since 2003. It inhibits bacteria through bactericidal activity and has a broad spectrum of activity against most Gram-positive bacteria (French, 2006; Rybak, 2006). It is often used as a second-line therapy after a glycopeptide or an oxazolidinone antibiotic. This drug is widely preferred as a prolonged treatment option in cases of endovascular or osteoarticular infections due to toxicity concerns which arise with the use of its counterparts (Gonzalez-Ruiz et al., 2011).

1.5.5 Quinupristin-Dalfopristin (Synercid)

Quinupristin and dalfopristin are streptogramin antibiotics which, used in combination, act together to bind to different sites on the large bacterial ribosomal subunit to inhibit protein synthesis synergistically. The first injectable streptogramin antibiotic, it demonstrates consistent *in vitro* activity against MRSA (Drew et al., 2000). This antibiotic has been used in cases where vancomycin therapy failed in invasive MRSA infections. However, due to side effects of myalgias and arthralgias (muscle and joint pains), its usage in MRSA treatment is limited (Saravolatz & Eliopoulos, 2003). It has also been reported that quinupristin-dalfopristin does not show superior efficacy over vancomycin or ß-lactam antibiotics in any clinical trial (Anstead, Quinones-Nazario, & Lewis, 2007).

1.5.6 Rifampin

Rifampin was first approved for tuberculosis treatment in 1971, and due to its low toxicity, its use has expanded towards staphylococcal infections especially those caused by *S. aureus* where is it used in combination with another antibiotic, which is active against staphylococcus, for better eradication (Forrest & Tamura, 2010). This drug offers potent bactericidal activity and it is able to penetrate cells and certain tissues especially when used as adjunctive treatment together with vancomycin (Deresinski, 2009). However, the role of this antibiotic as a combination therapy for MRSA has not been well established due to a shortage of clinical trials in the literature (Liu et al., 2011).

1.5.7 Telavancin (Vibativ)

Telavancin is a lipoglycopeptide with rapid bactericidal activity which functions by more than one mechanism, including inhibition of bacterial cell wall synthesis and disruption of bacterial membrane function which eventually results in bacterial death. It is also active against almost all gram-positive bacteria including MRSA (Stryjewski et al., 2008). However, it can only be administered via the parenteral route just like its glycopeptide counterpart, vancomycin. Telavancin's improved potency and bactericidal activity have prompted its approval for its use in treating complicated skin and skin structure infections (cSSSIs) especially those caused by pathogenic MRSA (Saravolatz, Stein, & Johnson, 2009).

1.5.8 Vancomycin (Vancocyn)

Vancomycin belongs to the antibiotic class of the glycopeptides and has a molecular weight of approximately 1500 Daltons. It is the drug of choice for severe infections caused by MRSA and is also the drug of choice for patients who are allergic to penicillins or cephalosporins (Wilhelm & Estes, 1999). This compound inhibits the synthesis of peptidoglycan, the major structural polymer of the bacterial cell wall where it disrupts the second stage of peptidoglycan synthesis at a site earlier than the targeted location of penicillin thus offering no chance of cross-resistance to occur (Reynolds, 1989).

Since its first use in 1958, this drug has been the drug of choice for treating gram-positive bacterial infections when all other antibiotics have failed. It was not until more than 3 decades later that the very first intermediately vancomycin-resistant *Staphylococcus aureus* (VISA) was discovered in Japan (Hiramatsu et

al., 1997). Modified cell expression of several marked genes and thickened cell wall due to genetic mutation have prevented vancomycin from reaching its target (Sievert et al., 2008). Horizontal gene transfer of *vanA* from vancomycin-resistant *Enterococci* (VRE) to MRSA has brought upon a new category of *S. aureus* strains called vancomycin-resistant *S. aureus* (VRSA) which demonstrates complete vancomycin resistance (Chang et al., 2003).

Its efficacy has come into question due to its slow bactericidal activity, poor tissue penetration and the increasing occurrence of 'MIC creep'. The latter term means there is a an observation of *S. aureus*'s MIC value experiencing a gradual increase against vancomycin treatment (Deresinski, 2007). Vancomycin has been compared to other newer antibiotics in a variety of randomised clinical trials. In a major trial which compared the efficacy of vancomycin and linezolid for bacterial eradication in cSSSIs caused by MRSA, linezolid performed better. The clinical trial found that linezolid treatment had 88.6% eradication of MRSA compared to vancomycin therapy's 66.9% (Weigelt et al., 2005).

1.5.9 Linezolid (Zyvox)

Emergence of microorganisms with reduced susceptibility towards vancomycin has necessitated the need for a new agent for our defence against MRSA (Caffrey, Quilliam, & LaPlante, 2010). Linezolid, an oxazolidinone antibiotic was first approved for use in April 2000 by the American Food and Drug Administration. This novel agent is the first in its class to prevent formation of the initiation complex (70S) by selectively binding to 23S ribosomal RNA at the peptidyl transferase center of the 50S ribosomal unit. The Clinical and Laboratory Standards Institute (CLSI) guidelines state that the

minimal inhibitory concentration (MIC) value of less than or equal to 4µg/ml is the break point for linezolid susceptibility in *Staphylococcus spp*. and most of the *Staphylococcus aureus* strains including MRSA have been found to be susceptible towards linezolid (Ikeda-Dantsuji, Hanaki, Nakae, et al., 2011).

Linezolid inhibits bacterial protein translation (Beibei et al., 2010). There is less chance for cross resistance to occur because the other protein synthesis inhibitors such as tetracyclines, aminoglycosides and macrolides often interferes in the elongation step of protein synthesis, which occurs much later in the protein synthesis process while linezolid inhibits the formation of initiation complex itself (Kloss, Xiong, Shinabarger, & Mankin, 1999). This antibiotic is bacteriostatic against *Staphylococcus spp*. and bactericidal against *Streptococci spp*. (Pankey & Sabath, 2004).

Unlike vancomycin, which has to be given only intravenously due its poor oral absorption rate and in frequently adjusted doses due to high nephrotoxicity, linezolid is 100% bioavailable in its oral form. It is available in the form of a tablet (400 and 600 mg), oral suspension (100 mg/5 ml) and a ready-to-use intravenous formulation (200 mg/100 ml and 600 mg/300 ml) (D. L. Stevens, Dotter, & Madaras-Kelly, 2004). The availability of linezolid's oral form accommodates switching of drug administration either parental or orally during the course of treatment without any changes to the drug dosage (Beringer et al., 2005; Welshman, Sisson, Jungbluth, Stalker, & Hopkins, 2001; Wunderink, Rello, Cammarata, Croos-Dabrera, & Kollef, 2003). Oral administration of linezolid compared to intravenous administration of vancomycin seems to have reduced the number of catheter-related infections. MRSA infected patients are usually switched from linezolid by intravenous route to oral administration

within 5 days of initial therapy. The usage of linezolid to treat MRSA will eventually reduce the pressure on excessive vancomycin use (Dennis L. Stevens et al., 2002).

A prospective, randomised and double blind multicentre trial comparing the efficacy of linezolid treatment in pneumonia caused by MRSA reports that the success rate for linezolid therapy was 57.6% and 46.6% for vancomycin therapy (Wunderink et al., 2012). The microbiological outcome collected from the respiratory sample in the trial showed 17% of cultures positive for MRSA in linezolid-treated patients and 46% for patients who received vancomycin. Besides that, a higher rate of nephrotoxicity was recorded for patients who received vancomycin (18.2%) than linezolid (8.4%) (Watkins et al., 2012).

In cases of SSTIs caused by MRSA, another randomised and controlled study has reported that linezolid treatment was well tolerated and the outcome was similar with vancomycin in treating the infections (Weigelt et al., 2005). Patients with proven MRSA SSTIs experienced a shorter length of hospital stay, better microbiological outcome and reduced duration of intravenous therapy when treated with linezolid compared to the patients treated with vancomycin (Itani, Biswas, Reisman, Bhattacharyya, & Baruch, 2012).

Osteomyelitis is often a tricky condition to treat due to the poor penetration of antibiotics into bone. In usual clinical practice, antibiotics are often prescribed for longer courses such as 6 to 8 weeks. However, linezolid is normally not prescribed for more than 4 weeks due its adverse effects of causing bone marrow suppression with long term usage (Liu et al., 2011). In a retrospective chart-review study which was conducted for 13 weeks where MRSA was the primary pathogen in osteomyelitis infection, a 79% cure-rate was recorded for linezolid

treatment even though 51.5% of patients reported adverse events during the treatment duration (Senneville et al., 2006).

One of the most common infections encountered by diabetic patients is diabetic foot infections (DFI). MRSA has become the most frequently occurring pathogen causing DFIs. Prevalence of this pathogen was found to be between 5% to 30% in a study conducted from year 1997 to 2007 (Eleftheriadou, Tentolouris, Argiana, Jude, & Boulton, 2010). Previous hospitalisation, excessive or inappropriate usage of antibiotics, prolonged duration of the foot wound and presence of osteomyelitis are some of the risk factors which prompts MRSA infections on the diabetic foot wounds (Liu et al., 2011). A previous randomised study reported that the clinical cure rate for linezolid-treated patients was 81% compared to 68% for patients treated with ampicillin-sulbactam or amoxicillin-clavunate in patients with infected foot ulcers. Whereas in patients without osteomyelitis, cure rate was 87% in linezolid-treated condition and 72% in patients treated with aminopenicillin/ß-lactamase inhibitors (Lipsky, Itani, Norden, & Group, 2004).

According to the clinical practice guidelines for MRSA treatment by the Infectious Disease Society of America (IDSA) published in year 2011, the usage of linezolid is recommended as an initial or alternative therapy for pneumonia, SSTIs, brain abscess, subdural empyema, spinal epidural abscess, septic arthritis, osteomyelitis, meningitis and septic thrombosis of the cavernous or dural venous sinus (Liu et al., 2011). Either linezolid or vancomycin was recommended by the American Thoracic Society and IDSA for treatment of hospital-acquired pneumonia, ventilator-associated pneumonia and healthcare-

associated pneumonia which was proven to be MRSA-infected (American Thoracic Society & Infectious Disease Society of America (IDSA), 2005).

The inclusion of linezolid as a treatment option in clinical practice guidelines proves that this drug is a valuable addition to the treatment for MRSA in the ever increasing resistance towards antibiotics. Although many linezolid-treated patients tolerate it well, caution should always be practiced by physicians where rare but serious adverse side effects of linezolid including anaemia, thrombocytopenia, peripheral neuropathy and optic neuritis could be experienced by patients in the event of over-dosage or prolonged usage (Watkins et al., 2012).

1.6 MECHANISMS OF LINEZOLID RESISTANCE

Increasing resistance towards antibiotics used to combat Gram-positive bacterial infections has prompted the need for new antibiotics which do not share the same mechanism of action as traditional antibiotics thus limiting the chances of cross resistance from occurring (D. L. Stevens et al., 2004). One such antibiotic is linezolid, the first of the oxazolidinones approved for treating MRSA infections. The synthetic nature of this antimicrobial meant that resistance was expected to occur only rarely in *Staphylococcus aureus* mainly through spontaneous mutations (Eliopoulos, Meka, & Gold, 2004).

1.6.1 Ribosomal resistance

(i) Point mutations in the peptidyl transferase center of 23S rRNA

A study conducted in year 1999, just before the approval of linezolid for clinical usage found that all laboratory-derived linezolid-resistant mutants of *Halobacterium halobium* had single point mutations in 23S ribosomal RNA

(rRNA). Seven of the mutations which originate from six different positions were localised in the central loop region of domain V in 23S rRNA, where the peptidyl transferase center (PTC) is situated, suggesting that possible linezolid mutations in clinical settings was more likely to originate from point mutations at linezolid target area in PTC (Kloss et al., 1999) (Figure 1.1).

Linezolid resistance mechanisms among Gram-negative bacteria have been investigated in *E. coli* which contained a randomly mutagenized plasmid-borne rRNA operon. Five linezolid resistant mutants were isolated with mutation G2032A found in all of the isolates. Engineered mutation of G2032A, G2032U and G2447U in the same microorganism rendered linezolid resistance at a high concentration (Xiong et al., 2000).

Mycobacterium smegmatis is a useful model for ribosome-drug interaction studies due its resemblance to other Gram-positive pathogenic bacteria. However, *M. smegmatis* with G2032C mutation have only 2-fold increase in linezolid MIC compared to 11-fold increase with the same mutation in *E. coli* (Long et al., 2010).

On the other hand, a G2447U mutation was found to confer linezolid resistance in genetically-derived *M. smegmatis* with a single functional *rrn* operon. Two classes of *M. smegmatis* mutants were isolated where one class of mutants had a uniform G2447U mutation. Changes associated with ribosomes were indicated when these class I mutants displayed high level of linezolid resistance *in vitro* for oxazolidinone assays (Sander et al., 2002). Class II mutants are described in section 1.6.2.

In summary, all the linezolid-resistant strains with their respective mutations localised to the peptidyl transferase center are shown in Figure 1.1. Apparently,

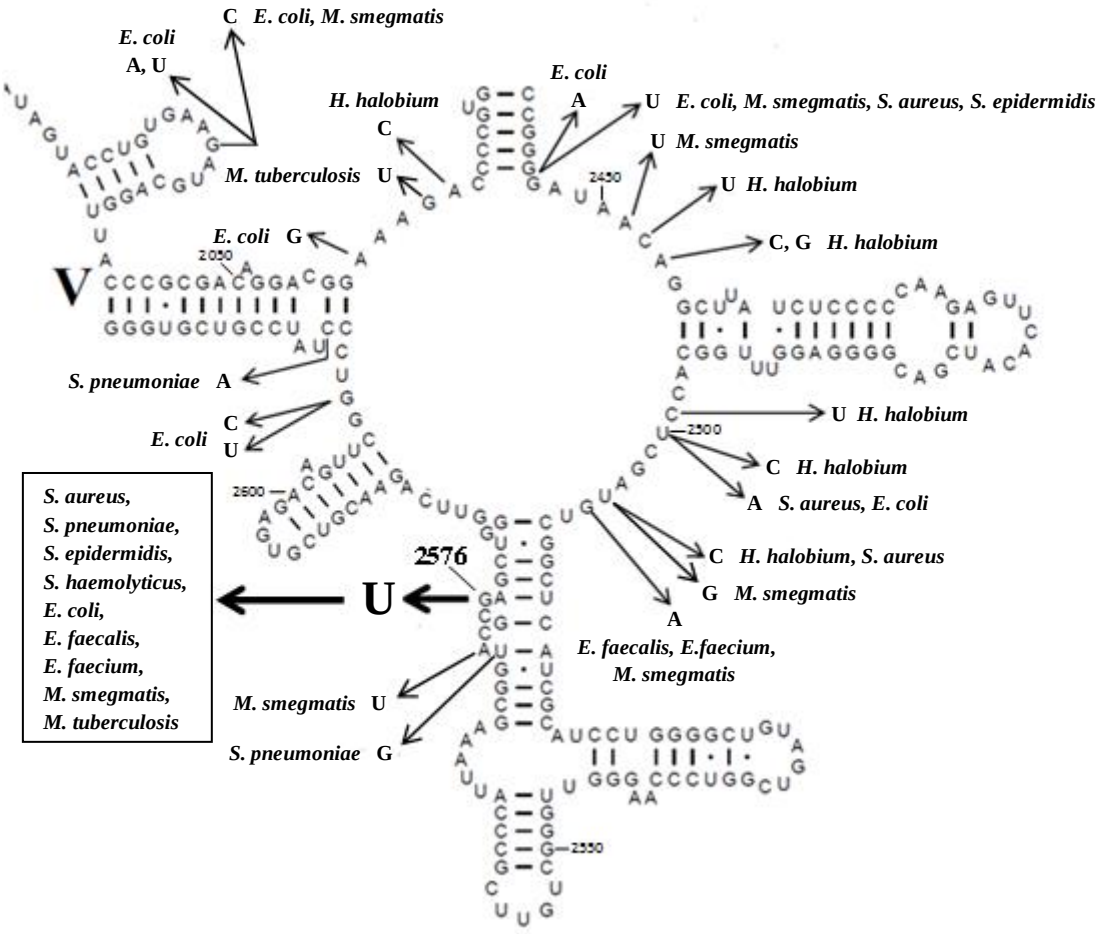

Figure 1.1: Linezolid mutations found in the central loop of domain V in 23S ribosomal RNA according to bacterial species. The most common G2576U mutation is shown with a bold arrow.

these linezolid-resistant strains express various types of mutations that may or may not be specific to each of their species alone in contrast to G2576U mutation which uniformly causes linezolid resistance in many bacterial species as shown in Figure 1.1.

The very first case of linezolid resistant MRSA in the clinical settings was reported in the year 2001 in an 85-year-old patient suffering from dialysis-related peritonitis in United States of America. It was discovered that the resistant MRSA strain isolated from the peritoneal fluid exhibited a G to U mutation in position 2576. All three replicates of the original linezolid-resistant-MRSA isolate showed G2576U mutation in the central loop of domain V in 23S rRNA. This spontaneous mutation was believed to emerge from selective pressure of linezolid therapy in the patient (Tsiodras et al., 2001).

The first linezolid resistant MRSA strain in the United Kingdom was reported in 2003, where the patient underwent linezolid therapy for thoracotomy and drainage of right-sided empyema. This was the second case of linezolid resistance in clinical settings worldwide. After 21 days of antimicrobial drug treatment, resistant MRSA strain was isolated from a wound swab of the drain site and empyema fluid. Again, the G2576U mutation in the central loop of domain V in 23S rRNA was detected as in the first case and was also shown to be the reason for resistance in this clinical isolate (Wilson et al., 2003).

As shown in laboratory as well as clinically-linezolid resistant strains, the G2576U mutation is the most common mechanism by which MRSA acquires resistance towards linezolid. However, it has been demonstrated *in vitro* that the frequency of acquiring linezolid resistance mutations is generally very low, i.e. $< 10^{-9}$ (Ikeda-Dantsuji, Hanaki, Sakai, et al., 2011).

The reason for the rarity of acquiring linezolid resistance via G2576U mutation is that *Staphylococcus aureus* has five or six copies of the ribosomal RNA (*rrn*) operon (Klappenbach, Saxman, Cole, & Schmidt, 2001). This mutation is generally dose-dependent, where more than one *rrn* operon copy is needed to be mutated for the bacteria to confer linezolid resistance. Therefore, the more number of *rrn* copies are mutated with this mutation (G2576U), the more resistant the bacteria towards linezolid.

Another interesting finding reported that despite 60 passages in antibiotic-free medium to eliminate G2576U mutation over a 75-day period, the tested linezolid-resistant MRSA isolate, maintained a single copy of mutant 23S rRNA (Meka et al., 2004). An existing single copy of mutant 23S rRNA would not yield elevated MIC for linezolid detectable by standard laboratory susceptibility testing. A case study of G2576U mutation reported that there are possibilities of homologous recombination of mutated and non-mutated copies of 23S rRNA of Enterococci sp. to survive under selective antibiotic pressure especially in hospitals (Marshall, Donskey, Hutton-Thomas, Salata, & Rice, 2002). Therefore, linezolid therapy for this type of clinical strains in hospital settings would quickly be unsuccessful as gene conversion of G2576U will occur via homologous recombination for survival of strain.

Besides *S. aureus*, *Enterococcus faecalis* and *Enterococcus faecium* have been found to utilise the same G2576U mutation to combat linezolid therapy in an Austrian hospital. These enterococcal isolates also expressed cross resistance towards another experimental oxazolidinone AZD2563, which is still in clinical trials, signifying that oxazolidinone resistance might be a class effect of this mutation (Johnson et al., 2002). AZD2563 is still in phase II clinical trials and