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MOLECULAR CHARACTERISATION OF METHICILLIN-RESISTANT *Staphylococcus aureus* STRAINS

PHUTI EDWARD MAKGOTLHO

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MOLECULAR CHARACTERISATION OF METHICILLIN-RESISTANT *Staphylococcus aureus* STRAINS

by

PHUTI EDWARD MAKGOTLHO

Submitted in partial fulfilment of the requirements for the degree

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University of Pretoria
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I, the undersigned, declare that the dissertation hereby submitted to the University of Pretoria for the degree MSc (Medical Microbiology) and the work contained therein is my own original work and has not previously, in its entirety or in part, been submitted to any university for a degree.

Signed:________________________ this ___________day of___________________2009

"As an adolescent I aspired to lasting fame, I craved factual certainty, and I thirsted for a meaningful vision of human life -- so I became a scientist. This is like becoming an archbishop so you can meet girls."

Anonymous

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LIST OF ABBREVIATIONS

AMP	Adenosine monophosphate
bp	Base pairs
BT	Bacteriophage
CA-MRSA	Community-associated methicillin-resistant *Staphylococcus aureus*
CDC	Center for Disease Control and Prevention
CLSI	Clinical and Laboratory Standards Institute
CNS	Coagulase-negative *Staphylococcus*
DNA	Deoxyribose nucleic acid
EDTA	Ethylene diamine tetraacetate
ET	Epidermolytic toxin
FnBP	Fibronectin-binding protein
h	Hour
hrs	Hours
HA-MRSA	Health-care associated methicillin-resistant *Staphylococcus aureus*
HIV	Human immunodeficiency virus
HVR	Hyper-variable region (*S. aureus* specific)
ICAM	Intercellular adhesion molecules
IgG	Immunoglobulin G
kDa	Kilodalton
kb	Kilobases
MH	Mueller-Hinton medium
min	minutes
MIC	Minimum inhibitory concentration
µl	Microlitre
MLEE	Multi-locus enzyme electrophoresis
M-PCR	Multiplex polymerase chain reaction
MSCRAMM	Microbial surface components recognising the adhesive matrix molecules
MSSA	Methicillin-susceptible *Staphylococcus aureus*
MRSA	Methicillin-resistant *Staphylococcus aureus*
NaCl	Sodium chloride
NAG	N-acetylglucosamine
NAM	N-acetylmuramic acid
NCCLS	National Committee for Clinical Laboratory Standards
PBP2a	Penicillin-binding protein 2a
PCR	Polymerase chain reaction

PFGE	Pulsed-field gel electrophoresis
PNSG	Poly-1- 6 β-$_D$-N-succinyl glucosamine
PVL	Panton-Valentine leukocidin
RAPD	Random amplified polymorphic DNA
s	seconds
SCC*mec*	Staphylococcal cassette chromosome
Spa	Staphylococcal Protein A
SSSS	Staphylococcal scalded skin syndrome
TNF-α	Tumor necrosis factor alpha
TSS	Toxic shock syndrome
UK	United Kingdom
US	United States
UPMGA	Unweighted pair group method with rrithmetic mean
VCAM	Vascular-cell adhesion molecules
VISA	Vancomycin-intermediate resistant *Staphylococcus aureus*
VRSA	Vancomycin-resistant *Staphylococcus aureus*

LIST OF ARTICLES SUBMITTED FOR PUBLICATIONS AND CONFERENCE CONTRIBUTIONS

PUBLICATIONS

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2. Makgotlho PE, Kock MM, Omar S, Dove MG, Hoosen AA and Ehlers MM (2009). Antibiotic susceptibility profiles of methicillin-resistant *Staphylococcus aureus* isolates from Steve Biko Academic Hospital. To be submitted for publication to: *South African Journal of Epidemiology and Hospital Infection.*

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1. Makgotlho PE, Kock MM, Dove MG and Ehlers MM (2007). Rapid DNA extraction method for the detection and identification of methicillin-resistant *Staphylococcus aureus*. University of Pretoria, Faculty of Health Sciences day, 20 August 2007. Poster presentation.
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4. Makgotlho PE, Kock MM, Omar S, Lekalakala R, Dove MG and Ehlers MM (2008). SCC*mec* type and subtype characterisation of methicillin resistant *Staphylococcus aureus*

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by

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SUMMARY

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a pandemic human pathogen accounting for most of health-care associated infections throughout the world. However, in recent years, a more virulent strain of MRSA has emerged in the community defined as community-associated MRSA (CA-MRSA). These emerging strains of CA-MRSA are described to have different antibiotic susceptibility profiles, possess the SCC*mec* type IV element and usually produce the Panton-Valentine leukocidin (PVL) toxin. The majority of these CA-MRSA strains are associated with skin and soft tissue infections and necrotising pneumonia, with a 34% mortality rate.

Identification and characterisation of MRSA isolates is mainly performed using phenotypic methods, which are time consuming. Little information exists on the prevalence and characteristics of MRSA isolates including antibiotic susceptibility patterns, PVL-producing CA-MRSA strains, the SCC*mec* types and genotypes that might be circulating in the Steve Biko

Academic Hospital. Identification and characterisation of MRSA isolates based on these criteria are important in controlling possible outbreaks in the clinical setting.

In this study, 97 clinical MRSA isolates from the Steve Biko Academic Hospital, South Africa were collected between April 2006 to February 2007. These isolates were analysed and characterised using multiplex PCR (M-PCR), real-time PCR as well as staphylococcal protein A (*spa*) and hyper-variable region (HVR) typing. The aim of this study was to determine the antibiotic profiles, prevalence of MRSA isolates, the SCC*mec* types and the genotypes.

Antibiotic susceptibility determination was performed using the disk diffusion susceptibility method as guidelined by the CLSI. Six distinct antibiotypes were identified with a total of 73%, 71%, 70% and 7% of MRSA isolates resistant to clindamycin, erythromycin, gentamicin and fusidic acid, respectively.

The presence of *Staphylococcus aureus* specific 16S rRNA, the *mecA* and PVL genes was determined using a modified M-PCR assay. A total of 4% of the MRSA isolates possessed the PVL gene. Real-time PCR analysis also showed a 100% prevalence of the PVL gene in the same 4% MRSA isolates confirming the results of the first M-PCR assay. The second M-PCR was used to determine the SCC*mec* type prevalence and to distinguish between health-care associated MRSA (HA-MRSA) and CA-MRSA. SCC*mec* typing showed 67% of the isolates belonged to SCC*mec* type II and 14.4% SCC*mec* type III, both types belonging to HA-MRSA. A total of 4% of the MRSA isolates were CA-MRSA belonging to SCC*mec* type IVd. Genotyping results showed three distinct *spa* clusters whilst HVR showed six distinct clusters.

Molecular-based assays proved to be useful tools to determine the prevalence and monitoring of MRSA outbreaks as well as to identify the SCC*mec* types, subtypes and genotypes of MRSA strains that might be circulating in the hospital. The determination of the different antibiotypes of MRSA can assist in the monitoring of the antibiotic resistant profile trends in the Steve Biko Academic Hospital, thus assisting with the correct implementation of antibiotic regimens for suspected MRSA infections. In an endeavour to assess the dissemination of MRSA strains

especially PVL expressing CA-MRSA strains, it is of paramount importance to continuously monitor the emergence of these strains in clinical settings.

CHAPTER 1

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a bacterium that belongs to the family of *Staphylococcaceae* (http://www.bacterio.cict.fr/allnamessz.html). The bacteria form part of the normal flora of the skin, intestine, upper respiratory tract and vagina (Lowy, 1998). *Staphylococcus aureus* can become pathogenic when conditions such as pH, temperature and nutrient availability are altered and become favourable for overgrowth (Mims *et al*., 2004). The pathogenicity of *S. aureus* is determined by the production of toxins, such as the 33-kd protein-alpha toxin, exfoliatin A, exfoliatin B and Panton-Valentine leukocidin (PVL) toxins (Lowy, 1998). These toxins can be harmful to the host and cause skin diseases (carbuncles, boils, folliculitis and impetigo) and other complications, such as endocarditis, meningitis as well as toxic shock syndrome (TSS) (Mims *et al*., 2004).

Since 1959, treatment of *S. aureus* infections included semi-synthetic penicillin drugs, such as methicillin (Livermore, 2000). However, in the 1960's the rise of methicillin-resistant *S. aureus* (MRSA) strains was apparent (Jevons, 1961). Due to the increase of MRSA strains every decade, these bacteria were identified in the early 1980's as a major cause of nosocomial infections (Boyce *et al*., 2004). The possibility of transmission of health-care associated MRSA (HA-MRSA) to the community was unavoidable. Since 1987, MRSA was increasingly found in the community (community associated- methicillin-resistant *S. aureus*) (CA-MRSA) presenting with severe skin and soft tissue infections and necrotising pneumonia (Hayani *et al*., 2008).

Health-care associated methicillin-resistant *S. aureus* consists of SCC*mec* types I-III, while CA-MRSA consists of type IV and V (Deresinski, 2005; Popovich and Weinstein, 2009). Staphylococcal cassette chromosome *mec* type IV differs from the other types because of its small size and absence of non-beta-lactam (clindamycin, tetracyclines and trimethoprim-sulfamethoxazole) genetic resistance determinants (File, 2008). Therefore, SCC*mec* type IV is susceptible to a broader array of antibiotics (File, 2008).

Community-associated MRSA is more virulent than typical HA-MRSA, due to the frequent production of Panton-Valentine leukocidin (PVL) toxin (Wannet *et al.,* 2005). Panton-Valentine leukocidin toxin is associated with deep skin infection, soft tissue infection and necrotising pneumonia (Lina *et al*., 1999). Panton-Valentine leukocidin toxin has been identified as a genetic marker for CA-MRSA (Vandenesch *et al*., 2003). Current diagnostic or phenotypic based methods of identifying the MRSA strains are time consuming and labour intensive (Reischl *et al*., 2000). These identification methods do not distinguish between the SCC*mec* element types and subtypes and can therefore not differentiate between the various HA-MRSA and CA-MRSA strains (Zhang *et al*., 2005).

The aim of this study was to determine the prevalence and to characterise HA-MRSA and CA-MRSA isolates obtained from clinical specimens. The use of molecular techniques were evaluated and compared to phenotypic methods to determine the diagnostic potential of these assays for the rapid identification and characterisation of MRSA isolates obtained from the Steve Biko Academic Hospital.

The objectives of this study were:

1. To determine the morphological and antibiotic resistant profiles of *S. aureus* isolates
2. To evaluate and optimise DNA extraction methods for *S. aureus* from pure culture
3. To evaluate and optimise M-PCR assays for the characterisation of MRSA isolates
4. To evaluate and optimise a real-time PCR assay for the detection of PVL from typed and subtyped *S. aureus* isolates
5. Data analysis

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

First described in the 1880s by Sir Alexander Ogston, a Scottish surgeon, staphylococci infections have progressively increased in hospitals and communities (Van Belkum *et al*., 2009) *Staphylococcus aureus* (*S. aureus*) causes infections in almost every organ and tissue of the human body (Lowy, 1998). The most commonly affected part of the body due to *S. aureus* infection is the skin (Lowy, 1998; Daum, 2007). More serious infections associated with *S. aureus* infections include endocarditis, mastitis, meningitis, osteomyelitis, phlebitis (inflammation of veins) and pneumonia (Lowy, 1998; Bhatia and Zahoor, 2007). *Staphylococcus aureus* has also been implicated in a number of acute food poisoning outbreaks worldwide due to the production of the heat-stable enterotoxin B that is pre-produced in food by the bacterium (Le Loir *et al*., 2003). Various other diseases can be linked to *S. aureus* specific toxins including staphylococcal scalded skin syndrome (SSSS) and toxic shock syndrome (TSS) (Salyers and Whitt, 2002).

Other species such as *Staphylococcus epidermidis* causes infections associated with indwelling medical devices (Vadyvaloo and Otto, 2008). *Staphylococcus saprophyticus* causes urinary tract infections commonly associated with young girls (Horowitz and Cohen, 2007). While *Staphylococcus lugdunensis, Staphylococcus haemolyticus, Staphylococcus warneri, Staphylococcus schleiferi* and *S intermedius* are infrequently associated with pathogenesis in health-care settings (Kloos and Bannerman, 1999). Amongst the staphylococcal species, coagulase-positive *Staphylococcus aureus* and coagulase-negative (CNS) *Staphylococcus epidermidis* (*S. epidermidis*) are of clinical importance (Waldvogel, 2000). *Staphylococcus aureus* is a coloniser of the nasal passages, causing skin infections, which range from boils, furuncles, impetigo and sties to more serious complications such as endocarditis, scalded skin syndrome, surgical-wound infections and toxic shock syndrome (Prescott, 2002). The transmission of *S. aureus* in hospitals is often a result of exposure of patients to health-care

workers who are *S. aureus* carriers or from infected patients (Lowy, 1998). *Staphylococcus aureus* and *S. epidermidis* are both important causes of nosocomial infections (Ziebuhr, 2001), with CNS accounting for 50% of catheter related infections worldwide (Murray *et al*., 2005).

Staphylococcus aureus is a significant pathogen because of the extracellular virulence factors that facilitate pathogenesis and colonisation of the host (Greenwood *et al*., 2002). Treatment of *S. aureus* has become difficult due to the ability of the bacterium to rapidly develop multi-drug resistance (Lowy, 1998). Initial treatment of *S. aureus* infections in the 1940s involved a beta-lactam antibiotic, penicillin (Geddes, 2008). However, by the end of the 1940s, 50% of *S. aureus* strains were resistant to penicillin in the USA (Lowy, 1998). In 2002, 90% of *S. aureus* strains isolates found in hospitals worldwide were resistant to penicillin (Greenwood *et al*., 2002).

Methicillin, a semisynthetic penicillin, was introduced in 1960 as an alternative to penicillin therapy for the treatment of *S. aureus* infection (Chambers, 2001). However, the identification of methicillin resistant *S. aureus* (MRSA) strains were reported in 1961, within a year after its introduction as an antistaphylococcal drug (Lowy, 2003). Methicillin resistant *S. aureus* strains were initially prevalent in hospitals before 1980; however, the spread of the resistant strains to the community followed soon (File, 2008). Between 1993 and 2003, novel strains of MRSA that were phenotypically and genotypically distinct from the parent health-care associated MRSA (HA-MRSA) were identified in the community suggesting evolution of the original MRSA (Naimi *et al*., 2003). These strains of MRSA became known as community-associated methicillin resistant *Staphylococcus aureus* (CA-MRSA) (Center for Disease Control (CDC), 2003a; Naimi *et al*., 2003). In Texas, USA, a 62% increase in CA-MRSA infections was reported in children between 2001 to 2003 (Heymann *et al*., 2005). In 2005, a study conducted in Texas, USA, reported more than 70% prevalence of CA-MRSA in 1 562 MRSA infections (Kaplan *et al*., 2005). Recently, CA-MRSA strains have been reported to cause infections in health-care facilities demonstrating the ecological fitness and emergence of these strains in different clinical settings (Popovich *et al*., 2008). The dissemination of these CA-MRSA strains in both the health-care facilities and communities has become a health-care concern worldwide (Ribeiro *et al.,* 2007). Due to this dissemination, monitoring of both HA-MRSA and CA-MRSA

in hospitals are essential in order to implement adequate and efficient infection control measures to prevent potential outbreaks of these strains.

The purpose of this study was to investigate 97 MRSA isolates collected from April 2006 to September 2007 according to their specific antibiotic susceptibility profiles, the prevalence of the *mecA* gene, the PVL producing CA-MRSA strains, the SCC*mec* types as well as the different genotypes. The results from this study gave us an indication of the specific characteristics and clinical importance of the MRSA strains that were circulating in the Steve Biko Academic Hospital.

2.2 Classification of *S. aureus*

Staphylococcus aureus is a bacterium, which belongs to the family *Staphylococcaceae* and the genus *Staphylococcus* (Table 2.1) (http://www.textbookofbacteriology.com). The genus *Staphylococcus* is Gram-positive bacteria that comprises of 41 known species and subspecies that are indigenous to humans (http://www.bacterio.cict.fr/allnamessz.html). These Gram-positive bacteria can grow under both aerobic and facultative anaerobic conditions and form grape-like staphylococci clusters on solid media (Lowy, 1998).

Table 2.1: Summary of the classification of *Staphylococcus aureus* (http://www.textbookofbacteriology.com)

Domain	*Bacteria*
Kingdom	***Eubacteria***
Phylum	***Firmicutes***
Class	***Bacilli***
Order	***Bacillales***
Family	***Staphylococcaceae***
Genus	**Staphylococcus**
Species (cause of human disease)	***S. aureus*** ***S. epidermidis*** ***S. saprophyticus*** ***S. haemolyticus*** ***S. lugdunensis***

Amongst the 41 species, only five are common in causing human disease such as *S. aureus*, *S. epidermidis*, *S. saprophyticus*, *S. haemolyticus and S. lugdunensis* (Trülzsch *et al*., 2007). *Staphylococcus aureus* is the most virulent species of the staphylococci (Murray *et al*., 2005). Other staphylococci can be human colonisers but rarely cause disease (Murray *et al*., 2005). In a 2000 study by Trülzsch and colleagues (2007), a novel coagulase negative *Staphylococcus* species, *Staphylococcus pettenkoferi*, isolated from blood specimens in Belgium and Germany were reported and proposed.

2.3 Morphology and characteristics of *S. aureus*

Staphylococcus aureus is a coagulase-positive, facultative anaerobic bacterium and can be microscopically characterised as single, pairs or clusters of Gram-positive cocci (Deresinski, 2005). *Staphylococcus aureus* is a non-motile, non-sporing and catalase positive bacterium, which can be differentiated from streptococci and other Gram-positive bacteria due to the production of catalase (Kloos and Schleifer, 1986). *Staphylococcus aureus* bacteria ferment glucose to produce lactic acid (Waldvogel, 2000). The cocci commonly form irregular clusters with a grape like appearance under the microscope (Figure 2.1) (http://www.life.umd.edu/CBMG /faculty/asmith/Staphylococcus.jpg; Todar, 2005). However, *S. aureus* cocci can appear as single

cells, in pairs or short chains (Waldvogel, 2000). The individual coccus size is approximately 0.5 to 1.5 μm in diameter (Wilkinson, 1983).

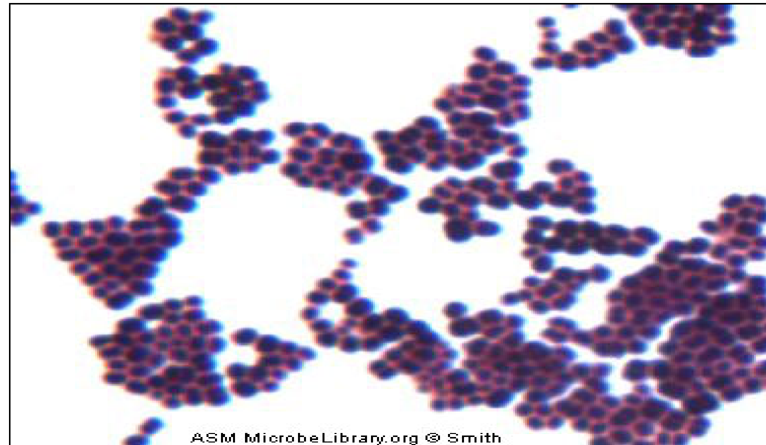

Figure 2.1: Gram-positive *S. aureus* cocci in clusters and short chains (http://www.life.umd.edu/CBMG/faculty/asmith/Staphylococcus.jpg)

Macroscopically, *S. aureus* is a facultative anaerobic bacterium, which grows rapidly on blood agar and non-selective solid media including nutrient agar under both aerobic and anaerobic conditions (Yu and Washington, 1985). Colonies appear smooth, convex and sharply defined on blood agar plates when grown at room temperature (20°C to 25°C) (Lowy, 1998). The colonies are gold pigmented due to carotenoids but this may not be apparent under certain conditions, such as anaerobic conditions or in liquid medium (Waldvogel, 2000). *Staphylococcus aureus* usually produces beta-haemolysis on horse, human or sheep blood agar plates (Figure 2.2), whereas *S. epidermidis* is non-haemolytic on blood agar plates when grown at 37°C (Todar, 2005).

Figure 2.2: ***Staphylococcus aureus*** **on a blood agar plate.** ***Staphylococcus aureus*** **colonies appear smooth, convex, cream white and haemolytic on blood agar (http://aapredbook.aappublications.org/content/images/large/2006/1/123_102. jpeg)**

Staphylococcus aureus produces coagulase whereas *S. epidermidis* strains do not produce this enzyme (Waldvogel, 2000). Coagulase is a surface enzyme that binds a blood protein, prothrombin, which is part of the coagulation cascade (Waldvogel, 2000). The binding of prothrombin causes blood to coagulate (Todar, 2005). Coagulation of blood is often used in the microbiology laboratories to differentiate between *S. aureus* and CNS strains (Brown *et al*., 2005).

2.4 Epidemiology of *S. aureus* infections

In healthy individuals, the carrier rate of *S. aureus* range between 15% to 35% with a risk of 38% of individuals developing infection followed by a further 3% risk of infection when colonised with methicillin-susceptible *S. aureus* (MSSA) (File, 2008). Certain groups of individuals are more susceptible to *S. aureus* colonisation than others including health-care workers, nursing home inhabitants, prison inmates, military recruits and children (CDC, 2003a; Kampf *et al*., 2003; Zinderman *et al*., 2004; Bogdanovich *et al*., 2007; Cardoso *et al*., 2007; Chen *et al*., 2007; Ben-David *et al*., 2008; Ho *et al*., 2008)

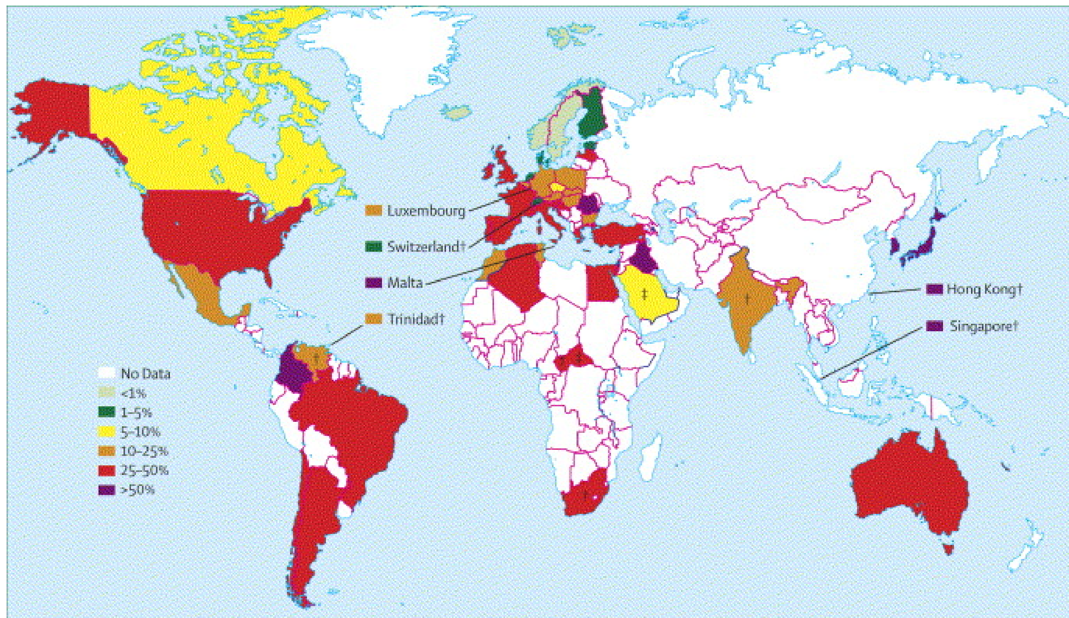

Figure 2.3: Global prevalence of MRSA (Grundmann *et al*., 2006)

In a review study, conducted in 2007 by the University of the Witwatersrand and the University Hospital of Geneva, health-care workers accounted for 93% of personnel to patient transmission of MRSA (Albrich and Harbath, 2008). Previously several outbreaks have been reported in Northern-Taiwan in 1997 that suggested MRSA transmission associated with health-care workers, including surgeons (Wang *et al*., 2001). Grundmann and colleagues (2006), reported a prevalence of > 50% in countries such as Singapore (1993-1997), Japan (1999-2000) and Colombia (2001-2002) while countries with a prevalence of 25% to 50% included South Africa (1993-1997), Brazil (2001), Australia (2003), Mexico and the United States (Grundmann *et al*., 2006). The lowest prevalence of less than 1% were found in Norway, Sweden and Iceland (1993-1997) (Grundmann *et al*., 2006). In 2007, a prevalence of more than 50% of MRSA strains isolated from Cyprus, Egypt, Jordan and Malta was reported by Borg and colleagues (2007). This high prevalence was attributed to overcrowding and poor hand-hygiene facilities in the hospitals (Borg *et al*., 2007).

2.5 Pathogenesis and virulence of *S. aureus* infections

It has been documented that there is probably no other bacterium that produces as many cellular components, enzymes, extracellular toxins and haemolysins as *S. aureus* (Todar, 2005). The cell wall of *S. aureus* is composed of a thick peptidoglycan layer, which contributes to the virulence of the bacterium (Lowy, 1998). The peptidoglycan stimulates the production of cytokines by macrophages resulting in complement system activation and platelet aggregation (Lowy, 1998). *Staphylococcus aureus* produces microcapsules including serotype 5, which is predominantly found in MRSA strains (Lowy, 1998).

Infections caused by *S. aureus* can occur in two stages: (i) *S. aureus* cells enter the body through damaged endovascular points of the host where platelet-fibrin-thrombi complex have formed and attach via microbial surface components that recognise adhesive matrix molecules (MSCRAMM) mediated mechanisms and (ii) the bacterial cells may attach to endothelial cells *via* adhesion-receptor interactions or by bridging ligands, including serum components such as fibrinogen (Todar, 2005). Upon entry into the host tissue, immune cells phagocytose *S. aureus* cells, which promotes the production of proteolytic enzymes and toxins (Table 2.2) that facilitate the spread to adjoining tissues and the release of the staphylococci into the bloodstream resulting in bacteraemia (Timbury *et al*., 2002). The infected endothelial cells produce tissue necrosis factor as part of the immune response to infection, which results in necrosis and abscess formation (Timbury *et al*., 2002).

Different strains of *S. aureus* produce different virulence factors (Table 2.2), which result in their ability to multiply and spread across adjacent tissue (Timbury *et al*., 2002). The virulence factors of *S. aureus* strains can be structured into various classes defined by their cellular location and their function (Todar, 2005). The extracellular components, MSCRAMMs, are surface proteins that bind to the extracellular matrix proteins in the pathogenesis of *S. aureus* (Projan and Novick, 1997). The microbial surface components are particularly important in clinical settings, since these molecules adhere to intravenous catheters, which are rapidly coated with serum constituents, such as fibrinogen (Lowy, 1998). Some of the best studied MSCRAMMs and other surface adhesins include: coagulase, collagen-binding protein, clumping factor, fibronectin-

binding protein (FnBP), poly-*n*-succynyl-β-1,6 glucosamine (PNSG) and protein A (Table 2.2) (Projan and Novick, 1997). Other important *S. aureus* proteins include enzymes such as beta-lactamase, which encodes resistance to beta-lactam antibiotics, thus facilitating invasion of viable cocci into the host (Deresinski, 2005).

Table 2.2: Summary of toxins and toxic components produced by *Staphylococcus aureus* (Timbury *et al*., 2002)

Toxin	Activity
Haemolysins α, β and δ	Cytolytic; lyse erythrocytes of various animal species
Coagulase	Clots plasma, also used in clinical microbiology laboratories to differentiate between *S. aureus* and CNS
Fibrinolysin	Digets fibrin
Leukocidin	Kills leukocytes
Hyaluronidase	Breaks down hyaluronic acid
DNAse	Hydrolyses DNA
Protein A	Lypolytic (produces opacity in egg-yolk medium
Capsule	Antiphagocytic
Epidermolytic toxins A and B	Epidermal splitting and exfoliation
Enterotoxin (s)	Food poisoning toxins that cause vomiting and diarrhoea
Toxic shock syndrome toxin-1	Shock, rash and desquamation

Although numerous studies have contributed to the current knowledge of these components and products responsible for the development of infection, little information regarding the interactions of the bacteria with each other exists (Marrack and Koppler, 1990). In a study by Viera-da Motta and colleagues (2001), the production of enterotoxins was shown to be partly regulated by a quorum sensing mechanism involving the *agr* gene. The mechanism involves intersignalling of *S. aureus* bacterial cells through chemical production of extracellular products which controls survival of the cell (Viera-da Motta *et al*., 2001). Several diseases may be caused by biofilm-associated *S. aureus* strains (Yarwood *et al*., 2004). The suppression of toxins is an important part in the treatment and management of *S. aureus* infections (Viera-da Motta *et al*., 2001). A thorough and complete understanding of the interaction of these *S. aureus* products and components is necessary to apply the correct treatment and to prevent infections (Marrack and Koppler, 1990).

2.5 Antimicrobial resistance of *S. aureus* strains

Staphylococcus aureus causes the greatest apprehension as a pathogen because of the intrinsic virulence that it has and the ability to rapidly adjust to different environmental conditions (Lowy, 1998). The trend of multidrug resistance in *S. aureus* is particularly alarming because of the severity and diversity of diseases caused by this pathogen (Waldvogel, 2000). Despite the availability of novel drugs as an approach to staphylococcal therapy, the bacteria seem to be able to rapidly develop resistance to these drugs (Diekema *et al*., 2004). Perhaps the most commonly known resistance of *S. aureus*, is methicillin resistance, which has caused alarming reports with regard to the spread of *S. aureus* in hospitals and the community (Kowalski *et al*., 2003, Carleton *et al*., 2004, Cepeda *et al*., 2008, Tattevin *et al*., 2008).

Chromosomes or plasmids can mediate antibiotic resistance in *S. aureus* through various mechanisms, including transduction and conjugation (Chambers, 1997). Although the mechanism of methicillin resistance in *S. aureus* is partly understood, there have been reports of low-level methicillin resistance in *mecA* negative strains of *S. aureus* (Ünal *et al*., 1994). These *mecA* negative MRSA strains possibly arose from the hyper-production of beta-lactamase (McDougal and Thornsberry, 1986).

2.5.1 Penicillin resistance

Penicillin was first introduced as an antistaphyloccal drug six decades ago in 1940 (Lowy, 2003). However, as early as 1942, Rammelkamp reported penicillin resistant staphylococci (Rammelkamp, 1942; Lowy, 2003). The penicillin resistant staphylococci were subsequently recognised in the community in 1942 (Lowy, 2003). In the late 1960s, more than 80% of both hospital and community staphylococcal isolates were reported to be resistant to penicillin (Lowy, 2003).

The inactivation of penicillin in *S. aureus* strains was first demonstrated in 1944 by Kirby (Gaze *et al*., 2008). Penicillin is inactivated by penicillinase, a beta-lactamase that hydrolyse the beta-lactam ring of penicillin (Figure 2.4) (Kernodle, 2000).

Figure 2.4: **Schematic representation (a) of the induction of staphyloccal beta-lactamase synthesis in the presence of penicillin (Lowy, 1998). (i) The BlaI DNA-binding protein binds to the operator region. The binding results in the repression of RNA transcription from both *blaZ* and the *blaR1-bla1* genes. The beta-lactamase is stimulated at low levels in the absence and exposure to penicillin. (ii) When the bacterial cell is exposed to penicillin, the penicillin binds to the transmembrane sensor-transducer, BlaR1. The binding of the penicillin to the BlaR1 in turn activates BlaR1 autocatalytic activation. (iii-iv) The active BlaR1 cleaves BlaI into inactive fragments directly or indirectly through a second protein known as BlaR2 allowing the commencement of the transcription of both *blaZ* and *blaR1-blaI*. (v-vi) The *blaZ* (v) encodes the extracellular enzyme, beta-lactamase which hydrolyse the beta-lactam ring of penicillin rendering it inactive. Figure (b): A schematic representation of the methicillin-resistance mechanism that follows a similar mechanism as that of the inactivation of penicillin. The MecR1 protein induces MecR1 synthesis when exposed to a beta-lactam antibiotic. The MecR1 protein inactivates MecI, which allows the synthesis of PBP2a. Beta-lactamase and PBP2a expression are therefore co-regulated by MecI and BlaI.**

The resistance to penicillin is mainly mediated by the *blaZ* gene, which encodes for beta-lactamase (Kernodle, 2000). Four types of *blaZ* genes, A,B, C and D, have been distinguished by serotyping and differences in hydrolysis of beta-lactam substrates (Olsen *et al*., 2006). The *blaZ* gene is a transposable gene located on a plasmid, *pBW15* and transposon, *Tn4002* (Gillespie *et al*., 1988, Olsen *et al*., 2006). The *pBW15* is a 17.2-kb beta-lactamase plasmid that is present in 96% of *S. aureus* strains (McMurray *et al*., 1990). The transposon, *Tn4002* is 6.7 kb in size (Gillespie *et al*., 1988). The beta-lactamase enzyme is produced by staphylococci when the bacterial cells are exposed to beta-lactam antibiotics including penicillin and its derivatives

(Kernodle, 2000). The *blaZ* gene is regulated by two adjacent regulatory genes, namely the *blaR1*, an antirepressor and *blaI,* a repressor (Kernodle, 2000). When a *S. aureus* cell is exposed to a beta-lactam, a protein which functions as a transmembrane sensor-transducer is cleaved (Chambers, 2001). The cleaved protein functions as a protease, that cleaves the BlaI directly or indirectly (Figure 2.4) (Chambers, 2001).

2.5.2 Methicillin resistance in *S. aureus* strains

The *mecA* gene present in MRSA strains encodes the altered protein (PBP2a), which is not inactivated by methicillin (Berger-Bach, 1994, Gaze *et al*., 2008). The *mecA* gene resides on the staphylococcal cassette chromosome *mec* (SCC*mec*) and is expressed by the regulator genes *mecR1* and *mecI* (Lowy, 1998). The regulator gene *mecR1* is activated by beta-lactam antibiotics and serves as a signal transducer that inactivates the *mecI* repressor gene product (Lowy, 1998). Some SCC*mec* types contain genetic elements for other antibiotic resistance, such as *Tn554*, a transposon responsible for resistance to macrolides, clindamycin and streptogramin B, while the *pT181* plasmid accounts for resistance to tetracyclines (Oliveira and De Lencastre, 2002).

There are five different types of SCC*mec* with varying sizes, including SCC*mec* type I, II, III, IV and V with sizes 34, 53, 67, 21-24 and 28 kb respectively (Figure 2.5) (Deresinski, 2005). These five types (I-V) have been used to classify and distinguish between HA-MRSA and CA-MRSA strains (Deresinski, 2005). Staphylococcal cassette chromosome *mec* types I-IV were demonstrated to have alleles *ccrA* and *ccrB*, which is different from type V that contains the *ccrC* allele (Deresinski, 2005). The *ccr* gene complex encodes for site-specific recombinases responsible for the mobility of SCC*mec* (Ito *et al*., 2001). *Staphylococcus aureus* has different *mec* complexes, which are classified into class A, class B, class C and class D (Ito *et al*., 2004). The *mec* gene complexes are structured as follows: class A, *IS431-mecA-mecR1-mecI*; classB, *IS431-mecA*-Δ*mecR1-IS1271*; class C, *IS431-mecA*-Δ*mecR1-IS431* and class D, *IS431-mecA*-Δ*mecR1* (Ito *et al*., 2004). In a study done by Katayama and colleagues (2000), class C strains were found to have an intermediate level of methicillin resistance (MIC 16 to 64 mg/ml) when compared to other classes. Strains found to have neither the *IS431* nor the *IS127* were classified as class D *mec*

strains (Katayama *et al*., 2000). These four *mec* complexes together with the *ccr* gene complexes classify the different SCC*mec* types (Ito *et al*., 2001).

Figure 2.5: **Diagram of the SCC*mec* type I, II, III, IV and V elements (Grundmann *et al*., 2006). SCC*mec* type I-V with sizes 34, 53, 67, 21-24 and 28 kb and additional genes carried by each SCC*mec*. SCC*mec* II and III encode several genes conferring resistance to additional antibiotics such as tetracyclines and erythromycin. SCC*mec* type IV and V representing the smallest SCC*mec* types, cannot harbour other additional genes.**

Staphylococcal cassette chromosome *mec* type IV differs from the other types because of its small size and absence of non beta-lactam (clindamycin, tetracyclines and trimethoprim-sulfamethoxazole) genetic resistance determinants (Ito *et al*., 2001). In 2006, a new SCC*mec* type VI has been proposed, which is characterised clinically by low-level resistance to methicillin and being dominantly present as a paediatric strain (Sa-Leao *et al*., 1999; Oliviera *et al*., 2006).

2.5.2.1 Community-associated MRSA strains

A community-associated MRSA isolate is defined as an MRSA isolate recovered from a clinical specimen from a patient residing in a surveillance area who had no established risk factors for

MRSA infection (Kluytmans-Vanden Bergh and Kluytmans, 2006). These established risk factors included the isolation of MRSA two or more days after hospitalisation, a history of hospitalisation, dialysis, surgery or residence in a long-term care facility within a year before the MRSA-culture date, presence of a permanent indwelling catheter or percutaneous medical device at the time of laboratory culture (Kluytmans-VandenBergh and Kluytmans, 2006).

The etiologies of CA-MRSA are debatable; some studies proposed the possibility of CA-MRSA descending from hospital isolates, whilst other studies proposed that CA-MRSA arose as a consequence of horizontal transfer of the methicillin resistance-determinant, *mecA*, into a methicillin-susceptible *S. aureus* strains (Chambers, 2001). Community associated MRSA is more virulent than typical HA-MRSA due to the frequent production of PVL toxin (Wannet *et al.,* 2004). Health-care associated MRSA is associated with bloodstream, urinary and respiratory tract infections (File, 2008). Conversely, CA-MRSA infections are associated with deep skin infection, soft tissue infection and necrotising pneumonia (Table 2.3) (File, 2008). The severity of CA-MRSA infections can result in hospitalisation and even death due to the release of the PVL toxin (Roberts *et al*., 2008). In a study conducted by Davis and colleagues (2007), in 100 inpatients with MRSA infection, SCC*mec* type IV was detected in 71% of the MRSA strains isolated between 2003-2005 with 54% of these possessing the PVL genes.

The SCC*mec* element of MRSA isolates has diversified because of novel genes that have been discovered (Chongtrakool *et al*., 2006). New nomenclature has been proposed by Chongtrakool and colleagues (2006). Staphylococcal cassette chromosome *mec* elements studied in MRSA strains from 11 Asian countries (615 MRSA isolates), were classified as SCC*mec* type 3A based on their structures (Chongtrakool *et al*., 2006). These strains were isolated from eight countries including Thailand, Sri Lanka, Indonesia, Vietnam, Philippines, Saudi Arabia, India and Singapore (Chongtrakool *et al*., 2006). The diversity of the SCCmec elements found in this study prompts further investigation and renaming of SCC*mec* types and subtypes by roman numerals as new SCC*mec* types are constantly evolving (Chongtrakool *et al*., 2006).

Table 2.3: Characterisation of the different SCC*mec* types and suggested clinical presentations of MRSA infections (File, 2008)

Strain	SCC*mec* type	Antibiotic resistance	PFGE type	Toxins	PVL genes	Infection spectrum
HA-MRSA	Types I, II and III	Multi-drug resistant	USA 100	Few	Rare	Bloodstream, respiratory tract and urinary tract infections
CA-MRSA	Type IV and V	Resistance is typically limited to beta-lactam and erythromycin although multi-drug resistance can occur	USA 300	More, usually PVL presence	Common	Skin and soft-tissue infections and necrotising pneumonia

CA-MRSA community-associated methicillin-resistant ***Staphylococcus aureus***
HA-MRSA health-care associated methicillin-resistant ***Staphylococcus aureus***
PFGE pulsed field gel electrophoresis
PVL Panton-Valentine leukocidin
SCC*mec* staphylococcal cassette chromosome ***mec***

The CA-MRSA strains have a great potential to cause an epidemic in the community, which can spread to the hospital (Kluytmans-Vanden Bergh and Kluytmans, 2006). Methicillin-resistant *Staphylococus aureus* in the hospital may be reduced by restricting MRSA and other pathogens through the implementation of appropriate infection control measures, such as frequent screening of colonisers, implement effective decolonisation treatment and practising basic hygiene methods by health-care personnel (Albrich and Harbath, 2008). However, eradication of community-associated pathogens tends to be more difficult because of the high frequency of transmission by asymptomatic colonised individuals and high cost in screening all heatlh-care workers (Albrich and Harbath, 2008).

2.5.3 Vancomycin-resistance in *S. aureus* strains

The increased prevalence of MRSA strains in the community resulted in the increased usage of the glycopeptide, vancomycin (Appelbaum, 2006). However, the increased usage of vancomycin to treat MRSA infections lead to the emergence of vancomycin-resistant staphylococci (Hiramatsu *et al*., 2001b). The first case of vancomycin resistance among staphylococci was reported in 1987 and was identified in a *Staphylococcus haemolyticus* strain (Schwalbe *et al*., 1987). In 1997, the first report of a vancomycin-intermediate resistant *S. aureus* (VISA) strain was reported from Japan, with reports subsequently following from other countries including France (Ploy *et al*., 1998; Chesneau *et al*., 2000), Scotland (Hood *et al*., 2000) and two isolates in South Africa (Ferraz *et al*., 2000). These VISA isolates were all MRSA strains (Smith *et al*., 1999). Complete resistance to vancomycin was reported in Michigan in the United States in 2002 and subsequently in Pennsylvania two months later (CDC, 2002; Tenover *et al*., 2004).

Identification of two forms of vancomycin resistance have been demonstrated (Walsh and Howe, 2002). The first form involves the VISA strains with a minimum inhibitory concentration of 8 to 16 µg/ml (Walsh and Howe, 2002). The reduced susceptibility to vancomycin by *S. aureus* is hypothesised to be a result of changes in peptidoglycan synthesis (Walsh and Howe, 2002). There is a visible irregularly shaped and thickened cell wall in these VISA strains due to increased amounts of peptidoglycan (Hiramatsu, 2001a). Evidently, there is a decrease in cross-linking of the peptidoglycan strands (Walsh and Howe, 2002) resulting in the exposure of more D-alanyl-D-alanine residues (Figure 2.6) (Hiramatsu *et al*, 1998).

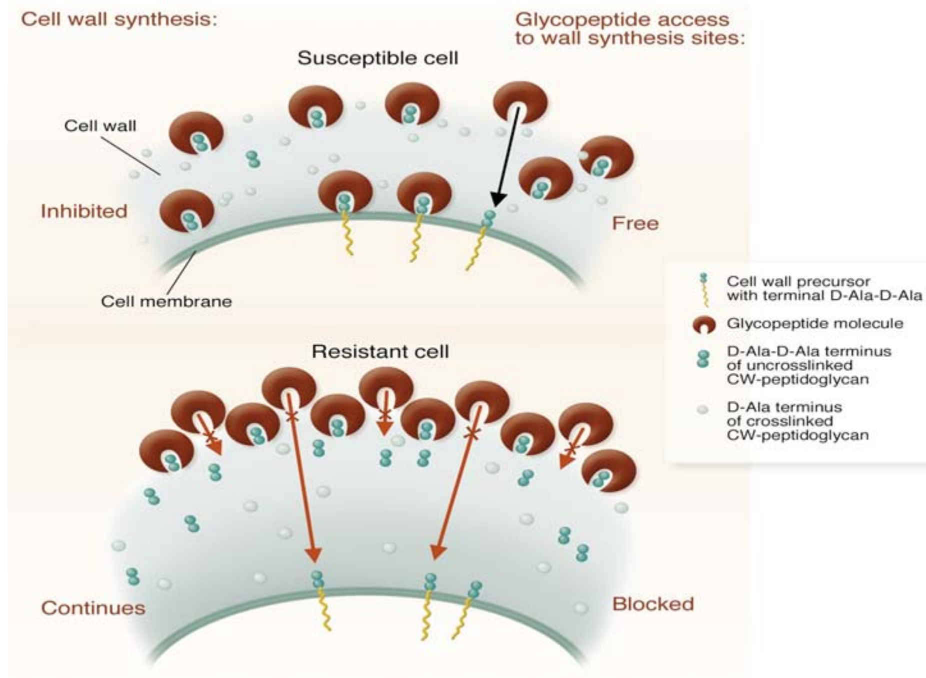

Figure 2.6: **Schematic representation of the mechanisms of *S. aureus* intermediate resistance to vancomycin (Lowy, 1998). The vancomycin-intermediate *S. aureus* strains synthesise additional quantities of peptidoglycan with increased numbers of D-Ala-D-Ala residues that bind vancomycin, thus preventing the molecule to bind to its bacterial target (cell wall) (Lowy, 1998).**

The second form of vancomycin resistance involves vancomycin-resistant *S. aureus* (VRSA) with a minimum inhibitory concentration (MIC) of ≥128 µg/ml (Walsh and Howe, 2002). The mechanism is hypothesised to be due to conjugation with vancomycin resistant *Enterococcus faecalis* (VRE) (Showsh *et al*., 2001). The process of conjugation results in the transfer of the *vanA* operon of the *E. faecalis* bacterium to the MRSA strain (Showsh *et al*., 2001). The *vanA* gene together with its regulator genes, *vanSR*, from VRE is carried by a transposon, *Tn1546*, which is integrated into the plasmid (pLW1043) and conjugatively transferred into *S. aureus* (Hiramatsu *et al*., 2004). Vancomycin-resistant *S. aureus* is therefore, an MRSA with a pLW1043 carrying the *vanA* gene (Hiramatsu *et al*., 2004). The pLW1043 also carries other resistance mediating genes against gentamycin, penicillin and trimethoprim (Hiramatsu *et al*., 2001b).

The mechanism of resistance in VRSA is caused by the alteration of the terminal peptide to D-Ala-D-Lac instead of D-Ala-D-Ala (Gonzalez-Zorn and Courvalin, 2003). The D-Ala-D-Lac synthesis occurs with minimal or low concentration of vancomycin (Gonzalez-Zorn and Courvalin, 2003).

2.5.4 Fluoroquinolone resistance in *S. aureus* strains

Fluoroquinolones are broad spectrum and bacteriocidal antibiotics (Hooper, 2002). The fluoroquinolone drugs kill bacteria by inhibiting bacterial DNA synthesis (Hooper, 2002). Important examples of the fluoroquinolone group include ciprofloxacin, ofloxacin and norfloxacin (Ng *et al*., 1996). Introduced in the 1980s, fluoroquinolones were initially developed for the treatment of Gram-negative bacteria, such as *Pseudomonas* species with limited activity against Gram-positive bacteria (Hooper, 2002). Over the years, new fluoroquinolones with increased activity against Gram-positive cocci were developed including grepafloxacin, levofloxacin, moxifloxacin, sparfloxacin and trovafloxacin (Hooper, 2002). However, the use of these drugs have been highly regulated because of increased development of resistance by bacteria to this group of drugs (Hooper, 2002).

Fluoroquinolone resistance of *S. aureus* emerged rapidly in US hospitals in 1988 after the introduction of ciprofloxacin with 80% of the infections identified as MRSA (Blumberg *et al*., 1991). Ciprofloxacin was initially developed for the treatment of Gram-negative and Gram-positive bacteria other than *S. aureus*, thus exposure of *S. aureus* to fluoroquinolones was minimal (Blumberg *et al*., 1991). *Staphylococcus aureus* resistance to fluoroquinolones is suggested to be as a result of exposure of the bacteria to fluoroquinolones in the mucosal and cutaneous surfaces in the nasal cavity (Blumberg *et al*., 1991). In 2005, MacDougall and colleagues reported a 38% resistance in 616 *S. aureus* strains from 17 US hospitals isolated in 2000 (MacDougall *et al*., 2005). Recently, a study reported a 85% fluoroquinolone-resistance in 1 846 MRSA strains isolated from Kuwaiti hospitals between March and October 2005 (Udo *et al*., 2008).

The DNA gyrase and topoisomerase, which are responsible for DNA replication are the two enzymes targeted by fluoroquinolones (Drlica and Zhao, 1997). The DNA gyrase alters the supercoiling of the DNA whilst the topoisomerase IV separates DNA strands, which are interlocked to allow separation of the daughter chromosomes into daughter cells (Drlica and Zhao, 1997). The activity of the fluoroquinolone drugs differs with the different types of drugs on the level of inhibitory activity against the two enzymes (Takei *et al*., 2001).

2.6 Diseases caused by *S. aureus*

Staphylococcal diseases are usually a result of the production of a toxin or through the invasion and destruction of tissue (Murray *et al*., 2005). Diseases that arise from exclusively staphylococcal toxins include staphylococcal scalded skin syndrome (SSSS), staphylococcal food poisoning and toxic shock syndrome (TSS) (Murray *et al*, 2005). Other staphylococcal diseases include suppurative infections, wound infections and catheter related infections (Murray *et al*., 2005).

2.6.1 Bacteraemia

Staphylococcus aureus remains a common cause of community onset bloodstream infections (Collignon *et al*., 2005). Staphylococcal bacteraemia mortality rate was approximately 20% to 50% between 1992 and 1998 in Belgium (Blot *et al*., 2002). The increased risk in staphylococcal bacteraemia is mostly attributed to catherisation and patients with a high nasal carriage (85%) of *S. aureus* in hospital settings (Morin and Hadler, 1998). It is estimated that more than 50% of *S. aureus* associated bacteraemia are acquired in the hospital after surgical operation or resulting from constant use of contaminated intravascular catheters (Mylotte and Tayara, 2000). Other risk factors for HA-MRSA bacteremia include immunosuppressive diseases, such as cancer; diabetes; human immunodeficiency virus (HIV) and the extensive use of cortocosteroids and foreign bodies, which include prosthetic heart valves as well as central and peripheral venous catheters (Jensen *et al*., 1999).

2.6.2 Endocarditis

Staphylococcus aureus related endocarditis has accounted for 25% to 35% of cases worldwide between 1985 to 1993 (Sandre and Shafran, 1996). The infection is abundant in elderly patients, children (Valente *et al*., 2005), prosthetic valve patients, intravenous drug users and hospitalised patients (Chambers *et al*., 1983). Infective endocarditis is a complication often arising from *S. aureus* associated bacteraemia with a 12% incidence in infants and children in North Carolina, USA, between 1998 and 2001 (Valente *et al*., 2005). Echocardiography is one way of exploring the heart valves thus diagnosing endocarditis (Kim *et al*., 2003). Prognosis of *S. aureus* related endocarditis is worsened in patients with HIV infection, as it usually presents as an advanced infective endocarditis (Fernandez-Guerrero *et al*., 1995). The mortality rate for hospital-infective endocarditis between 1972 and 1992 in Spain was 40% to 56% and it has been demonstrated that the mortality is even higher in patients when the isolated bacteria was *S. aureus* (Fernandez-Guerrero *et al*., 1995). In a cohort study study conducted from June 2000 until December 2003 by Fowler and colleagues (2005), *S. aureus* accounted for 25.9% and 54.2% of infective endocarditis in Australia/New Zealand and Brazil, respectively.

2.6.3 Toxic shock syndrome

Toxic shock syndrome was first described by Todd and his collaborators (1978) in Denver, USA, in children aged 8 to 17 years (Freedman and De Beer, 1991). The disease is characterised by diarrhoea, erythroderma, high fever, hypotension, mental confusion and renal failure (Freedman and De Beer, 1991).

In the 1980's, the disease was frequently observed in women with the onset of menstruation (Chesney *et al*., 1981). In 1980 and 1981, TSS reached epidemic proportions and the sudden increase was attributed to the introduction of hyper-absorbable tampons (Chesney *et al*., 1981). The prevalence of TSS decreased moderately when the tampons were removed from the market, with 3 to 15 per 100 000 women of menstrual age/year subsequently (Chesney *et al*., 1981). Non-menstrual cases have been associated with localised infections, surgery or insect bites (Chesney *et al*., 1981). Researchers suggested that cases of non-menstrual toxic shock syndrome

have a higher mortality rate compared to cases of menstrual involved toxic shock syndrome (Waldvogel, 2000). These female cases have been associated with caesarean section surgeries and long-term diaphragm use (Waldvogel, 2000). Initial symptoms include diarrhoea, fever, myalgias and vomiting (Waldvogel, 2000). Hypovolemic shock develops due to loss of colloids and fluids (Chuang *et al*., 2005). A sunburn-like rash develops within a few hours with the involvement of conjuctival inflammation (Waldvogel, 2000).

Diagnosis and treatment of TSS includes identification of the *S. aureus* strain and resistance profiling of the identified strain (White *et al*., 2005). Electrolytes and fluid replacement should be given to the patient as part of the overall therapy (White *et al*., 2005). An adjuvant treatment approach included agents that can block TSS superantigens, such as intravenous immunoglobulin that contains superantigen neutralizing antibodies (Chuang *et al*., 2005).

2.6.4 Food poisoning

Two-thirds of the 250 foodborne diseases described in the literature are caused by bacteria (Le Loir *et al*., 2003). *Staphylococccus aureus* is the leading cause of gastroenteritis resulting from the consumption of contaminated food (Le Loir *et al*., 2003). *Staphylococcus aureus* food poisoning is due to the release of toxins in the food during its growth, causing symptoms ranging from abdominal pain to nausea, vomiting and sometimes diarrhoea but never diarrhoea alone (Wieneke *et al*., 1993). The onset of *S. aureus* food poisoning is rapid, ranging from 30 min to 8 h after ingestion, with spontaneous remission after 24 hrs (Jay, 1992).

Staphylococcus aureus enterotoxins (SEs) involved in food poisoning are highly stable and resistant to neutralisation by proteolytic enzymes, such as pepsin or trypsin (Bergdoll, 1989). To date, there are 14 different SE types, which have similar structures (Le Loir *et al*., 2003). *Staphylococcus aureus* enterotoxins are small proteins that are produced in food, soluble in water and are rich in lysine, aspartic acid and glutamic acid (Le Loir *et al*., 2003). These SE's are more heat resistant in food than in laboratory medium (Bergdoll, 1989).

Various high sugar, protein and salt content foods are involved with *S. aureus* food poisoning including milk and milk products (cheeses and ice creams), sausages, canned meat, salads (potato salads) and sandwich fillings (Bergdoll, 1989). The foods that are involved in *S. aureus* food poisoning differ from one country to another (Wieneke *et al*., 1993). The main sources of contamination of these foods are food-handlers by manual contact, coughing or sneezing since up to 50%-70% of the human population are *S. aureus* carriers (Solberg, 2000; Le Loir *et al*., 2003). In a study by Gadaga and colleagues (2007), 32% of food handlers were found to be carriers of *S. aureus* in Zimbabwe compared to 6.4% food handlers carrying *E. coli* between April 2004 to March 2005. Other sources involve contamination from animal origins either by animal carriage or zoonosis (Le Loir *et al*., 2003; http://www.sva.org.sg/en/sva_admin/upload/journal_ article/Slides_Zoonoses%20in%20Australia%).

2.6.5 Staphylococcal scalded skin syndrome

Staphylococcal scalded skin syndrome was first described in 1878 by Ritter von Rittershain as a disease manifested by a bullous exfoliative dermatitis in infants less than 1 month old (Rogolsky, 1979). Later in 1956, Lyell described a syndrome similar to SSSS in infants and in children with which the skin looks and feels as though it had been scalded by hot water (Figure 2.7) (Gemmel, 1995). The disease presents occasionally with an onset of general localised erythema and spreads to the entire body in less than two days (Rogolsky, 1979).

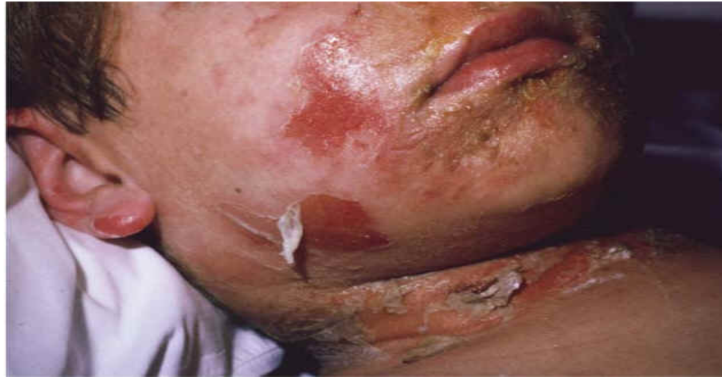

Figure 2.7: Staphylococcal scalded skin syndrome (skin looks scalded by hot water) (http://www.uv.es/~vicalegr/CLindex/Clpiodermitis/ssss.htm)

The symptoms are usually followed by an upper respiratory infection or a purulent conjunctivitis (Elias *et al*., 1977). The disease has been attributed to the production of an exotoxin known as epidermolytic toxin (ET) (Arbuthnott, 1981). In a study conducted by Mockenhaupt and colleagues (2005), SSSS accounted between 0.09 and 0.13 of cases per 1 million patients with a 51% mortality rate in Germany between 2003 to 2004. In the study by Mockenhaupt and colleagues (2005), 11% and 40% was observed in children and adults, respectively. Staphylococcal scalded-skin syndrome has been shown to be due to exfoliative toxins (Yamasaki *et al*., 2005). The exfoliative toxin genes, *eta* and *etb*, have been detected in 30% and 19% of SSSS presenting patients by polymerase chain reaction (PCR) (Yamasaki *et al*., 2005).

2.7 Treatment and prevention of *S. aureus* infections

Penicillin is still the main drug of choice for staphylococcal infections as long as the isolate is sensitive to it (Kowalski *et al*., 2003). In patients with histories of a delayed-type penicillin allergy a cephalosporin, such as cefazolin or cephalothin can be administered as an alternative choice of treatment (Lowy, 1998). A semisynthetic penicillin, such as methicillin, is indicated for patients with beta-lactamase producing staphylococcal isolates (Lowy, 1998). Patients who have an MRSA infection are treated with a glycopeptide known as vancomycin (Michel and Gutmann, 1997). Vancomycin is the empirical drug of choice for the treatment of MRSA (Michel and Gutmann, 1997). Patients who are intolerable to vancomycin are treated

with a fluoroquinolone (ciprofloxacin); lincosamide (clindamycin); tetracycline (minocycline) or trimethoprim-sulfamethoxazole, which is also known as co-trimoxazole (Lowy, 1998).

Novel quinolones, such as ciprofloxacin with increased antistaphyloccal activity are available but their use may become limited due to the rapid development of resistance during therapy (Lowy, 1998). Several antimicrobial agents with activity against MRSA are currently evaluated and include: (i) oritavancin, a semisynthetic glycopeptide (Guay, 2004); (ii) tigecycline, a monocycline derivative (Guay, 2004) and (iii) DW286, a fluoroquinolone (Kim *et al*., 2003). Amongst these three antibiotics, tigecycline has been approved by the Food and Drug administration (FDA) in June 2005 (Stein and Craig, 2006).

Recently, an evaluation of glycosylated polyacrylate nanoparticles showed to have *in vitro* activities against methicillin-resistant *S. aureus* and *Bacillus anthracis* (Abeylath *et al*., 2007). Other recent investigative drugs include, silver nano particles, oleanolic acid from extracted *Salvia officinalis* (Sage leaves) (Horiuchi *et al*., 2007; Yuan *et al*., 2008). Two novel antibiotics, neocitreamicins I and II, isolated from a fermentation broth of a *Nocardia* strain have shown to have *in vitro* activity against *S. aureus* and vancomycin-resistant *Enterococcus faecalis* (VRE) (Peoples *et al*., 2008). Accurate empirical therapy against *S. aureus* infections would be an important step towards the reduction of the development of resistance in the different strains (Lowy, 1998).

2.7.1 Vaccine developments for the prevention of *S. aureus* infections

There is no vaccine available, which stimulates active immunity against staphylococcal infections in humans (Todar, 2005). Currently, under investigation is a vaccine called Staph Vax, composed of *S. aureus* type 5 and 8 capsular polysaccharides conjugated to non-toxic recombinant *Pseudomonas aeruginosa* exotoxin A (Welch, 1996; Fattom *et al*., 2003; Schaffer and Lee, 2009). In a study evaluating a higher dose of this bivalent vaccine in *S. aureus* infected patients, favourable results were obtained proving partial protection against *S. aureus* (Shinefield *et al*., 2002). In 2006, Kuklin and colleagues investigated the potential of the *S. aureus* surface protein iron surface determinant B (IsdB) as a prophylactic vaccine against *S. aureus* infection in

mice. The vaccine was highly immunogenic, with reproducible and significant protection in animal models of infection (Kuklin *et al*., 2006). Recently, a study investigating the *S. aureus* clumping factor and FnBPA as vaccine was proposed, which showed that these antigenic properties resulted in increased protection in *S. aureus* infected mice (Arrecubieta *et al*., 2006). Vaccine development against TSS are being researched and might be a feasible approach in the prevention of the disease (Hu *et al*., 2003). However, since TSS is a multi-toxin mediated disease, development of these vaccines is difficult (Hu *et al*., 2003).

2.8 Diagnostic identification of MRSA from clinical specimens

Methicillin-resistant *Staphylococcus aureus* identification is based on phenotypic and genotypic investigations (Fluit *et al*., 2001). Phenotypic identification of *S. aureus* includes Gram-staining, catalase, coagulase, DNAse, culture on mannitol salt agar or blood agar and sugar fermentation tests (Waldvogel, 2000). Upon identifying *S. aureus* by Gram-staining (Gram-positive cocci), catalase (positive), fermentation tests (oxidase positive) and tube coagulase (positive) or DNase (positive), the sample is grown on mannitol salt agar or blood agar at 37°C for 18 to 24 h (Brown *et al*., 2005). The colonies appear yellow on mannitol salt agar and creamy white on blood agar (Brown *et al*., 2005). *Staphylococcus aureus* colonies are subjected to antimicrobial susceptibility testing by the Kirby Bauer disk diffusion method, automated methods such as the Vitek (bioMérieux, France) and Microscan (Dade Microscan, West Sacramento, CA) systems or other commercially available methods including latex agglutination assay kits (Brown *et al*., 2005).

Various molecular techniques have been implemented for the rapid identification and charaterisation of MRSA strains. These include genotypic identification of MRSA strains is based on the amplification of the *mecA* gene, which confers resistance to methicillin (Murakami *et al*., 1991, Chongtrakool *et al*., 2006, McClure *et al*., 2006)

2.8.1 Antimicrobial susceptibility testing

Determination of antimicrobial susceptibility testing of clinical isolates is not only necessary for the optimal antimicrobial therapy of infected patients but for the monitoring of the spread of MRSA strains or resistance genes throughout the hospital and the community (Fluit *et al*., 2001). Routine antibiotic resistance determination of MRSA strains includes the determination of the MIC using a traditional reference dilution method such as the agar dilution method and broth dilution method (CLSI, 2006). Nevertheless, there is conflicting recommendations regarding the most reliable method of identification of MRSA antibiotic susceptibility (Brown *et al*., 2005). This is because different strains of diverse heterogeneity are included in various studies and perform differently under specific test conditions (Brown *et al*., 2005). Several other antimicrobial susceptibility testing methods include automated methods such as the Vitek/Vitek2 (bioMérieux) and Microscan (Dade Behring) (Brown *et al*., 2005).

2.8.1.1 Kirby Bauer disk diffusion method

The disk diffusion methods including the Kirby-Bauer disk diffusion (Figure 2.8) method are the most routinely used detection methods for methicillin resistance in *S. aureus* in clinical laboratories despite the increasing development of commercial methods and automated systems (Jureen *et al*., 2001). The Kirby Bauer disk diffusion method is a standardised antimicrobial susceptibility test, which is recommended by the Clinical and Laboratory Standards Institute (CLSI) (Shoeb, 2008).

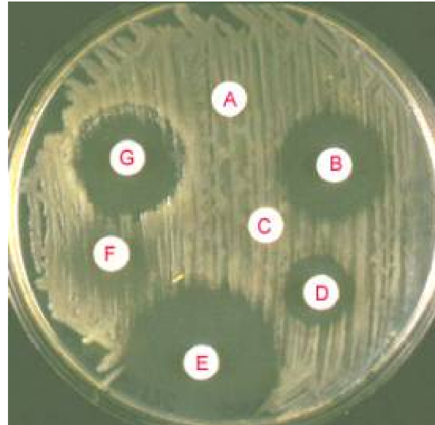

Figure 2.8: **Mueller-Hinton agar plate showing antibiotic disks A-G. Disks B, E and G have clear zones indicating susceptibility to these antibiotics. Discs A, C, D and F show the resistance of the bacterium to these antibiotics (http://jameslunsford.com/microunknown2_exp_explain2_files/image002.jpg).**

Staphylococcus aureus colonies grown on Mueller-Hinton agar plates in the presence of thin wafers (disks) containing relevant antibiotics at standardised concentrations (CLSI, 2003b). Susceptibility of *S. aureus* is demonstrated by a clear zone around the disk known as the zone of inhibition (Figure 2.8). Minimum inhibitory concentration is determined according to the breakpoints guidelined by the CLSI (Brown *et al*., 2005). The expression of methicillin resistance in *S. aureus* is affected by a number of *in vitro* conditions (Brown *et al*., 2005). These conditions include the type of test, medium of growth, inoculum size, the period and temperature of incubation (CLSI, 2003b).

Other susceptibility testing methods include the (i) E-test method, which is performed according to the manufacturer's recommendations; (ii) the breakpoint methods involving both the agar and the broth methods by testing only the breakpoint concentrations and (iii) the agar screening method, which is recommended for routine screening of colonies isolated and for confirmation of suspected resistance seen in the disc diffusion tests (CLSI, 2003a).

2.8.2 Automated systems for the detection and identification of *S. aureus* strains

Automated detection, identification and antibiotic resistant profiles for MRSA strains includes the Vitek and Microscan systems (Shetty *et al*., 1998; Swenson *et al*., 2001). These automated systems have revolutionised the diagnostic element of microbiology because of their ease of use, speed and accuracy (Shetty *et al*., 1998). The Vitek system (bioMerieux, France) for automated detection of *S. aureus* strains is accomplished by biochemical characterisation of these strains (Swenson *et al*., 2001). Suspended pure colonies of *S. aureus* in saline are inoculated in specific identification cards containing biochemical broths in wells that include catalase, coagulase and oxidase tests and incubated in the Vitek system (Shetty *et al*., 1998). The Vitek software determines the results of the wells by measuring the light attenuation with an optical scanner (Shetty *et al*., 1998). Determination of methicillin resistance in *S. aureus* is performed by inoculating the isolates into wells with dilution of antimicrobials and measuring the MIC values with the Vitek software (Shetty *et al*., 1998).

The Microscan system (Dade Microscan, West Sacramento, CA) uses plastic trays that contain 96 micro-wells (Figure 2.9), which carry dried biochemical and antibiotics (Jorgensen and Ferraro, 1998). The Microscan panels are inoculated with a defined concentration of the suspension of the isolate in question that are incubated at appropriate temperature in a Autoscan Walk/Away system (Sung *et al*., 2000). Various Microscan systems use fluorescent markers to determine the identity of the bacterium (Lindsey *et al*., 2008). Antimicrobial susceptibility and resistance is measured by a turbidometer with the use of Windows software (Microsoft; Redmond, WA) (Jorgensen and Ferraro, 1998).

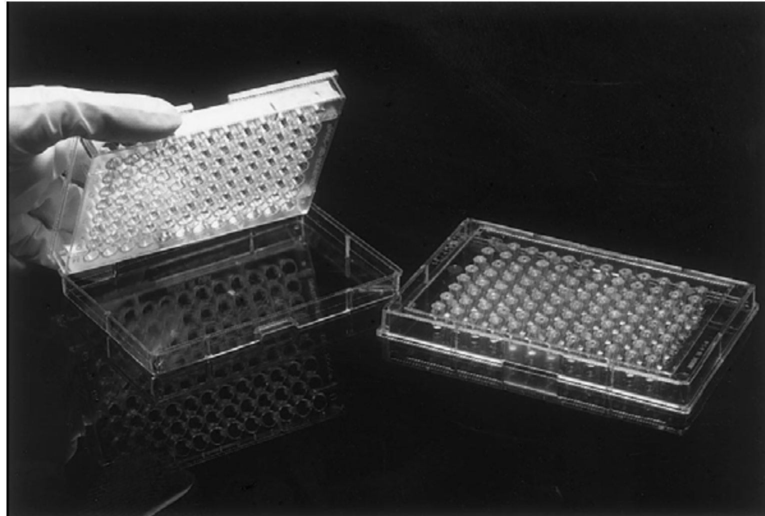

Figure 2.9: **Microscan panel tray (*right*) with a disposable tray inoculator (*left*) used for the identification and antimicrobial susceptibility testing by the Microscan system (Jorgensen and Ferraro, 1998).**

In MRSA studies, the Microscan-based susceptibility testing has been shown to be a rapid and sensitive technique in MRSA identification (Lindsey *et al*., 2008). In a study conducted by Dillard and colleagues (1996), 252 isolates of *S. aureus* were tested for oxacillin susceptibility by MicroScan Gram positive overnight and rapid MIC panels (Swenson *et al*., 2001). The results of these method were compared with those of standard disk diffusion testing (Kirby-Bauer) and found to have a 100% agreement (Dillard *et al*., 1996). Although the Microscan system is a rapid automated technique used by some laboratories for routine MRSA identification, shortcomings of this technique have been shown to have a lessened ability to detect some forms of inducible antimicrobial resistance such as in MRSA strains (Swenson *et al*., 2001).

2.8.3 The latex agglutination assay for the detection of *S. aureus* and MRSA strains

The latex agglutination assay (Oxoid, Ltd) is a rapid Food and Drug administration (FDA) approved test for the detection of *S. aureus* and MRSA isolates (Malhotra-Kumar *et al*., 2008). Earlier latex agglutination assays detected the *S. aureus* specific protein A and clumping factor (Kuusela *et al*., 1994). Later, the latex assay was developed to detect other *S. aureus* specific surface antigens (Brown *et al*., 2005). The assay is based on the detection of the PBP2a in

approximately 20 minutes (Chapin and Musgnug, 2004). Penicillin binding protein 2a is mediated by the *mecA* gene (Chapin and Musgnug, 2004). Monoclonal antibodies against PBP2a sensitise latex particles of isolated MRSA colonies (Van Griethuysen *et al*., 1999). The PBP2a assay is a rapid and sensitive method of detecting MRSA isolates compared to other phenotypic methods such as the standard agar disk diffusion test (Cavassini *et al*. 1999). In 2004, Lee and colleagues reported a 100% sensitivity and specificity for the detection of MRSA using the MRSA-Screen latex agglutination test (Lee *et al*., 2004). In another study by Cuevas and colleagues (2003), the latex agglutination PBP2a test had a sensitivity of 100% and a specificity of 98% for evaluating 137 MRSA isolates. However, several reviews showed that any test involving the clumping factor may give false positive results (Brown *et al*., 2005). Chapin and Musnug (2004), also showed that the latext agglutination test has poor sentitivity when applied directly to blood cultures for MRSA detection.

2.9 Molecular identification and characterisation assays of MRSA strains

Since conventional identification and antibiotic resistance detection often take more than 48 h, molecular based detection techniques, including conventional PCR and real-time PCR, have been developed for the rapid and accurate identification and characterisation of MRSA isolates (Fluit *et al*., 2001; Huletsky *et al*., 2004). Molecular techniques are often applied for the routine diagnostic MRSA detection along with antimicrobial susceptibility testing methods, partly because susceptibility testing alone is not enough to confirm MRSA presence due to the sensitivity of the test conditions (Trindade *et al*., 2003). The identification of MRSA was simplified by the polymerase chain reaction (PCR) technique (Van Pelt-Verkuil *et al*., 2008).

2.9.1 Application of PCR assays for identification of MRSA

Polymerase chain reaction is a process that allows amplification of pre-determined DNA regions (genes) by the use of small target specific DNA primers (Van Pelt-Verkuil *et al*., 2008). The PCR amplification system can turn a few molecules of specific target nucleic acid into as much as a microgram of DNA (Hoffmann *et al*., 2009). Two oligonucleotide primers flank and define

the target sequence that is to be amplified (Hoffman *et al*., 2009). These primers hybridise to opposite strands of the DNA and serve as initiation points for amplification (Hoffman *et al*., 2009). A thermostable enzyme, DNA *Taq* polymerase catalyses this synthesis (Hoffman *et al*., 2009).

Polymerase chain reaction is a rapid, powerful and reliable molecular method for MRSA typing compared with all other MRSA typing techniques such as bacteriophage and capsular typing (Jaffe *et al*., 2000). In addition, PCR typing methods are better to distinguish between MRSA isolates (Zhang *et al*., 2005). The use of PCR for the detection of the *mecA* gene (Barski *et al*., 1996) and SCC*mec* typing has been described previously by Oliveira and colleague (2002), and used as a reference method for SCC*mec* typing (Zhang *et al*., 2005). In the case of MRSA, PCR-based assays detect the *mecA* gene responsible for mediating methicillin resistance in staphylococci (Fluit *et al*., 2001). Other genes such as *femA*, *femB* and *nuc* genes may be detected in MRSA isolates but these genes may be absent in some MRSA strains (Jonas *et al*., 1999).

Polymerase chain reaction-based methods have been shown to have shortened the turn-around time (2 h to 4 h) in identifying MRSA isolates resulting in prompt treatment for MRSA associated infection (Van Hal *et al*., 2007). These PCR-based methods are able to detect multiple other *S. aureus* specific genes including the 16S rRNA, PVL and *fem* genes (Fluit *et al*., 2001; McClure *et al*., 2006). This PCR method of detecting multiple genes in *S. aureus* simultaneously is called multiplex PCR (M-PCR).

2.9.1.1 Multiplex PCR assays in MRSA strain identification and characterisation including SCC*mec* typing and subtyping

Multiplex PCR (M-PCR) utilises multiple oligonucleotide primers all included in the same PCR reaction mix to simultaneously amplify several target genes (Van Pelt-Verkuil *et al*., 2008). The number of the PCR primers is greatly influenced by the PCR conditions including the annealing temperature, dNTP concentration, Mg^{2+} concentration and DNA template concentration

(Van Pelt-Verkuil *et al*., 2008). Usually, M-PCR primers are longer in base pairs than single PCR primers because of potential cross-reaction in a PCR amplification process (Van Pelt Verkuil *et al*., 2008). Although M-PCR is an ideal DNA amplification method, several disadvanges including contamination; cross reactions due to non-specific binding due to the number of primers and extended pre-optimisation time prior to actual reaction greatly impacts on the use of this PCR method in laboratories (Berg *et al*., 2000; Van Pelt-Verkuil *et al*., 2008).

In MRSA studies, M-PCR has been used to target several MRSA genes including the *S. aureus* specific 16S rRNA gene; the *S. aureus* methicillin resistance gene, *mecA* and the PVL conferring *luxS-PV* and *luxF-PV* (McClure *et al*., 2006). Other *S. aureus* M-PCR protocols include the amplification of *S. aureus* specific toxin genes such as *etaA* and *etaB* responsible for exfoliative disease and *TSST-1* for TSS disease (Mehrotra *et al*., 2000). Pyrogenic toxin genes detection by M-PCR has also been described (Monday and Bohach, 1999).

Multiplex PCR typing methods of MRSA have been previously described (Ito *et al*., 2001, Oliviera *et al*. 2002, Zhang *et al*., 2005, Boye *et al*., 2007, Kondo *et al*., 2007). The M-PCR typing method is based on the characterisation of MRSA's specific *ccr* gene complex, which encodes for site-specific recombinases responsible for the mobility of SCC*mec* (Ito *et al*., 2001). The *ccr* gene complex together with *mec* complexes which are classified into class A, class B, class C and class D (Ito *et al*., 2004) can type MRSA isolates into the different SCC*mec* types thus enabling researcher to distinguish between HA-MRSA and CA-MRSA (Zhang *et al*., 2005). Recently, another M-PCR assay was developed for the subtyping of the SCC*mec* type IV into eight subtypes (Milheirico *et al*., 2007). The "SCC*mec* IV" M-PCR is important to trace clones of CA-MRSA characterised by SCC*mec* type IV to understand the mechanism of SCC*mec* assembly and acquisition in these clones (Milheirico *et al*., 2007). The M-PCR assays can be useful in infection control strategies and be implemented for epidemiological studies to determine clonal relatedness during outbreaks in clinical settings (Chongtrakool *et al*., 2006).

2.9.1.2 Real-time PCR assay for the identification of MRSA

Although automated systems such as the Vitek and Microscan lessen the laborious aspect of MRSA detection, the turnaround time is not significantly different from manual antimicrobial susceptibility assays (Stratidis *et al*., 2007). Studies reported that some of these systems do not detect heteroresistant MRSA strains (Swenson *et al*., 2001).

Real-time PCR is an automated single step closed PCR system that can rapidly amplify genes in *S. aureus* and other microorganisms using various flourescent chemistries (Huygens *et al*., 2006). Amplification and detection of target DNA are coupled in a single vessel, eliminating the need for laborious post-amplification processes (Yang and Rothman, 2004). Real-time PCR uses fluorescent intercalating dyes, such as SYBR-Green I, which bind non-specifically to double-stranded DNA during amplification (Yang and Rothman, 2004). A more specific alternative approach is the use of fluorescent-labelled probes, such as hydrolysis probes (Taqman probes), hybridisation probes and molecular beacons (Klein, 2002). Probe chemistry is based on the transfer of energy between two adjacent dye molecules known as a flourophore and a quencher (Klein, 2002). This process is known as fluorescence resonance energy transfer (FRET) (Klein, 2002). Apart from the three described real-time PCR based fluorescence principles, scorpion primers with fluorescent-labelled tails can be used (Whitcombe *et al*., 1999). These scorpion primers hybridise to an amplified target DNA (Whitcombe *et al*., 1999). A scorpion primer consists of a specific probe sequence that is held in a hairpin loop configuration by complementary stem sequences on the 5' and 3' sides of the probe (Thelwell *et al*., 2000).

The real-time assays for MRSA are based on the amplification and detection of specific MRSA genes (Stratidis *et al*., 2007). A real-time PCR assay that can detect MRSA directly from clinical specimens was developed by Huletsky and colleagues in 2004 (Huletsky *et al.,* 2004). This novel real-time PCR detected MRSA strains from a mixture of staphylococci in non-sterile clinical specimens in less than an hour (Huletsky *et al.,* 2004). The technique could also identify the various SCC*mec* types (I-V) (Huletsky *et al*., 2004). However, the cost of of real-time PCR is higher than conventional culture methods for MRSA identification (Conterno *et al*., 2007). Real-

time PCR seems less sensitive and specific than conventional culture methods in some studies (Otter *et al*., 2007; Van Hal *et al*., 2007). Recently, Van Hal and colleagues (2009), evaluated a commercial real-time PCR kit (*Easy*-Plex, MRSA Easy-Plex Gene Disc, AusDiagnostics, Australia), which targets the *mecA* gene to identify 200 MRSA strains in Australia. The sensitivities and specificities of the PCR assay was 84% and 90%, respectively (Van Hal *et al*., 2009). In contrast, McClure and colleagues (2006), showed a 100% sensitivity for the real-time PCR detection of the *mecA* and PVL genes in Canada.

2.10 Typing assays of MRSA strains

Following the development of PCR, various techniques became available for the typing of MRSA and MSSA strains including random amplified polymorphic DNA (RAPD), variable-number tandem repeat (VNTR) typing techniques including coagulase (*coa*), hyper-variable region (HVR) and staphylococcal protein A (*spa*) typing methods (Stranden *et al*., 2003). However, prior to the development of PCR, several molecular techniques were used for identification and typing of *S. aureus* and MRSA strains. The section below discusses the different non-PCR based and PCR based techniques used in the genotyping of MRSA.

2.10.1 Non-PCR based typing techniques of MRSA strain typing

Before the development of PCR, several efficient typing methods were used for *S. aureus* strain typing. These methods including bacteriophage typing (1952), capsular typing (1984), PFGE (1984) and zymotyping have been applied for discriminating between *S. aureus* and MRSA strains (Schlichting *et al*., 1993; Weller, 2000; Grady *et al*., 2001; Basim, 2001). Amongst these methods, PFGE is the most extensively used method to date for the typing of MRSA strains as it is the “gold standard”. Most novel MRSA typing studies couple PFGE as a reference method for MRSA strain typing as it is the most sensitive and specific MRSA strain typing method to date (Molina *et al*., 2008; Stranden *et al*., 2008; Ibrahem *et al*., 2009; Pu *et al*., 2009).

2.10.2 Bacteriophage typing

Bacteriophage typing (BT) has been used for over 30 years by the Center for Disease Control and Prevention (CDC) to discriminate among outbreak related *S. aureus* strains (Schlichting *et al*., 1993). Several MRSA outbreaks have been defined with this technique and its discriminatory power is greater than phenotypic tests such as capsular typing and zymotyping (Weller, 2000). Zymotyping is based on the differentiation of electrophoretic properties of the bacterial esterase enzymes of *S. aureus* by multilocus enzyme electropheresis (MLEE) (Weller *et al*., 2000). Bacteriophage typing has several limitations and weaknesses, which include characterising isolates on the basis of a phenotypic marker that has poor reproducibility, e.g. in the characterisation of *S. aureus*, some *S. aureus* isolates do not have the bacteriophage receptor which restricts the infection of prophage isolates (Schlichting *et al*., 1993). Bacteriophage typing is a time-consuming and technically demanding procedure, which is most efficiently done on large batches, thus the technique requires maintenance of a large number of phage stocks and propagating strains, which confines its use to a few reference laboratories (Schlichting *et al*., 1993). Bacteriophage typing of *S. aureus* has since been replaced by the pulsed-field gel electrophoresis (PFGE) typing technique (Bannerman *et al*., 1995).

2.10.3 Capsular typing

Staphylococcus aureus is classified into 11 serotypes based on the capsular polysaccharide (Verdier *et al*., 2007). Capsular polysaccharide is a component of the cell wall of *S. aureus* that increases the bacterial virulence and protects the cell from phagocytosis (Verdier *et al*., 2007). Capsular typing is based on the reactivity of monoclonal antibodies to specific *S. aureus* antigenic capsular polysaccharides (Von Eiff *et al*., 2007). Although 11 capsular polysaccharides are described, only types 5 and 8 are clinically important presenting in 70% to 80% of the *S. aureus* infections globally (Verdier *et al*., 2007).

2.10.4 Pulsed-field gel electrophoresis for genotyping of MRSA strains

Pulsed-field gel electrophoresis is often considered the "gold standard" of molecular typing methods (Olive and Bean, 1999). The PFGE technique was developed in 1984 by Schwartz and Cantor (Trindade *et al*., 2003). The PFGE technique is based on the digestion of bacterial DNA with restriction enzymes that recognises specific sites along the chromosome (Trindade *et al*., 2003). The restriction enzyme digestion generates large DNA fragments that cannot be separated by conventional electrophoresis (Olive and Bean, 1999). The electric field is pulsed at different angles across the gel allowing the DNA fragments to separate in order of size (Maslow *et al*., 1993). The PFGE system can separate DNA fragments of up to 10 megabase pairs (Kiadó, 2006). The PFGE system consists of a power supply system, with a voltage of up to 750 volts, a switch unit that can alternate current at different directions of, a computer system to control the resolution in PFGE and cooler system that regulates the PFGE system since DNA is temperature sensitive (Basim, 2001). The PFGE technique has replaced the traditional BT technique in that it does not have the same limitations as BT (Bannerman *et al*., 1995).

Pulsed-field gel electrophoresis has been found to have a high discriminatory power and was illustrated in epidemiologic studies done in Brazil (Trindade *et al*., 2003). A high discriminatory power is an important characteristic of a typing technique defined as the probability that isolates with related and identical phenotypic and genotypic profiles are clonal and part of the same transmission (Trindade *et al*., 2003).

The PFGE technique has been extensively used for MRSA typing compared to other techniques (Trindade *et al*., 2003). A common MRSA strain was found in eight of nine hospitals in Sao Paulo, Brazil, using the PFGE technique and this confirmed the presence of an endemic clone (Trindade *et al*., 2003). However, the limitation of PFGE is the extended time before the results are available (Stranden *et al*., 2003). The procedural steps in the technique are straight forward, however, the time needed to complete analysis can take up to a week, which reduces the ability of the laboratory to analyse large numbers of samples (Stranden *et al*., 2003). The DNA fragments are run on a electrophoresis gel with alternating electrical current. In addition, PFGE requires

high cost reagents and specialised equipment including restriction enzymes, specialised power supply and high molecular weight markers (Weller, 2000).

2.11 PCR-based typing methods for MRSA typing

Following the development of PCR, typing of MRSA strains evolved to the detection of polymorphic regions of the MRSA genome (Schmitz *et al*., 1998). Polymerase chain reaction-based typing techniques have increased the understanding of MRSA strains by identifying the different genotypes and related MRSA strains (Trindade *et al.*, 2003). These PCR-based methods include random amplified polymorphic DNA (RAPD) (Tambic *et al*., 1997), VNTR-based typing techniques such as *coa* (Tiwari *et al*., 2008), *spa* (Schmitz *et al*., 1998) and hypervariable region (HVR) typing (Senna *et al*., 2002).

2.11.1 Random amplified polymorphic DNA of MRSA strains

Random amplified polymorphic DNA (RAPD) or amplified fragment length polymorphism (AP-PCR) technique uses a short primer of 10 base pairs (bp) (Farber, 1996). The short 10 bp primer with random sequences of nucleotides randomly amplifies DNA targets producing fragments, which serve as genetic markers (Tambic *et al*., 1997). The fragments produced in the PCR assay are separated by gel electrophoresis (Tambic *et al*., 1997). Random amplified polymorphic DNA is a rapid and simple to perform technique that can be applied for any organism (Power, 1996). In a study conducted in 1997, the RAPD method was shown to have the ability to type non-phage typeable MRSA strains in an outbreak setting proving a higher discriminatory power than phage-typing (Tambic *et al*., 1997). The RAPD technique has also been shown to be suitable for routine genotyping of hospital-acquired staphylococci (Van Belkum, 1994; Van Belkum *et al*., 1995). However, the RAPD technique has been documented to have a inferior discriminatory power when compared with PFGE (Saulnier *et al*., 1993).

2.11.2 Variable-numbers of tandem repeat based typing techniques of *S. aureus* strains

The variable-numbers of tandem repeat genotyping is based on the number of repeat units at the same locus of the *S. aureus* genomic DNA (Wichelhaus *et al*., 2001). The number of repeat units differs in *S. aureus* strains (Wichelhaus *et al*., 2001). The number of repeat units can be detected with flanking primers similar to the method used in DNA fingerprinting of eukaryotic and prokaryotic species (Farlow *et al*., 2001). Several genes including the *coa* and *spa* genes have various numbers of degenerated repeats of 81 bp and 24 bp, respectively (Sabat *et al*., 2003). Based on this polymorphism, *S. aureus* strains can be genotyped using flanking primers specific for these two genes, *coa* and *spa* genes (Sabat *et al*., 2003). In MRSA strains, the locus between the *mecA* and the *IS*431 can also be used to genotype *S. aureus* strains, however, this is only limited to MRSA strains and not MSSA strains (Kim and Oh, 1999).

2.11.2.1 Coagulase typing of MRSA strains

The coagulase protein of *S. aureus* is an important virulence factor, which causes plasma to clot (Waldvogel, 2000). In *S. aureus*, the *coa* gene has a polymorphic repeat region that can be used for differentiating *S. aureus* isolates (Mlynarczyk *et al*., 1998; Shopsin *et al*., 2000). The polymorphic region of the *coa* gene has 81-bp tandem short sequence repeats, which are variable in number and sequence when determined by restriction fragment length polymorphism (RLFP) analysis of PCR products (Van Belkum *et al*., 1995). Recently, a study conducted in India by Tiwari and colleagues (2008), evaluating the *coa* gene typing method on 84 *S. aureus* clinical isolates, 33 different *coa* types could be identified in the hospital and community. The technique proved to be relatively inexpensive and is simple to perform and analyse (Tiwari *et al*., 2008). However, the discriminatory power of the *coa* gene typng method has shown to be lower than the PFGE method (Schmitz *et al*., 1998).

2.11.2.2 Staphylococcal protein A typing of MRSA strains

Staphylococcal protein A (*spa*) typing is based on the characterisation of the *spa* gene, which encodes for the *S. aureus* specific surface protein A (Gao and Stewart, 2004). The *spa* gene consists of different functional regions including the Fc binding region and the X-region, which have between five and 15 repeat sequences respectively (Sakurada *et al*., 1994). The *spa* typing for *S. aureus* is easy to implement in the laboratory and is as reproducible as the RAPD method (Schmitz *et al*., 1998). Repetitive DNA sequences or insertion elements are not consistent tools to assess relatedness because of the predisposition for rapid modification (Schmitz *et al*., 1998). However, the repetitive sequence (RS) region of the *spa* gene was reported to be a satisfactory stable repetitive DNA sequence to be used for typing purposes of MRSA strains (Frenay *et al*., 1996). In a study by Harmsen and colleagues (2003), 107 and 84 strains were studied during two periods of 10 and 4 months, respectively and 10 *spa* types could be identified (Harmsen *et al*., 2003). Similar to the *coa* gene typing method, the *spa* typing method is a rapid and inexpensive technique, but the discriminatory power of the technique is lower than the PFGE method (Harmsen *et al*., 2003).

2.11.2.3 Hyper-variable region typing of MRSA strains

The hyper-variable region (HVR) is positioned between the *mecA* and the *IS*431 genes, where the is a high heterogenecity in MRSA strains (Schmitz *et al*., 1998). The HVR contains direct repeat units of 40 bp each (Senna *et al*., 2002). The HVR typing technique has been evaluated in various studies for the characterisation of MRSA isolates (Stranden *et al*., 2003). In one study, HVR discriminatory power was shown to be similar to *spa* typing (Schmitz *et al*., 1998). The HVR technique is rapid, reproducible and simple to perform when compared to PFGE, however, the HVR has a lower reproducibility than PFGE (Stranden *et al*., 2003). In a study evaluating the reproducibility between the *spa*, *coa* and HVR typing techniques, the reprodubility was 100%, 97% and 76% to 89% respectively (Stranden *et al*., 2003). However, hyper-variable region typing is a rapid and inexpensive method of typing, which can be used during outbreaks and as

well as for infection control implementation, results were comparable with PFGE (Stranden *et al*., 2003).

The rate of mutations and genetic rearrangements of strains control the consistency of the various PCR based typing techniques (Stranden *et al*., 2003). Using these typing techniques in combination will provide better results when compared to using one technique. The sensitivities and specificities can thus be compared when more than one technique is used.

Differentiation between HA-MRSA and CA-MRSA is primarily based on the haboured SCC*mec* element (Deresinski, 2005). Several M-PCR assays have been proposed to distinguish between these two types of MRSA (Oliviera and De Lencastre, 2002; Zhang *et al*., 2005; Boye *et al*., 2007, Kondo *et al*., 2007). Methicillin-resistant *S. aureus* classification and subtyping is important for recognising MRSA outbreaks, determining the source of outbreak and recognising virulent strains that might be circulating in the clinical setting (Oliviera and De Lencastre, 2002). The monitoring of multi-drug resistant MRSA strains (HA-MRSA) and virulent strains (CA-MRSA) is essential in enforcing the correct and adequate control measures and adjusting guidelines for antimicrobial chemotherapy in different hospital settings (Baba *et al*, 2002).

The aim of this study was to determine the prevalence and characterise HA-MRSA and CA-MRSA isolates obtained from clinical specimens using molecular techniques. The molecular techniques can further be used as rapid tools for identification and characterising MRSA isolates in epidemiological investigations and outbreaks.

2.12 Summary

Staphylococcus aureus, a Gram-positive facultative anaerobic cocci remains the world's foremost pathogen accounting for the majority of health-care associated infections (Cercenado *et al*., 2008). *Staphylococcus aureus* infections are diverse, ranging from necrotising pneumonia, skin and soft tissue infections to severe diseases *S. aureus* specific such as TSS and SSSS (Waldvogel, 2000). In addition to health-care associated infections, *S. aureus* has become

adaptable to other community enviroments, such as military schools, kindergarten and beauty salons (Cercenado *et al*., 2008).

It is estimated that over 95% of patients with staphylococcal infections do not respond to penicillin therapy (Olsen *et al*., 2006). Even more concerning, is the continuing rise of MRSA strains, since 1961 accounting for 25% of health-care associated infections in the USA in 2001 (Shopsin and Kreiswerth, 2001). In contrast to HA-MRSA, a more virulent MRSA strain was identified in the community setting in the 1980s in children without any established risk factors for MRSA acquisition and it was defined as CA-MRSA (Cercenado *et al*., 2008). Community-associated MRSA strains have been reported to be more virulent than HA-MRSA due to the frequent production of the PVL toxin (Baba *et al*, 2002). Patients associated with PVL-positive CA-MRSA infections have a higher mortality rate than PVL-negative MRSA infected patients (Baba *et al*, 2002), emphasising the rapid identification of these CA-MRSA strains in clinical settings (Cercenado *et al*., 2008).

The SCC*mec* is a genomic island, which harbours the *mecA* gene (Deresinski, 2005). The *mecA* gene is the primary methicillin-resistance mediating gene, which codes for an altered PBP2a with reduced affinity to beta-lactam antibiotics such as, benzylpenicillin, cloxacillin and methicillin as well as some cephalosporins and monobactams (Deresinski, 2005). Treatment of multi-drug resistant MRSA strains is therefore limited to linezolid and vancomycin (Kohno *et al*., 2007). Resistance to these antibiotics has already been reported in MRSA strains in Brazil in 2002 for linezolid and VRSA in Michigan, USA in 2002 (Tsiodras *et al*., 2001; Goldrick, 2002; Wilson *et al*., 2003).

Molecular epidemiology investigations showed that CA-MRSA strains carry the SCC*mec* type IV or V element, which is not haboured by HA-MRSA strains (File, 2008). Health-care associated MRSA strains habour SCC*mec* types I, II and III elements, which contain other antibiotic resistant genes, such as *Tn554* and *pT181* (Deresinski, 2005). Therefore, CA-MRSA strains are susceptible to non-beta-lactam antibiotics, such as clindamycin, erythromycin and tetracycline (File, 2008).

Several microbiology laboratories use the Kirby-Bauer disk diffusion method to demonstrate MRSA; however, other microbiology laboratories use commercially available kits, such as the latex agglutination assays (Brown *et al*., 2005). Automated systems including the Vitek and MicroScan systems are used for routine identification and susceptibility testing for MRSA isolates (Shetty *et al*., 1998; Swenson *et al*., 2001). Discrepancies with regard to sensitivities, reproducibility and discriminatory power exist when using these methods as indicated in the literature (Trindade *et al*., 2003).

Multiplex PCR has been shown to be a useful rapid and robust technique for multiple detection of virulence genes as well as for SCC*mec* typing and subtyping (Mehrotra *et al*., 2000; Zhang *et al*., 2005).

Pulsed-field gel electrophoresis is the "gold standard" in genotyping and characterising MRSA strains (Trindade *et al*., 2003). The PFGE technique is restriction-enzyme based method, which separates the products of the restriction-enzyme reaction by alternating the current during gel electrophoresis (Olive and Bean, 1999). Although PFGE remains the "gold standard" in MRSA genotyping, the technique is laborious, needs highly specialised equipment and the interpretation of results is difficult (Weller, 2000). Therefore, other rapid, easy to perform and robust PCR-based techniques have been proposed for genotyping MRSA including *coa*, HVR and *spa* typing techniques (Schmitz *et al*., 1998). The techniques are based on amplifying the tandem repeats in the gene sequences of MRSA resulting in unique amplification patterns between MRSA strains (Schmitz *et al*., 1998).

This study was aimed at identifying and characterising 97 MRSA isolates collected from April 2006 to September 2007 in the Steve Biko Academic Hospital. The antibiotic susceptibility profiles, the prevalence of the *mecA* gene, the presence of the PVL genes, the SCC*mec* types as well as the genotypes were determined using conventional methods (Kirby Bauer disc diffusion), multiplex PCR, real-time PCR, the *spa* and HVR typing techniques.

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CHAPTER 3

MOLECULAR IDENTIFICATION AND GENOTYPING OF MRSA ISOLATES

The editorial style of the FEMS Immunology and Microbiology was followed in this chapter

3.1 ABSTRACT

Aims: The aim of this study was to identify and characterize 97 MRSA isolates.

Methods: Two conventional multiplex PCR assays, a real-time PCR assay and two PCR based genotyping techniques (*spa* and HVR typing methods) were used to identify and characterize 97 MRSA strains isolated between April 2006 to September 2007 from the Steve Biko Academic Hospital.

Results: All MRSA isolates were positive for 16S rRNA, 99% were positive for the *mec*A gene and 4% positive for the PVL gene. SCC*mec* typing showed 67% of isolates were SCC*mec* II (HA-MRSA), 14% were SCC*mec* III (HA-MRSA) and 4% were SCC*mec* IVd (CA-MRSA). These CA-MRSA isolates showed a prevalence of 100% for the PVL gene. Using *spa* typing, three distinct clusters could be identified while HVR typing revealed six different clusters. CA-MRSA isolates were clustered together using *spa* and HVR typing.

Significance and Impact of study: This study showed the prevalence of the CA-MRSA strains, PVL genes, the SCC*mec* types and the clonality of the MRSA strains. The high prevalence of the PVL gene in CA-MRSA isolates already residing in ICUs was alarming and indicated the emergence of new MRSA lineages with a particular fitness for community and hospital transmission.

Keywords: Methicillin-resistant *S. aureus*, health-care associated MRSA, community-associated MRSA, Panton-Valentine leukocidin toxin, *mecA*

3.2 INTRODUCTION

Amongst all known bacteria, *S. aureus* is possibly the greatest concern of all health-care associated pathogens due to its ability to cause a wide variety of life-threatening infections (Lowy, 1998). *Staphylococcus aureus* has the ability to rapidly adapt to different environmental conditions (Lowy, 1998). In 2003, *S. aureus* was reported to be the leading cause of health-care associated infections globally (NNISS, 2004). Numerous anti-staphylococcal agents exist including linezolid, daptomycin, tetracyclines and fluoroquinolones, but these are rapidly becoming of less value due to the ability of the bacterium to develop efficient mechanisms to neutralize these agents (Lowy, 2003). The methicillin resistance mechanism is the most recognized in methicillin-resistant *S. aureus* (MRSA) strains (Lowy, 2003).

Methicillin-resistant *S. aureus* has been recognized as an important health-care associated infection (Jevons, 1961). Treatment as early as 1959 included semi-synthetic penicillin drugs such as methicillin for *S. aureus* infection (Jevons, 1961). The rise of MRSA strains became apparent as early as 1960, approximately a year after methicillin introduction (Jevons, 1961). In the early 1980's MRSA strains were identified as a major cause of nosocomial infections due to the increase every decade (Rubins *et al*., 1999). The possibility of transmission of the health-care associated MRSA (HA-MRSA) to the community was unavoidable (Kluytmans-Vanden Bergh and Kluytmans, 2006). Since 1987, MRSA was increasingly found in the community and referred to as community-acquired MRSA (renamed community-associated MRSA) (CA-MRSA) (Moreno *et al.,* 1995; Millar *et al*., 2007).

The development of methicillin resistance in *S. aureus* strains can be ascribed to the altered penicillin binding protein (PBP2a), which has a reduced affinity to penicillin and beta-lactam antibiotics (Hartman and Tomasz, 1981). The *mec*A gene that encodes the altered protein (PBP2a) is not inactivated by methicillin during treatment (De Lencastre *et al.,* 1994). The *mecA* gene resides on a genomic island termed the staphylococcal cassette chromosome *mec* (SCC*mec*) (Ito *et al*., 2001).

Methicillin-resistant *S. aureus* strains that have been clinically identified as community-associated have been shown to be more virulent with a high degree of severity of disease when compared to HA-MRSA (Baba, 2002). This is due to the production of the Panton-Valentine leukocidin toxin (PVL) (Dufour *et al*., 2002). Panton-Valentine leukocidin is a toxin associated with deep skin infection, soft tissue infection and necrotizing pneumonia (Lowy, 1998). Panton-Valentine leukocidin toxin stimulates pore formation in the leukocyte membrane resulting in death of the cell, thus promoting tissue necrosis (Ebert *et al*., 2008). Since, the PVL-associated genes of CA-MRSA are harboured by a bacteriophage, φSLT, these toxin genes may be transmitted easily to other HA-MRSA strains (Narita *et al*., 2001; Enright *et al*., 2002).

Panton-Valentine leukocidin toxin has been identified as a genetic marker for CA-MRSA strains (Vandenesch *et al*., 2003). However, the prevalence rate of the PVL toxin in CA-MRSA strains varies with different studies and countries (McClure *et al*., 2006). Some studies reported a prevalence of between 77% to 100% for the PVL toxin in Minnesota, USA in 2000 (Naimi *et al*., 2003) whilst a prevalence of less than 5% were reported in Western Europe (Prevost *et al*., 1995).

The presence of PVL toxin in CA-MRSA strains can be confirmed by co-amplification of the *lukS/F-PV* genes (Deresinski, 2005).

In comparison to previous detection methods such as Southern blotting and pulsed-field gel electrophoresis (PFGE), PCR assays such as multiplex-PCR (M-PCR), real-time PCR, hyper-variable region (HVR) and *spa* typing techniques can provide a rapid amplification, detection and typing tool for MRSA strains (Stranden *et al*., 2003). The *spa* method is based on the amplification of the protein A mediating gene (*spa* gene) which generates a staphylococcal strain specific amplification pattern, which can be used to classify MRSA strains (Schmitz *et al*., 1998). Whereas, the HVR typing method is based on the amplification of the 40 bp repeat unit elements between the IS*431mec* and *mecA* genes, which are situated on the staphylococcal cassette chromosome (Senna *et al*., 2002). Both the *spa* and HVR typing methods have been reported to provide a rapid and inexpensive method for the genotyping of MRSA strains (Stranden *et al*., 2003; Koreen *et al*., 2004)

The purpose of this study was to evaluate and optimize a M-PCR assay for the simultaneous detection of the *S. aureus* specific 16S rRNA, *mecA* and PVL genes. The MRSA strains were also SCC*mec* typed and subtyped to differentiate between HA-MRSA and CA-MRSA strains using a second M-PCR assay. Furthermore, a real-time PCR method was evaluated to detect the PVL genes in MRSA isolates and compare these results with the first M-PCR assay. Genotyping of these strains were conducted using the *spa* and HVR typing methods to show the clonal relatedness of these MRSA strains. New and relevant information concerning the prevalence of these MRSA

strains were obtained and PCR based methods proved to be useful tools to monitor the circulation of these strains in this clinical setting.

3.3 MATERIALS AND METHODS

3.3.1 Sample analysis

Ninety seven (97) MRSA isolates were obtained from clinical specimens sent from the Steve Biko Academic Hospital for analysis to the diagnostic laboratory at the Department of Medical Microbiology, University of Pretoria/NHLS. With a sample size of 97, a two-sided 95% confidence interval for a single proportion using the large sample normal approximation will extend 0.1 from the observed proportion for an expected proportion of 0.5, the latter choice being conservative in the absence of prevalence estimates for the *mecA* and the PVL gene from previous studies. These bacterial isolates were identified as *S. aureus* based on their morphology, Gram-staining and catalase properties. Coagulase and DNase tests were performed to identify *S. aureus* isolates. A community-acquired MRSA strain (ATCC CA05) was used as a reference strain.

3.3.2 Antibiotic resistance determination

Resistance to methicillin was determined using the Kirby-Bauer disc diffusion method (Mougeot *et al*., 2001). Briefly, *S. aureus* colonies were isolated from sheep blood agar plates (Oxoid, England) and inoculated in 0.85% (5 ml) of saline (Mougeot *et al*., 2001). Turbidity was

adjusted to 0.5 McFarland standards (1.5 x 10^8 cfu.ml^{-1}). Fifteen minutes after turbidity adjustment, the swab was dipped into the inoculum and streaked evenly onto Mueller-Hinton agar plates (Oxoid, England) and left to dry for 15 min. Within 15 min of drying the plates, oxacillin/methicillin discs with a concentration of 1 µg (Abtek, Liverpool, UK) were applied aseptically. The plates were incubated (Horo incubator) at 37°C for 18 hrs. After incubation, the diameter of the inhibition zones was measured with a ruler. Results were interpreted according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2003).

3.3.3 Total bacterial DNA extraction

The bacterial DNA extractions were performed using a modified phenol-based DNA extraction method (Sambrook and Russel, 2001). A single pure *S. aureus* colony that was grown on a sheep blood agar plate (Diagnostic Media Products, South Africa) was inoculated in 2 ml of Brain Heart Infusion (Biolab, Wadeville, Gauteng) broth and incubated (Sunvic Controls LTD, England) at 37°C for 24 hrs. One millilitre of the culture was pelleted in a 2 ml Eppendorf tube (Merck, Wadeville, Germiston, South Africa) by centrifugation (Spectrafuge 24D, Labnet International, Inc., New Jersey, USA) at 4 930 x *g* at 4°C for 5 min. The supernatant was discarded and the pellet was placed on ice. The pellet was resuspended in 1 ml STE buffer [0.1 M of NaCl (Promega, Madison, USA), 1 mM EDTA (pH 8.0) (Merck, Wadeville, Germiston, South Africa) and 10 mM Tris-HCl (Merck, Wadeville, Germiston, South Africa) (pH 7.5)] (Sambrook and Russell, 2001) by up and down aspiration with a pipette. Five microlitre of lysozyme (50 mg.ml^{-1}) (Roche Molecular Diagnostics, Germany) was added to the cell suspension and incubated (Horo incubator) at 37°C for 1 h. Following incubation, 50 µl of 20% sodium dodecyl sulphate stock solution (SDS) (Promega, Madison, USA) was added. Ten

microlitre of proteinase K (20 $mg.ml^{-1}$) (Finnzymes, South Africa) was added and the suspension was incubated (Sunvic Controls LTD, England) at 50°C overnight or until the suspension was clear.

Several gentle extractions with equal volumes of mixed phenol:chloroform:isoamylalcohol (25:24:1) (Sigma, St. Louis, USA) were performed. The suspension was centrifuged (Spectrafuge 24D, Labnet International, Inc., New Jersey, USA) at 4 930 *x g* for 5 min at room temperature (25°C). The upper phase was transferred to a new 1.5 ml Eppendorf tube (Whitehead Scientific, Brackenfell, Cape Town) and centrifuged (Spectrafuge 24D, Labnet International, Inc. New Jersey, USA) at 4 930 *x g* for 5 min at room temperature (25°C). An equal volume of chloroform:isoamylalcohol (24:1) (Sigma, St. Louis, USA) was added and the suspension was centrifuged (Spectrafuge 24D, Labnet International, Inc. New Jersey, USA) at 4 930 *x g* for 20 min at 15°C to remove any traces of phenol from the DNA suspension. The top layer was removed and added to a new 1.5 ml eppendorf tube (Whitehead Scientific, Brackenfell, Cape Town) and 0.3 M sodium acetate (3 M stock solution) (Sigma, St. Louis, USA) was added to the DNA suspension. Equal volumes of ice-cold absolute ethanol (-20°C) (Saarchem, Wadeville, Gauteng) was added, mixed and placed overnight at -20°C for the precipitation of the DNA.

The DNA suspension was pelleted by centrifugation (Spectrafuge 24D, Labnet International, Inc. New Jersey, USA) at 2 862 x *g* for 10 min at room temperature (25°C) and the aqueous phase was discarded. The pellet was desalted with 400 µl of 70% ice cold ethanol (-20°C) (Saarchem, Wadeville, Gauteng) and the tubes were inverted to dry. The pellet was resuspended in 200 µl of TE buffer (pH 8) (Promega, Madison, USA) [10 mM of Tris-HCl (pH 7.5) (Merck, Wadeville,

Germiston, South Africa) and 1 mM of ethylene diamine tetra-acetate (EDTA) (Merck, Wadeville, Germiston, South Africa) (pH 8.0)] and stored at -20°C until further analysis.

3.3.3 Multiplex PCR assay for detection of the 16S rRNA, *mecA* and PVL genes (McClure *et al*., 2006)

The M-PCR assay included 2 µl of the prepared DNA template in a 25 µl final reaction mixture which consisted of 12.5 µl of 2X GoTaq® Green Mastermix (Promega, Madison, USA). The 2X GoTaq® Green Mastermix consisted of GoTaq® DNA polymerase supplied in 2X GoTaq® Green Reaction buffer (pH 8.5) and 400 µM of each dNTP (Promega, Madison, USA). A concentration of 0.2 µM of each primer specific for the 16S rRNA, 0.24 µM of each primer specific for the amplification of the *mecA* gene and 0.2 µM of each primer specific for the *lukS/F-PV* was added (McClure *et al*., 2006).

The reaction tubes were spun down for 5 s and placed in a PX2 Thermal cycler (Thermo Electron Corporation, MA distributed by The Scientific Group, SA). The PCR conditions were modified as follows: denaturation at 94°C for 10 min, followed by 10 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s and extension at 72°C for 75 s and another 25 cycles of 94°C for 45 s, 50°C for 45 s and a final extension step at 72°C for 10 min (McClure *et al*., 2006).

3.3.4 Real-time PCR for the identification of PVL producing MRSA

The real-time PCR reaction for the detection of PVL producing CA-MRSA was done on all 97 isolates including the PVL positive type IV isolates obtained from the previous M-PCR assays by

optimizing and modifying the method described by Deurenberg *et al*. (2004). The reaction included the forward primer PVL-FP, a reverse primer PVL-RP (Sigma-Genosys, Cambridge, UK) and a minor groove binding probe (MGB) (Roche Diagnostics, Germany) (Table 3.2).

The following reaction conditions were used: the assay included 5 µl of DNA template in a final volume of 20 µl consisting of 4 µl of the LightCycler® Taqman® Master reaction mix (Roche Diagnostics, Germany). The LightCycler® Taqman® Master reaction mix is a ready-to-use hot start reaction mix supplied as a kit for real-time PCR on the LightCycler® version 2.0 (Roche Diagnostics, Germany). In addition to the reaction mix, 1 µl (0.5 µM) of each primer, 0.4 µl (0.2 µM) of the MGB Taqman probe and 8.6 µl PCR grade nuclease free water (Promega Corporation, Madison, WI) were added to obtain a final reaction volume of 20 µl in the LightCycler® 20 µl capillaries (Roche Diagnostics, Germany).

The following programme was used on the LightCycler® version 2.0 (Roche Diagnostics, Germany) for the amplification cycles: pre-incubation at 95°C for 10 min, denaturation at 95°C for 10 s, annealing at 60°C for 15 s, extension at 72°C for 10 s and a final extension step at 72°C for 10 min for 30 cycles. A single acquisition for fluorescence signals was done at the end of each extension step. Following the completion of the cycles, fluorescence signals were analyzed and quantified by amplification curves using the LightCyler® Software version 4.0 (Roche Diagnostics, Germany).

3.3.5 Multiplex PCR assay for typing and subtyping of SCC*mec* element

This M-PCR assay contained nine pairs of primers that were specific for typing the SCC*mec* types and subtypes and internal control primers for the confirmation of the *mecA* gene of the 97 isolates (Zhang *et al*., 2005). The types and subtypes that were to be amplified included type: I, II, III, IVa, IVb, IVc, IVd and type V (Table 3.3).

A volume of 2.5 µl of the extracted DNA was added to 22.5 µl PCR reaction mixture. The reaction mixture contained 12.5 µl of the QIAGEN Multiplex PCR Mastermix (Qiagen, USA). The QIAGEN Multiplex PCR Mastermix (Qiagen, USA) consisted of HotStarTaq DNA polymerase supplied in a 10X PCR buffer with 3 mM $MgCl_2$ and 400 µM of each dNTP (Qiagen USA). The assay included nine sets of primers prepared in a 10X primer mix. Five microlitre of the primer mix was added to the QIAGEN M-PCR Mastermix. In addition, 2.5 µl of the QIAGEN Q-solution was added to the reaction mixture. To obtain a final volume of 25 µl, 2.5 µl of RNase-Free water supplied with the QIAGEN M-PCR Mastermix kit was added to the reaction mixture.

The M-PCR amplification was performed using a PX2 Thermal cycler (Thermo Electron Corporation, MA distributed by The Scientific Group, SA) with an initial denaturation step at 94°C for 15 min followed by 10 cycles of 94°C for 30 s, 60°C for 90 s and extension at 72°C for 90 s followed by another 25 cycles of 94°C for 45 s, annealing at 55°C for 45 s, extension at 72°C for 90 s and a final extension step at 72°C for 10 min (Zhang *et al*., 2005).

3.3.6 PCR assay for *spa* typing of MRSA isolates

The PCR assay included two primers that amplified the repetitive sequence region of the *S. aureus* specific protein A gene. The PCR assay was modified by adjusting the primer concentrations and TaKaRa *Taq* DNA polymerase (Separations, Gauteng) from a previously published study (Schmitz *et al*., 1998).

The PCR assay included previously published primers SPA2 and SPA3 (Table 3.4) and included 2.5 µl of DNA template in a 25 µl final reaction mixture. The reaction mixture consisted of 0.125 µl of TaKaRa *Taq* DNA polymerase (Separations, Gauteng), 2 µl of dNTPs (2.5 mM each), 4 µl of 10X PCR buffer (10 mM Tris-HCl (pH 8.3), 500 mM KCl and 15 mM $MgCl_2$) and 16.75 µl of water.

The PCR amplification was performed using a Perkin Elmer thermocycler (Thermo Electron Corporation, MA distributed by The Scientific Group, SA) according to a method by Schmitz and colleagues (1998), with an initial activation step at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 40 s and extension at 72°C for 50 s followed by a final extension step at 72°C for 5 min (Schmitz *et al*., 1998).

3.3.7 PCR assay for HVR typing of MRSA isolates

The assay for HVR typing consisted of one pair of primers (Table 3.5) which included the HVR2 and HVR3 oligonucleotides (Schmitz *et al*., 1998). The PCR assay was performed to amplify the

hyper-variable region between the *mecA* and the *IS431* of MRSA strains as previously described (Schmitz *et al*., 1998).

The PCR assay (HVR2/HVR3) included 2.5 µl of the prepared DNA template in a 25 µl final reaction mixture which consisted of 12.5 µl of 2X GoTaq® Green Mastermix (Promega, Madison, USA). The 2X GoTaq® Green Mastermix consisted of GoTaq® DNA polymerase supplied in 2X GoTaq® Green Reaction buffer (pH 8.5) and 400 µM of each dNTP (Promega, Madison, USA). A concentration of 0.25 µM of each primer specific for flanking the tandem repeat sequence was added to the reaction mixture. The PCR amplification was performed using a Perkin Elmer thermocycler (Thermo Electron Corporation, MA distributed by The Scientific Group, SA) according to the method by Schmitz and colleagues (1998), with the following modifications of the PCR cycles: an initial activation step at 94°C for 5 min followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 20 s and extension at 72°C for 50 s followed by a final extension step at 72°C for 5 min.

3.3.8 Analysis of the reaction products

The amplicons of all the PCR reactions were visualised using a UltraViolet light box (UVP products, USA) following electrophoresis on a 2% (m/v) agarose gel (Whitehead Scientific, Brakenfell, Cape town), which contained 5 µl of 20 µg.ml^{-1} of ethidium bromide stock solution (Promega, Madison,USA). A molecular weight marker of a 100 bp ladder (Promega, Madison, USA) was included as a reference to specify lanes on each electrophoresis gel.

The *spa* and HVR typing amplification product gels were analyzed using the GelCompar II software (Applied-Math, Belgium) and Unweighted Pair Group Method with Arithmetic mean (UPGMA) dendrogrammes were constructed to show the clonal relatedness of the MRSA strains. The distance matrix was constructed using the dice coefficient with band optimization of 2.0% and tolerance of 1.6% was used.

3.4 RESULTS

The deoxyribose nucleic acid extraction method was effective for all 97 MRSA isolates. To optimize the amplification of the DNA templates for the M-PCR assay, single-target PCR reactions were performed using each primer pair to detect the specific target gene (Figure 3.1). Each of the primer pairs amplified single-target genes including the 16S rRNA, the *mecA* and PVL genes (Table 3.1). Gel electrophoresis results indicated the following sizes of the amplicons: 756 bp (16S rRNA), 310 bp (*mecA*) and 433 bp (PVL genes). The results of the M-PCR assay were compared with those obtained from the single-target PCR assay as previously reported by McClure and colleagues (2006).

The first M-PCR assay showed that all 97 MRSA isolates (100%) were positive for the *S. aureus* specific 16S rRNA gene confirming that these isolates were *S. aureus* strains (Figure 3.2). The *mecA* gene was detected in 96/97 (99%) of the MRSA isolates. One isolate which did not show the presence of the *mecA* gene was, however, phenotypically identified as a MRSA strain by the Kirby-Bauer disc diffusion method. Four MRSA isolates were positive for the PVL gene and SCC*mec* typing confirmed that these isolates were CA-MRSA strains (Figure 3.3).

All 97 MRSA isolates were further SCC*mec* typed and subtyped (Figure 3.4) using a second M-PCR assay (Zhang *et al*., 2005). This SCC*mec* typing M-PCR assay included primers specific for SCC*mec* types and a *mecA* internal control primer. This second M-PCR assay showed the same prevalence of 99% for the *mecA* gene as the first M-PCR. The same MRSA isolate was also negative for the *mec*A gene using the second M-PCR.

The real-time PCR assay concurs with the previously multiplex assay for detection of PVL genes in MRSA. This study reported a 100% PVL gene prevalence detected by M-PCR and real-time PCR.

The prevalence of the different SCC*mec* types was determined using a second M-PCR assay. The prevalence of the different SCC*mec* types is indicated in Table 3.6. The SCC*mec* typing revealed that the four PVL positive MRSA isolates belonged to SCC*mec* type VId (881 bp) (Table 3.6). No SCC*mec* type V (CA-MRSA) isolates were detected. The four PVL positive MRSA isolates were isolated from the following hospital wards: two from the neonatal intensive care unit (ICU), one from medical ICU and one from the casualty unit (Table 3.6, Table 3.7). Two of the CA-MRSA isolates (one from neonatal ICU and one from medical ICU) had a clinical presentation of pneumonia, which is typical of PVL positive CA-MRSA strains (Table 3.6) (File, 2005). The other two (2%) PVL-positive MRSA isolates were from neonatal ICU and casualty and had clinical presentations of sepsis and polytrauma.

Genotyping to determine the relatedness of these MRSA strains was done using the *spa* typing method, which revealed three distinct types designated type A, B, C with a prevalence of 16%, 77% and 1%. All CA-MRSA strains were clonally related using *spa* typing (100% clonality) and grouped

as cluster B. Although these CA-MRSA strains were clustered together in cluster B, 95% of cluster B isolates did not possess the same SCC*mec* type as these strains. In the total of 97 isolates, five MRSA isolates could not be typed using the *spa* typing method.

Hyper-variable region typing revealed six distinct clusters. These types were designated type A, B, C, D, E, and F, each with a prevalence of 25%, 3%, 25%, 16%, 4% and 24%, respectively. Three of the four CA-MRSA isolates could be grouped into the same cluster C (Isolate 15, 63 and 67). The other CA-MRSA isolate was grouped into cluster E (Isolate 29). Three percent (3%) MRSA isolates could not be typed using the HVR typing technique (Isolates 54, 90 and 91).

3.5 DISCUSSION

The Kirby Bauer disc diffusion method identified all *S. aureus* isolates (100%) as methicillin resistant. The one isolate which did not show the presence of the *mecA* gene was, however, phenotypically identified as a MRSA strain by the Kirby Bauer disc diffusion method. Similar strains have previously been described, indicating that the resistance in these MRSA strains is not intrinsically mediated by the *mecA* gene (Bignardi *et al*., 1996). This non-*mecA* mechanism is hypothesized to be due to altered modifications to the penicillin binding proteins which, results in hyperproduction of beta-lactamase or methicillinase production (Bignardi *et al*., 1996). According to Swenson and colleagues (2007), there is no evidence that the non-*mecA*-mediated resistance is of less clinical relevance, since it remains a MRSA strain (Swenson *et al*., 2007). The four MRSA isolates that were positive for the PVL gene were SCC*mec* typed and confirmed that these isolates were CA-MRSA strains (Figure 3.4). This indicated that these CA-MRSA isolates were all 100%

positive for the PVL gene compared to the 93% prevalence reported from a similar study conducted in Turkey during 2004 to 2005 (Kilic *et al.,* 2006).

The 67% of the staphylococcal cassette chromosome *mec* type II (398 bp) showed the highest SCC*mec* prevalence compared to the other SCC*mec* types (Table 3.6). The SCC*mec* type II is haboured by HA-MRSA and usually presents multi-drug resistant MRSA strains (File, 2008). A similar study conducted in San Francisco in 2003 reported a prevalence of 40% of SCC*mec* type II in MRSA isolates (Carleton *et al*., 2004), while a prevalence of 34% SCC*mec* type II element was reported in Turkey MRSA isolates during 2006 (Kilic *et al*., 2006). Compared to these results, the prevalence of the HA-MRSA SCC*mec* type II detected in this study was considerably higher. Interestingly, these SCC*mec* type II MRSA strains accounted for 91% of all general surgery isolates, suggesting a possible recurrent clone of this MRSA strain.

The second highest prevalence of 14% observed for SCC*mec* type III (280 bp) (HA-MRSA) was significantly lower compared to a study conducted in Southern Brazil in 2007, which showed a prevalence of 52% (Machado *et al*., 2007). The 8% of the MRSA isolates investigated in this study that were SCC*mec* non-typeable is slightly higher compared to the 4% SCC*mec* non-typeable MRSA strains reported in 2007 from Belgian hospitals (Denis *et al*., 2005). These non-typeable MRSA strains have been described previously in the literature and can possibly be new undescribed types (Zhang *et al*., 2005). These strains were not further investigated. Methicillin-resistant *S. aureus* strains that contained multibands have been described by Zhang and colleagues (2005), where they found a similar strain with multiple bands for types II and III. After SCC*mec* typing using a M-PCR described by Oliveira and De Lencastre (2002) this strain was confirmed to

belong to SCC*mec* type III. However, no additional SCC*mec* typing methods were performed for the multiband strains detected in this study.

The *spa* typing method resulted in discrimination of three clusters (A, B and C). Genotyping of these 97 MRSA isolates by the *spa* typing method revealed that the CA-MRSA strains belonged to the same clone, however, 94% of the same cluster C isolates did not share the same SCC*mec* type IV as the CA-MRSA isolates. Forbes and colleagues (2008) conducted a similar study in Virginia, USA, which included 25 isolates (collected between September 2004 and January 2005) and their results revealed five distinct types. Furthermore, this study indicated five *spa* non-typeable MRSA strains. These five non-typeaple isolates may have been attributed to *S. aureus* isolates that do not produce the surface protein A, lacking the *spa* gene. *Staphylococcus aureus* strains that do not produce protein A have been previously described (Callegan *et al*., 1994).

Discrimination of the HVR technique differed with the *spa* typing technique. The HVR had a higher discriminatory power with six clusters identified (A, B, C, D, E and F) than the *spa* typing technique (three). This might be due to the presence of HVR in MRSA strains because HRV is located between MRSA specific *mecA* gene and IS*431mec*A (Oliviera *et al*., 2000). A similar study conducted in Brazil, 2007, indicated eight types amongst 254 MRSA isolates (Cercenado *et al*., 2008). Although, HVR showed a higher discriminatory power for all 97 isolates, the technique could not discriminate one of the CA-MRSA isolates when compared with the *spa* typing technique (Isolate 29). No correlation between these non-typeable isolates was observed with regard to SCC*mec* types or clinical presentation of the patients.

According to the results, various typing methods revealed different genetic relatedness of the strains. The limitations of this study were the observed differences in the discriminatory powers between the two PCR-based genotyping assays for characterizing MRSA isolates. These PCR-based methods have been described as having lower discriminatory power than PFGE (Nishi *et al.*, 2002). Although PFGE is the "gold standard" method in genotyping MRSA isolates, Kilic and colleagues (2008) proposed that the SCC*mec* typing method should be used as an alternative to PFGE, *spa* typing and multilocus sequence typing (MLST) to determine the clonality of MRSA isolates recovered in the hospital and/or community for MRSA epidemiologic studies (Kilic *et al*., 2008a). In comparison with PFGE, these PCR-based typing methods are rapid, inexpensive and easy to perform. Future limitations will be undescribed new SCC*mec* types that are not included in current SCC*mec* typing assays. Sequencing of these non-typeable MRSA isolates is needed to further characterize these strains and include them in SCC*mec* nomenclature.

3.6 CONCLUSIONS

Methicillin-resistant *Staphylococcus aureus* remains a worldwide concern, even more worrying is the emergence of the PVL producing CA-MRSA that can be transmitted in hospital settings. The prevalence of CA-MRSA varies in different countries such as a prevalence of 30.2% in retrospective studies involving 5 932 MRSA isolates (2003), in Virginia, USA (Salgado *et al*., 2003) and Switzerland with a low prevalence of 0.0009% in 2005 (Harbath *et al*., 2005). Community-associated MRSA strains carry the SCC*mec* type IV or V element, which is shorter than other SCC*mec* elements (Deresinski, 2005). The short size of the SCC*mec* type IV element makes it easier to transmit to other MSSA strains (Deresinski, 2005). Most outbreaks of MRSA involves CA-MRSA rather than HA-MRSA, thus monitoring the

SCC*mec* type is important in determining the epidemiologic trend of the MRSA strains in clinical settings (Deresinski, 2005). No SCC*mec* type V element was detected in the MRSA isolates analyzed in this study.

In this study, the M-PCR assay could identify 4% PVL positive MRSA strains, which confirmed a 100% PVL presence in CA-MRSA after SCC*mec* typing. The real-time PCR assay detected a 100% PVL gene presence in the same CA-MRSA isolates, which is 100% in agreement with the first M-PCR assay. Staphylococcal cassette chromosome *mec* typing by M-PCR assay revealed three prevalent SCC*mec* types including SCC*mec* type II (67%), SCC*mec* type III (14%) and SCC*mec* type IV (4%) in the Steve Biko Academic Hospital MRSA isolates.

This study indicated CA-MRSA strains are prevalent in the Steve Biko Academic Hospital. These CA-MRSA isolates are problematic because most were isolated from ICU in-patients. Panton-Valentine leukocidin causes necrotizing pneumonia, which is usually severe and fatal in patients (Ebert *et al*., 2008). *Staphylococcus aureus* producing the PVL toxin carries a mortality rate of up to 75% and typically affects children and healthy patients (Ebert *et al*., 2008). The CA-MRSA strains identified in this study carried a 100% PVL presence posing a major health threat.

In order to eradicate the spread and transmission of these CA-MRSA strains, rapid diagnostic techniques such as M-PCR to detect these virulent strains of MRSA have to be implemented for the successful identification and treatment of these strains in hospitals and from communities. The correct implementation of hospital infection control procedures need to be taken to prevent spread and outbreaks in clinical setting. Panton-Valentine leukocidin producing CA-MRSA isolates are to be considered in the suspicion of pneumonia (Lina *et al*., 1999). Pneumonia associated with

PVL producing CA-MRSA has been reported to have a mortality rate of 37% (Ebert *et al*., 2008) within 48 h and some studies report even a higher mortality rate of 75% (Gillet *et al*., 2002).

The PCR-based identification and typing techniques used in this study proved to be rapid and accurate for the detection of MRSA strains. These techniques can enable diagnostic laboratories to determine the prevalence and to monitor possible HA-MRSA, CA-MRSA as well as PVL-positive strains that might be circulating in the hospital. Based on these data, surveillance programs can be set in place for the control of MRSA strains.

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TABLES

Table 3.1: Nucleotide sequences of the primers used in the first M-PCR for the simultaneous detection of the 16S rRNA gene, the *mecA* and the PVL virulence gene (McClure *et al*., 2006)

Primer	Oligonucleotide Sequence (5'-3')	Conc (µM)	Amplicon size (bp)	Specificity
Staph 756F Staph 750R	5'-AACTCTGTTATTAGGGAAGAACA-3' 5'-CCACCTTCCTCCGGTTTGTCACC-3'	0.2	756 bp	16S rRNA
MecA1-F MecA2-R	5'-GTAGAAATGACTGAACGTCCGATAA-3' 5'-CCAATTCCACATTGTTTCGGTCTAA-3'	0.24	310 bp	*mecA*
Luk-PV-1F Luk-PV-2R	5'-ATCATTAGGTAAAATGTCTGGACATGATCCA-3' 5'-GCATCAAGTGTATTGGATAGCAAAAGC-3'	0.2	433 bp	*lukS/F-PV*

Table 3.2: Nucleotide sequences of the primers and minor groove binding probe (MGB) used for the real-time PCR detection of the PVL genes (Deurenberg *et al*., 2004)

Primer/Probe	Oligonucleotide Sequence	Position	Primer/Probe
PVL-FP	5'-GCTGGACAAAACTTCTTGGAATAT-3'	2666- 2690	PVL-FP
PVL-RP	5'-GATAGGACACCATAAATTCTCTC-3'	2749-2723	PVL-RP
MGB Taqman probe	5'-AAAATGCCAGTGTTATCCA-3'	2694-2712	MGB probe

Table 3.3: Nucleotide sequences of the primers used for the second PCR for the typing and subtyping of MRSA SCC*mec* (Zhang *et al*., 2005)

Primer	Oligonucleotide Sequence (5'-3')	Conc (µM)	Amplicon size (bp)	Specificity
Type I-F Type I-R	5'-GCTTTAAAGAGTGTCGTTACAGG-3' 3'-GTTCTCTCATAGTATGACGTCC-5'	0.2	613	SCC*mec* I
Type II-F Type II-R	5'-CGTTGAAGATGATGAAGCG-3' 3'-CGAAATCAATGGTTAATGGACC-5'	0.2	398	SCC*mec* II
Type III-F Type III-R	5'-CCATATTGTGTACGATGCG-3' 3'-CCTTAGTTGTCGTAACAGATCG-5'	0.2	280	SCC*mec* III
Type IVa-F Type IVa-R	5'-GCCTTATTCGAAGAAACCG-3' 3'-CTACTCTTCTGAAAAGCGTCG-5'	0.2	776	SCC*mec* IVa
Type IVb-F Type IVb-R	5'-TCTGGAATTACTTCAGCTGC-3' 3'-AAACAATATTGCTCTCCCTC-5'	0.2	493	SCC*mec* IVb
Type IVc-F Type IVc-R	5'-ACAATATTTGTATTATCGGAGAGC-3' 3'-TTGGTATGAGGTATTGCTGG-5'	0.2	200	SCC*mec* IVc
Type IVd-F5 Type IVd-R6	5'-CTCAAAATACGGACCCCAATACA-3' 3'-TGCTCCAGTAATTGCTAAAG-5'	0.2	881	SCC*mec* IVd
Type V-F Type V-R	5'-GAACATTGTTACTTAAATGAGCG-3' 3'-TGAAAGTTGTACCCTTGACACC-5'	0.2	325	SCC*mec* V
MecA147-F MecA147-R	5'-GTGAAGATATACCAAGTGATT-3' 3'-ATGCGCTATAGATTGAAAGGAT-5'	0.2	147	*mec*A

Table 3.4: Nucleotide sequences for the *spa* typing of MRSA isolates (Schmitz *et al*., 1998)

Primer	Oligonucleotide sequence	Concentration	Specificity
SPA1	5'-CCACCAAATACAGTTGTACCG-3'	0.25 µM	1702-1682
SPA3	5'-CTTTGGATGAAGCCGTTGCGTTG-3'	0.25 µM	1088-1066

Table 3.5: Nucleotide sequences of the primers for the HVR typing of MRSA isolates (Schmitz *et al*., 1998)

Primer	Oligonucleotide sequence	Concentration	Target Positions
HVR2	5'-GGAGTTAATCTACGTCTCATC-3'	0.25 µM	892-912
HVR3	5'-CTAAGTAAAATTGCAGATAAGAGG-3'	0.25 µM	441-464

Table 3.6: Summary of the characteristics of SCC*mec* types and subtypes in this study including prevalence and origin of clinical wards

SCC*mec* type	CA-MRSA/HA-MRSA	Base pair size	Prevalence %	Ward	PVL
SCC*mec* type I	HA-MRSA	613 bp	-	-	-
SCC*mec* II	HA-MRSA	398 bp	67	90% isolated from general surgery ward	-
SCC*mec* type III	HA-MRSA	280 bp	14	CTS, INT-M, 2 from neonatal ICU, Casualty, 2 from M-ICU, ENT, ARV,Diabetic clinic, GS and PSS	-
SCC*mec* type IVa	CA-MRSA	776 bp	-	-	-
SCC*mec* type IVb	CA-MRSA	493 bp	-	-	-
SCC*mec* type IVc	CA-MRSA	200 bp	-	-	-
SCC*mec* type IVd	CA-MRSA	881 bp	4	2 from neonatal 1 from casualty 1 from M-ICU	All+
SCC*mec* type V	CA-MRSA	325 bp	-	-	-
Multibands SCC*mec* type II and III	Characterised to SCC*mec* type II HA-MRSA (Zhang *et al.*, 2005)	(398+280 bp)	3	KF, INT-M,PS	-
Multibands SCC*mec* type III and IVc	Unidentified	(280+200 bp)	1	-	-
Multibands SCC*mec* II and IVd	Unidentified	(398+881 bp)	2	Neonatal ICU and Neurosurgery	-
Non-typeable	-	Only *mecA* (147 bp)	8	CTS, Casualty,ARV	-

General abbreviations

CA-MRSA	Community-associated MRSA
HA-MRSA	Health-care associated MRSA
SCC*mec*	Staphylococcal cassette chromosome
PVL	Panton-Valentine leukocidin

Ward abbreviations

CTS	Cadiothoracic surgery
INT-M	Internal medicine
ARV	Antiretroviral clinic
GS	General surgery
PSS	Paediatric short stay
PS	Paediatric surgery
KF	Kalafong

Table 3.7: Clinical origin and presentaion of the MRSA isolates and their corresponding SCC*mec* types

Reference numbers		Clinical ward	Disease/Infection	Specimen type	SCC*mec* type
450061	UP1	6.5 General Surgery	Wound infection	BC	II
458408	UP2	Cardio-thoracic surgery	Not stated	Sputum	III
462103	UP3	Neonatal	Premature	BC	II
453044	UP4	Neurosurgery	Meningitis	CSF	II
479445	UP5	Casualty	H/S	Pus swab	II
460267	UP6	Internal medicine	Cystic fibrosis	Sputum	III
457531	UP7	Paediatric pulmonary	N/S	Sputum	II
450060	UP8	General surgery	N/S	BC	II
460269	UP9	Internal medicine	Sepsis	BC	II
502951	UP10	Orthopaedics	Multi-trauma (Wound infection)	Pus swab	II
450715	UP11	Neonatal ICU	diarrhea	Urine	III
449410	UP12	ICU	Burn wounds	BC	II
460188	UP13	General surgery	Vasculitis	BC	II
462399	UP14	Cardio-thoracic surgery	Post-tracheal repair	Luki	Non-typeable
487796	UP15	Neonatal	Pneumonia	BC	IVd
460268	UP16	Internal medicine	Sepsis	BC	II
454989	UP17	Casualty	Vomiting	Stool	III
452683	UP18	Neurosurgery	Head injury	Luki	II
460187	UP19	General surgery	Vasculitis	BC	II
466741	UP20	Medical isolation	Not stated	Luki	III
465750	UP21	Neuro out-patient department	Head injury	Luki	II
465028	UP22	General surgery	Hernia (abdominal)	Pus swab	II
468439	UP23	Neurosurgery	Head injury	ETA	II
467473	UP24	ENT	Not stated	Pus swab	III
462693	UP25	Cardio-thoracic surgery	Not stated	Luki	II
467497	UP26	ARV	Not stated	Pus swab	III
462116	UP27	Casualty	Bleeding tendencies	BC	Non-typeable
478105	UP28	High care	Head/Spinal injury	Sputum	II
454910	UP29	Neonatal	Sepsis	BC	IVd
467810	UP30	Diabetic clinic	Infected ingrown hair	Pus swab	III
465759	UP31	Paediatric ICU	Not stated	BC	II
478232	UP32	Orthopaedics	Human bite	Tissue	II
474977	UP33	Orthopaedics	H femur	Pus swab	II
470227	UP34	Kalafong	Sepsis	BC	Multiband (II and III)
473098	UP35	ARV	Recurrent MRSA	Urine	Non typeable
459677	UP36	Internal medicine	Sepsis	BC	Multiband (II and III)
480457	UP37	General surgery	Diabetes	Pus swab	II

Table 3.7: Clinical origin and presentaion of the MRSA isolates and their corresponding SCC*mec* types (continued)

467291	UP38	ARV	Not stated	Pus swab	II
494455	UP39	Casualty	Not stated	BC	II
512281	UP40	N/S	Not stated	Ear swab	II
520830	UP41	Medical ICU	Multilobar pneumonia	Luki	II
542213	UP42	Neurosurgery	Head injury	Luki	II
498511	UP43	Kalafong 2A	Diarrhea	BC	II
512993	UP44	Trauma ICU	Not stated	Luki/ETA	II
510561	UP45	Trauma ICU	Renal failure	BC	II
512188	UP46	Paediatric surgery	Not stated	BC	II
482464	UP47	Casualty	Not stated	Tissue	II
497415	UP48	Neurosurgery	Brain tumor	Luki	II
482363	UP49	Neurosugery	Not stated	Luki	II
493743	UP50	Kalafong	Meningitis	BC	Non-typeable
491203	UP51	Trauma ICU	Not stated	ETA	II
505609	UP52	Paediatric short stay	Not stated	Stool	III
538713	UP53	Renal	Not stated	Peritoneal-dialysis fluid	II
659156	UP54	Neonatal	Not surgery	BC	III
487087	UP55	Paediatric surgery	Duodenal adhesions	BC	Multiband (II and III)
524112	UP56	General surgery	Not stated	Pus swab	II
523448	UP57	General surgery	Abdominal pain	CVT Tip	III
492778	UP58	Neurosurgery	Not surgery	Luki	II
542213	UP59	Neurosurgery	Head injury	Luki	Multiband (II and IVd)
540645	UP60	General surgery	Burn wound	Pus swab	II
659699	UP61	Occupational health	For MRSA screening health-care worker	Nasal swab	II
657260	UP62	MICU	Conjestive heart failure	BC	II
659156	UP63	Medical ICU	Pneumonia	BC	IVd
492778	UP64	Neurosurgical	Not stated	Luki	II
495138	UP65	Neurosurgical	Head injury	Luki	II
490574	UP66	Kalafong	PCP pneumonia	BC	III
461777	UP67	Casualty	Polytrauma	Sputum	IVd
541232	UP68	Neonatal ICU	Premature	BC	II
540645	UP69	General surgery	Burn wounds	Pus swab	II
500936	UP70	Casualty	Abscess	Pus swab	Multiband (II and IVd)

Table 3.7: Clinical origin and presentaion of the MRSA isolates and their corresponding SCC*mec* types (continued)

UP71, UP79, UP90 and U96	Clinical data not available	-	-	Non-typeable
UP72, UP74-UP78, UP80-UP85, UP87, UP88, UP91-UP94, UP96 and UP97	Clinical data not available	-	-	II
UP73	Clinical data not available	-	-	Multiband (III and IVc)
UP74, UP86 and UP89	Clinical data not available	-	-	III

4	**IVd**
2	**II and IVd**
3	**II and III**
14	**III**
65	**II**
8	**non-typeable**
1	**III and IVc**

FIGURES

Figure 3.1: **Single PCR for the detection of the 16S rRNA, *mecA* and PVL genes. Lane M, (433 bp) represents the 100 bp molecular weight marker. Lane 1, represents the PVL positive band of CA-MRSA. Lane 2, the *S. aureus* specific 16S rRNA gene (756 bp) and Lane 3, the *mecA* (310 bp) (methicillin-resistant gene). Lane, 4 and 5 represents the negative and positive control respectively.**

Figure 3.2: **Gel electrophoresis results of MRSA isolates 1-16 for the detection of 16S rRNA, *mecA* and PVL genes. This M-PCR simultaneously amplified the 16S rRNA (756 bp), the *mecA* (310 bp) and the PVL genes (433 bp). Lane 1-8 are 16S rRNA (756 bp) and *mecA* (310 bp) positive isolates. Lane 9-12 are 16S rRNA, *mecA* and PVL positive MRSA isolates. In Lane 17, the negative water control was included. The positive control MRSA strain (ATCC CA05) was included in Lane 18. Lanes M represents the molecular weight marker (100 bp DNA ladder, Promega, Madison,USA)**

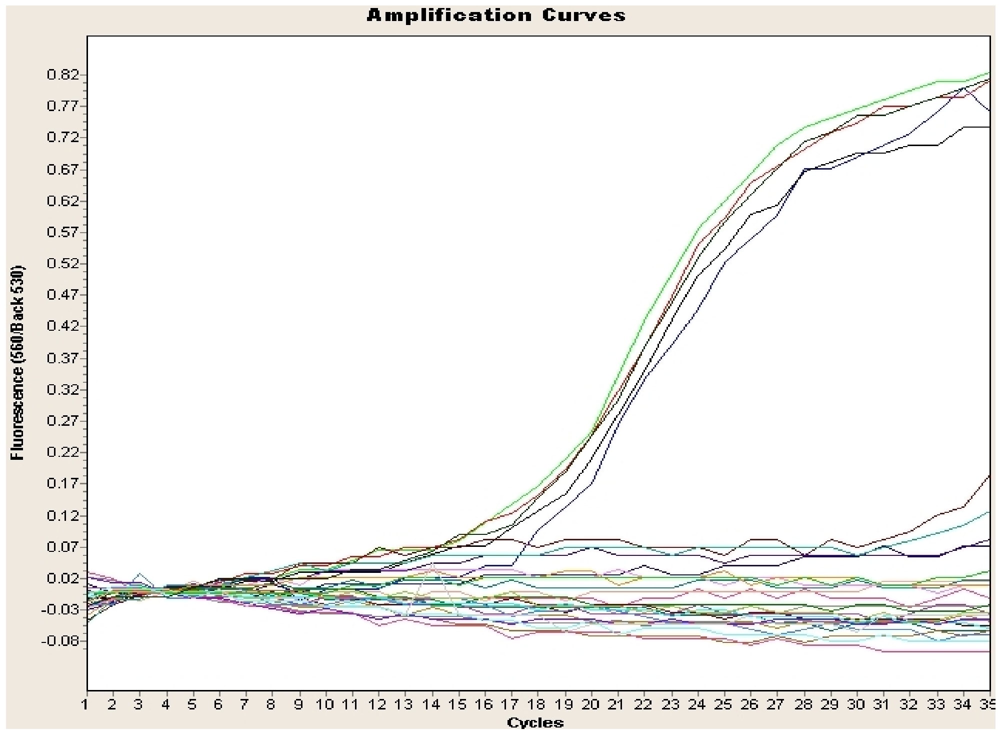

Figure 3.3: **Real-time PCR fluorescence amplification curves of MRSA isolates showing five amplified PVL genes including a positive control (ATCC CA05).**

Figure 3.4: **Gel electrophoresis results for the multiplex PCR for the SCC*mec* type characterisation of MRSA isolates. Lane 1 an 19 represents molecular weight marker. Lane 2, 3, 4 and 5 represent SCC*mec* type IV (881 bp) isolates. Lane 6 and 7 represent the multiband (398 and 881 bp) MRSA isolates. Lane 8 and 9 represent SCC*mec* type III (280 bp) isolates. Lane 10 and 11 represent multiband (398 and 280 bp) isolates. Lane 12 represent multiband (398 and 200 bp) isolate. Lane 13, 14 and 15 represent SCC*mec* type III (398 bp) isolates. Lane 16 and 17 represent the non-SCC*mec* typeable strains of MRSA strains.**

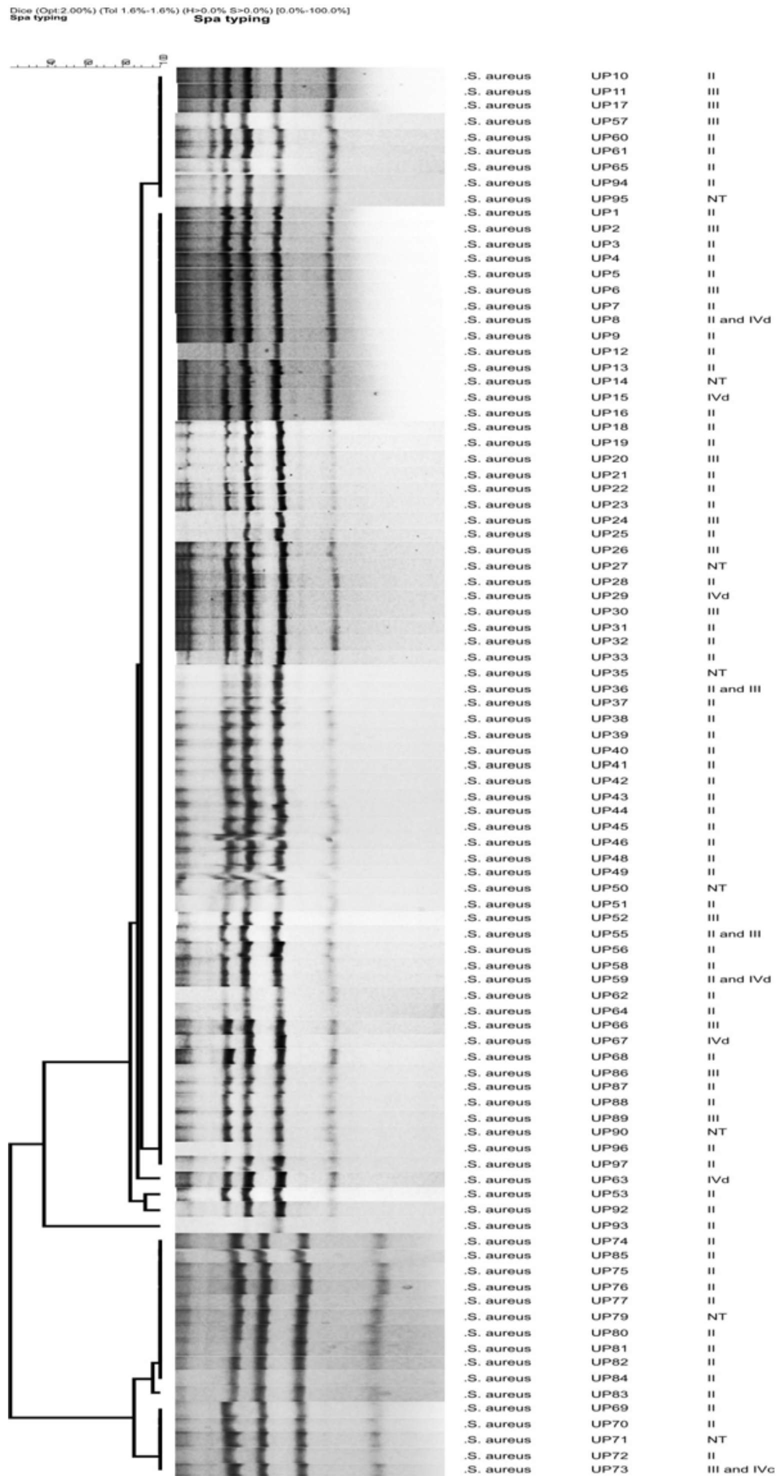

Figure 3.5: **Dendrogram obtained for *spa* typing, depicting clonal relationship (three clusters A, B and C) and SCC*mec* types of 97 MRSA clinical isolates obtained from the Steve Biko Academic Hospital, Gauteng, South Africa**

Dice (Opt:5.00%) (Tol 1.0%-1.0%) (H>0.0% S>0.0%) [0.0%-100.0%]

HVR typing **HVR typing**

.S. aureus	UP53	II
.S. aureus	UP92	II
.S. aureus	UP55	II and III
.S. aureus	UP76	II
.S. aureus	UP78	II
.S. aureus	UP80	II
.S. aureus	UP84	II
.S. aureus	UP86	III
.S. aureus	UP88	II
.S. aureus	UP22	II
.S. aureus	UP27	NT
.S. aureus	UP28	II
.S. aureus	UP32	II
.S. aureus	UP33	II
.S. aureus	UP77	II
.S. aureus	UP79	NT
.S. aureus	UP82	II
.S. aureus	UP83	II
.S. aureus	UP85	II
.S. aureus	UP87	II
.S. aureus	UP89	III
.S. aureus	UP26	III
.S. aureus	UP70	II
.S. aureus	UP52	III
.S. aureus	UP21	II
.S. aureus	UP23	II
.S. aureus	UP69	II
.S. aureus	UP3	II
.S. aureus	UP4	II
.S. aureus	UP5	II
.S. aureus	UP6	III
.S. aureus	UP7	II
.S. aureus	UP8	II and IVd
.S. aureus	UP9	II
.S. aureus	UP12	II
.S. aureus	UP1	II
.S. aureus	UP2	III
.S. aureus	UP67	IVd
.S. aureus	UP56	II
.S. aureus	UP65	II
.S. aureus	UP60	II
.S. aureus	UP61	II
.S. aureus	UP62	II
.S. aureus	UP64	II
.S. aureus	UP66	III
.S. aureus	UP15	IVd
.S. aureus	UP16	II
.S. aureus	UP57	III
.S. aureus	UP58	II
.S. aureus	UP59	II and IVd
.S. aureus	UP63	IVd
.S. aureus	UP68	II
.S. aureus	UP39	II
.S. aureus	UP40	II
.S. aureus	UP43	II
.S. aureus	UP46	II
.S. aureus	UP47	II
.S. aureus	UP48	II
.S. aureus	UP49	II
.S. aureus	UP51	II
.S. aureus	UP50	NT
.S. aureus	UP72	II
.S. aureus	UP17	III
.S. aureus	UP35	NT
.S. aureus	UP36	II and III
.S. aureus	UP37	II
.S. aureus	UP38	II
.S. aureus	UP25	II
.S. aureus	UP29	IVd
.S. aureus	UP24	III
.S. aureus	UP31	II
.S. aureus	UP18	II
.S. aureus	UP19	II
.S. aureus	UP20	III
.S. aureus	UP73	III and IVc
.S. aureus	UP75	II
.S. aureus	UP95	NT
.S. aureus	UP96	II
.S. aureus	UP97	II
.S. aureus	UP13	II
.S. aureus	UP14	NT
.S. aureus	UP41	II
.S. aureus	UP42	II
.S. aureus	UP30	III
.S. aureus	UP45	II
.S. aureus	UP34	II and III
.S. aureus	UP54	III
.S. aureus	UP81	II
.S. aureus	UP93	II
.S. aureus	UP94	II
.S. aureus	UP71	NT
.S. aureus	UP74	II
.S. aureus	UP11	III
.S. aureus	UP44	II

Figure 3.6: Dendrogram obtained for HVR typing, depicting clonal relationship (six clusters A, B, C, D, E and F) and SCC*mec* types of 97 MRSA clinical isolates obtained from the Steve Biko Academic Hospital, Gauteng, South Africa

CHAPTER 4

Antibiotic susceptibility profiles of methicillin-resistant *Staphylococcus aureus* isolates from Steve Biko Academic Hospital

The editorial style of the Southern African Journal of Epidemiology and Infection was followed in this chapter

4.1 ABSTRACT

Background: Methicillin-resistant *Staphylococcus aureus* was first identified in European hospitals in the 1960s, a year after the introduction of methicillin and since then, it has been a progressive pathogen with a prevalence of 54% or higher globally. Community-associated methicillin-resistant *S. aureus* (CA-MRSA) strains are increasingly reported with a presentation of severe pneumonia, which can be fatal. The aim of the study was to investigate the antibiotic susceptibility profiles of MRSA strains including previously identified CA-MRSA strains.

Methods: Ninety-seven MRSA collected from April 2006 to September 2007 were profiled according to their antibiotic susceptibility pattern using the Kirby Bauer disk-diffussion method. Included in the study was the prevalence of the clinical diseases associated with MRSA infections. The MRSA isolates were previously subjected to a multiplex PCR (M-PCR) for *mecA* and PVL detection and SCC*mec* characterisation to distinguish CA-MRSA from health-care associated MRSA (HA-MRSA)

Results: Antibiotic susceptibility revealed six distinct susceptibility types. All isolates were resistant to penicillin and cloxacillin and all isolates were sensitive to vancomycin. A total of 73%, 71%, 70%, and 7% of the isolates were resistant to gentamycin, erythromycin, clindamycin and fusidic acid, respectively. Lung, bloodstream, skin and soft tissue, gastrointestinal infections accounted for 31%, 30%, 23% and 7% of all diseases.

Conclusion: The high resistance rates of MRSA to clindamycin, erythromycin, gentamycin render the use of these antibiotics in MRSA infections unreliable. Monitoring and surveillance of

the antibiotic susceptibility trends for MRSA is of utmost importance in revision and amendment of antibiotic regimen guidelines for MRSA infections.

4.2 INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a pandemic human pathogen accounting for most of the nosocomial infections worldwide (Oliviera *et al*., 2002). The virulence of methicillin-resistant *Staphylococcus aureus* is no more than that of methicillin-susceptible *Staphylococcus aureus* (MSSA) (Karauzum *et al*., 2008). Methicillin-resistant *Staphylococcus aureus* is associated with a wide range of diseases including pneumonia, sepsis, food poisoning, skin and soft tissue infections (Shittu and Jin, 2006). There is a high morbidity and mortality rate associated with MRSA infections, this can be attributed to failure in treatment often as a result of antibiotic resistance variation of MRSA (Shittu and Jin, 2006).

Methicillin-susceptible *Staphylococcus aureus* strains convert to MRSA by acquiring a mobile genetic element known as the staphylococcus cassette chromosome (SCC) (Chongtrakool *et al*., 2006). The SCC element harbours the methicillin-resistance conferring gene, *mecA* (Katayama *et al*., 2000). The *mecA* encodes the altered penicillin-binding protein 2a (PBP2a), which has reduced affinity to beta-lactam antibiotics (Fuda *et al*., 2004). The acquisition of the *mecA* gene complex confirms resistance in *S. aureus* (Chongtrakool *et al*., 2006). Five different SCC*mec* types (I-V) can be distinguished among the respective MRSA strains (Ito *et al*., 2001). The SCC*mec* types I-III are found in HA-MRSA whilst type IV and V are present in CA-MRSA strains (Naimi *et al*., 2003). The SCC*mec* elements also carry other resistance genes such as *Tn554*, a transposon responsible for resistance to macrolides, clindamycin and streptogramin B while the *pT181* plasmid accounts for resistance to tetracyclines (Oliviera *et al*., 2002).

The earliest report of MRSA in Africa was in South Africa in 1978 (Shittu and Jin, 2006). Since then, the rise of MRSA in hospitals and communities has thrived to epidemic proportions (Hart and Kariuki, 1998). In 2005, Sein and colleagues reported a prevalence of 76% of all *S. aureus* isolates resistant to methicillin in Dr George Mukhari Hospital (Sein *et al*., 2005). The study also reported a MRSA prevalence of 57% and 83% in 2004 and 2002, respectively in the Steve Biko Academic Hospital previously known as the Pretoria Academic Hospital. The high prevalence of MRSA prompts adequate treatment for MRSA infections globally.

To complicate the treatment of MRSA infections further, was the emergence of a non-beta lactam susceptible CA-MRSA which carries the short SCC*mec* type IV element (File, 2008). The non-beta lactam antibiotics includes clindamycin and erythromycin (Deresinski, 2005). Community-associated MRSA strains are problematic because they are more virulent than HA-MRSA strains (Kilic *et al*., 2008). The increased virulence in CA-MRSA is due to the production of the Panton-Valentine leukocidin toxin, which is carried by 77%-100% of CA-MRSA strains (Ebert *et al*., 2008; Kilic *et al*., 2008). The PVL toxin is frequently associated with skin and soft tissue infections and necrotising community-associated pneumonia, which can be fatal (Hsu *et al*., 2005).

Treatment of MRSA infections includes clindamycin, erythromycin, fusidic acid and gentamycin. However, high MRSA resistance rates to these antibiotics have been reported in South Africa as well as worldwide (Sein *et al*., 2005; Kilic *et al*., 2006), thus treatment of MRSA infections has become problematic (Takizawa *et al*., 2005). Vancomycin and linezolid has been used as empirical treatment for MRSA infections (Stevens, 2006). The aim of this study was to determine the prevalence and susceptibility patterns of MRSA strains from the Steve Biko Academic Hospital.

4.3 MATERIALS AND METHODS

4.3.1 Bacterial isolates

Ninety seven (97) MRSA isolates were obtained from clinical specimens sent from an academic hospital for analysis to the diagnostic laboratory at the Department of Medical Microbiology, University of Pretoria/NHLS, South Africa. These 97 MRSA isolates were collected between April 2006 to February 2007. The isolates were collected randomly from all specimens including blood, sputum, pus swabs, stool, urine and luki. These bacterial isolates were identified as *S. aureus* based on their morphology, Gram-staining and catalase properties according to the CLSI guidelines, (2006). Coagulase and DNase tests were performed to identify *S. aureus* isolates.

4.3.2 Antibiotic susceptibility determination of the MRSA isolates

Resistance to methicillin was detected using the Kirby-Bauer disc diffusion method (CLSI, 2006). Briefly, *S. aureus* colonies were isolated from sheep blood agar plates (Oxoid, England) and inoculated in 0.85% (5 ml) of saline (CLSI, 2006). Turbidity was adjusted to 0.5 McFarland standards (1.5 x 10^8 $cfu.ml^{-1}$). Fifteen minutes after turbidity adjustment, the swab was dipped into the inoculum and streaked evenly onto Mueller-Hinton agar plates (Oxoid, England) and left to dry for 15 min. Within 15 min of drying the plates, the following antibiotic discs: clindamycin (2 µg), cloxacillin/cefotaxime (30 µg), erythromycin (15 µg), fusidic acid (10 µg), gentamycin (10 µg), penicillin-G (10 U) and vancomycin (30 µg) (MAST Diagnostics, Merseyside, UK) were applied aseptically. The plates were incubated at 37°C (Horo, incubator) for 18 hrs. After incubation, the diameter of the inhibition zones was measured with a ruler. Results were interpreted according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2006) previously known as the National Committee for Clinical Laboratory Standards. In addition, all 97 MRSA isolates were confirmed as MRSA by the detection of the *mecA* gene in the previous study

4.3.3 Statistical analysis

With a sample size of 97, a two-sided 95% confidence interval for a single proportion using the large sample normal approximation will extend 0.1 from the observed proportion for an expected proportion of 0.5. The latter choice being conservative in the absence of prevalence estimates for the *mecA* from the previous study, the antibiotics and clinical diseases. The prevalence of antibiotypes, resistance to each antibiotic and clinical diseases were expressed as percentages.

4.4 RESULTS

All 97 isolates (100%) were resistant to penicillin and cloxacillin indicating methicillin resistance, but all (100%) isolates were sensitive to vancomycin. The percentage of the MRSA isolates that were resistant to gentamycin, erythromycin, clindamycin and fusidic acid were 73%, 71%, 70%, and 7%, respectively. All 97 MRSA isolates including the four previously identified CA-MRSA strains were grouped into antibiotypes (Table 4.1).

Table 4.1: Antibiotic susceptibility profiles of MRSA isolates

Antibiotic susceptibility type	Antibiotics							% of MRSA isolates	% PVL isolates
	PEN	GM	EM	CD	CLX	FA	Van		
1	R	R	R	R	R	S	S	60	2
2	R	S	S	S	R	S	S	24	2
3	R	S	R	R	R	S	S	4	-
4	R	R	R	R	R	R	S	6	-
5	R	R	S	S	R	S	S	4	-
6	R	R	R	S	R	R	S	1	-

PEN Penicillin
GN Gentamicin
EM Erythromycin
CD Clindamycin
CLX Cloxacillin
FA Fusidic acid
Van Vancomycin

S Susceptible
R Resistant

All 97 MRSA isolates were analysed into clinical diseases they caused as well as origin of isolation. The prevalence of each disease is summarised in Table 4.2. The majority of the MRSA infections were isolated from the general surgery ward, neuro-surgery ward and neonatal ward, each with a prevalence of 14%, 14% and 9%, respectively.

Table 4.2: Prevalence of the clinical diseases and infections associated with MRSA isolates

Diseases/Infections	Prevalence %
Lung infections (including pneumonia)	31
Blood stream infection (sepsis and vasculitis)	30
Skin and soft tissue infections (wounds, abscesses and infected grown hair)	23
Gastro-intestinal infections (vomiting with or without diarrhoea, abdominal cramps)	7
Other (including meningitis, UTIs)	9

Pneumonia contributed to 18% of all lung infections and 6% of the total infections. A total of 38%, 19%, 19%, 7%, and 14% of the specimens were isolated from blood, pus swabs, luki, sputum, and others including stool, urine, CSF, tissue and peritoneal dialysis fluid, respectively.

4.5 DISCUSSION

Community-associated MRSA strains are reported to be susceptible to non-beta-lactam antibiotics; however, this study showed a prevalence of 50% of CA-MRSA strains that are resistant to all drugs, excluding fusidic acid and vancomycin. The multi-drug resistance in these CA-MRSA isolates could be mediated by other resistance mediating mechanisms, such as plasmids instead of the SCC*mec* element. The other 50% of CA-MRSA strains depict the “classic” non-beta-lactam susceptibility pattern. These two CA-MRSA strains belonged to antibiotype 2, which describes susceptibility to all antibiotics, except penicillin and cloxacillin.

Antibiotype 1 represented resistance to all drugs, except fusidic acid and vancomycin. The multi-drug resistance in antibiotype 1 accounted for 60% of the MRSA isolates predominating in this

clinical setting. The emergence of this pattern is of utmost concern because these two antibiotics, fusidic acid and vancomycin are expensive and not readily available (Yves and Paul, 2001; Penel and Yazdanpanah, 2008). Furthermore, this study reported fusidic resistant MRSA isolates (7%) circulating in the clinical setting. Even more concerning is the emergence of antibiotype 4 which represents resistance to all antibiotics except vancomycin with a prevalence of 6%, which is relatively low at this stage.

Gentamicin (aminoglycoside) resistance is accepted in MRSA isolates (Barada *et al*., 2007). A study conducted on MRSA isolates between 2000 and 2002 in Japan showed that 47% of the isolates were gentamicin resistant (Barada *et al*., 2007). Another study reported a 95% susceptibility of MRSA isolates to gentamicin in Besançon hospital, France (Thouverez *et al*., 2003). In this study, a 73% prevalence of gentamicin resistance was found in MRSA isolates. Gentamicin resistance in MRSA is mainly by aminoglycoside degrading enzymes encoded by the *aph*, *aac* and *aad* genes (Gardam, 2000).

Macrolide resistance in MRSA strains was first reported in the early 1970s (Khare and Jenkins, 2006). Erythromycin (macrolide) resistance in MRSA has been reported to be as high as 76% in a study conducted in Istanbul in 2005 (Nurittin *et al*., 2005). Erythromycin prevalence was 71% in this study. However, some countries report a decline in the trend of erythromycin susceptible MRSA, as in Germany where a decrease from 94% in 1994 to 68% in 1998 was reported (Khare and Jenkins, 2006). The reason for this decrease could not be determined.

The antibiotic, fusidic acid, has been shown to have excellent antimicrobial activity against *S. aureus* isolates for the past 20 years (Ravenscroft *et al*., 2000). Fusidic acid is widely used for MRSA skin infections and is available as a topical cream/ointment (Shah and Mohanraj, 2003). The resistance rate of fusidic acid in *S. aureus* was 10% in Yorkshire, UK in 2001 (Shah and Mohanraj, 2003) when compared with this study, which indicated a prevalence of 7%. Although the fusidic resistance prevalence is lower than the other reported antibiotics such as gentamicin and erythromycin, resistance is higher (50%) in dermatological patients (Shah and Mohanraj, 2003). Fusidic acid treatment of MRSA skin infections should be used for short periods to minimise the development of resistance (Shah and Mohanraj, 2003).

4.6 CONCLUSIONS

The emergence of community-associated MRSA is not limited to the community anymore but these strains are progressively introduced into the hospital setting. The inclined usage of these antibiotics promotes resistance in bacteria such as *S. aureus* due to selective pressure (Khare and Jenkins, 2006). The correct susceptibility pattern of the MRSA isolates needs to be determined rapidly for accurate treatment of MRSA infections. This study highlighted the prevalence of the resistance profiles and clinical presentations of MRSA strains circulating in the Steve Biko Academic Hospital, thus can be used as a reference for antibiotic resistance trends of MRSA strains in South Africa.

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CHAPTER 5

5.1 CONCLUDING REMARKS

The incidence of methicillin-resistant *Staphylococcus aureus* in the hospital and community continues to rise globally (Tenover, 2006). Methicillin-resistant *S. aureus* causes high rates of morbidity and mortality in hospitals in many geographical areas (Griffiths *et al*., 2004). Clinical diseases associated with community-associated MRSA (CA-MRSA) are even more concerning because the residence of CA-MRSA strains is now in health-care facilities including intensive care units (ICUs) (Klevens *et al*., 2005). The increasing shift in the epidemiology of CA-MRSA greatly affects antibiotic usage globally because health-care associated MRSA (HA-MRSA) and CA-MRSA have different antibiotic resistant profiles (Tenover, 2006).

The introduction of MRSA into non-health-care associated community is hypothesised to be two mechanisms of transmission (Gardam, 2000). The first mechanism is possibly due to the inevitable spread of these health-care associated strains as a result of the release of colonised patients back into the community (Gardam, 2000). Earlier studies described CA-MRSA cases that involved patients that have been hospitalised previously (Gardam, 2000). The second mechanism of CA-MRSA transmission and spread involves novel CA-MRSA strains that are genetically distinct from HA-MRSA strains (Gardam, 2000). This variation in genetic material is shown in the different antibiotic susceptibility profiles of these two MRSA strains. (Klevens *et al*., 2005).

In this study, two multiplex-PCR (M-PCR) assays, a real-time PCR assay and two PCR based genotyping techniques were evaluated for the identification and characterisation of 97 MRSA isolates obtained from the Steve Biko Academic Hospital between April 2006 and September 2007. Antibiotic susceptibility profiles of these MRSA isolates was also determined.

The first M-PCR used three pairs of primers to detect the *Staphylococcus aureus* specific 16S rRNA, the *mecA* and the PVL genes. All (100%) MRSA isolates were positive for the 16S rRNA gene, which confirmed that the isolates were *S. aureus* strains. This M-PCR determined the

prevalence of the *mecA* gene by a M-PCR assay in MRSA and revealed a 99% presence. This suggested that not all MRSA strains are *mecA* resistance-mediated. The non-*mecA* methicillin-resistant mechanism was previously observed in other strains (Mongkolrattanothai *et al*., 2004). Suprisingly, these strains carried the SCC*mec* elements, indicating the possible loss of the *mecA* genes in the SCC*mec* element. Simillar non-*mecA* containing SCC elements have previously been described in the literature, but the *mecA*-loss mechanism has not been described (Hanssen and Sollid, 2006). The PVL gene was detected in 4% of the MRSA strains.

Furthermore, a real-time PCR assay was used to determine the effective detection of the PVL gene associated with CA-MRSA strains, which causes necrotising pneumonia that is usually severe and fatal in patients (Ebert *et al*., 2008). The real-time PCR assay detected a 100% prevalence presence in the same CA-MRSA isolates, which is in agreement with the first M-PCR assay for the PVL gene detection. *Staphylococcus aureus* producing the PVL toxin is responsible for a mortality rate of up to 75% and typically affects children and healthy patients (Ebert *et al*., 2008). In this study, the PVL gene was detected in SCC*mec* types IV isolates using the real-time PCR. The dissemination of the PVL toxin gene in MRSA strains is important and should be monitored for the purposes of observing the trends in MRSA virulence in this clinical setting (Ebert *et al*., 2008).

All MRSA isolates were further subjected to a M-PCR assay for the characterisation of the SCC*mec* element. Genetic variation between CA-MRSA from HA-MRSA is primarily based on the specific staphylococcal cassette chromosome *mec* (SCC*mec* element) (Kilic *et al*., 2008). Using a second conventional M-PCR assay for SCC*mec* typing of 97 MRSA isolates, HA-MRSA strains accounted for 96% of all the MRSA isolates. The major problem with HA-MRSA strains is multi-drug resistance, due to the carriage of additional genetic material in SCC*mec* type II and III (Deresinski, 2005). The SCC*mec* type II (67%) (HA-MRSA) was the predominating SCC*mec* element in this study, followed by SCC*mec* type III (14%). Eight of the 97 isolates could not be SCC*mec* typed using the M-PCR, thus other methods such as sequencing of these non-typeable strains have to be performed to better characterise these isolates. These non-typeable strains pose an important challenge in clinical settings emphasising the molecular evolution of the SCC*mec* element of emerging MRSA strains to adapt to enviromental changes. This M-PCR showed a 4%

CA-MRSA prevalence with a 100% PVL prevalence in these CA-MRSA strains. These CA-MRSA strains carried the SCC*mec* type IVd element, which is "classic" for CA-MRSA strains (Deresinski, 2005). No SCC*mec* type I and IV was detected in any of the MRSA isolates analysed in this study.

The M-PCR assay is advantaged because three genes could be detected simultaneously. The same PVL prevalence (4%) was obtained in the same isolates, thus the sensitivities of the first M-PCR and the real-time PCR assays were the same. However, the real-time PCR assay proved to be a more rapid PCR assay for the detection of the PVL genes. Future researchers such as developing a real-time M-PCR assay need to be investigated for the detection of multiple genes in MRSA strains.

Genotyping by the *spa* typing method showed three clusters of MRSA strains, whilst HVR grouped six clusters in the 97 MRSA isolates. All (100%) PVL-positive CA-MRSA isolates were clustered together using the *spa* typing method. Two of the related CA-MRSA strains were isolated in patients from the neonatal ICU indicating a possible transmission of the same clone in that unit, however, the clinical presentations of the patients were different (pneumonia and sepsis). Using the HVR typing method, six clusters were identified in the 97 MRSA isolates. Similar to the *spa* typing method, HVR typing revealed that three CA-MRSA strains were clonally related. However, Kilic and colleagues (2006) proposed that genotyping of MRSA strains specifically for outbreak analysis should be done by SCC*mec* typing instead of other PCR-based typing methods such as *spa* and HVR because of discrepancies between the different methods. In this study, differences with regard to discriminatory power and typeability between the *spa* and HVR typing methods was observed.

Antibiotic susceptibility profiling confirmed six different antibiotypes. The majority of the MRSA strains, were resistant to non-beta-lactam antibiotics, such as gentamycin (73%), erythromycin (71%) and clindamycin (70%). These non-beta-lactam antibiotics are used secondary to vancomycin for MRSA infections. The monitoring and profiling of the antibiotic susceptibility trend of MRSA strains in the Steve Biko Academic Hospital is important for the revision and improvement of antibiotic guidelines. A total of 2% of the CA-MRSA isolates were

susceptible to non-beta-lactam antibitiotics including gentamycin, erythromycin, clindamycin, fusidic acid and vancomycin. Community-associated MRSA strains are described to usually be susceptible to non-beta-lactam antibiotics (Deresinski, 2005). This is due to the length size of the SCC*mec* type IV element, which does not carry additional resistant genes except for the *mecA* gene (Okuma *et al*., 2002). The resistance coding genes in *S. aureus* are not only habored by the SCC*mec* elements (Hanssen and Sollid, 2005), thus this might explain multi-drug resistance in the other 2% of CA-MRSA strains. In a study by Takizawa and colleagues (2005), a multi-drug resistant PVL-positive CA-MRSA isolate obtained from an athlete was described in Japan. It is therefore necessary that appropriate treatment of CA-MRSA isolates should include non-beta-lactam antibiotics, such as erythromycin and gentamicin. Resistance for these drugs may be habored by other mobile genes, such as plasmids (Takizawa *et al*., 2005). The administration of vancomycin for all MRSA infections should be limited in CA-MRSA suspected infections due to the *de novo* appearance of vancomycin-intermediate *Staphylococcus aureus* (VISA) and vancomycin-resistant *Staphylococcus aureus* (VRSA strains). Misuse of vancomycin could lead to the emergence of these resistant strains (CDC, 2002).

Genotyping techniques are essential when used in epidemiologic studies; however, in an outbreak situation, SCC*mec* typing would be the appropriate method of MRSA characterisation (Kilic *et al*., 2008). Staphylococcal cassette chromosome *mec* typing is essential because it can distinguish between HA-MRSA and CA-MRSA (Kilic *et al*., 2008). As a result of the different antibiotic susceptibility profiles between HA-MRSA and CA-MRSA, discrimination of these two MRSA strains is important in patient and antibiotic management (Kilic *et al*., 2008). Rapid and accurate identification and characterisation is needed for the detection of these strains, as they are ecologically fit to reside both in the community and health-care facilities (Klevens *et al*., 2005). Molecular methods evaluated in this study proved to be useful rapid tools that can be implemented for screening and monitoring these strains to ensure outbreaks are prevented by the correct infection control principles such as isolation of infected patients and good hand washing procedures.

5.2 FUTURE RESEARCH

Future research regarding MRSA should be directed towards improvement of surveillance programmes, monitoring of the prevalence and transmission of both HA-MRSA strains and CA-MRSA strains in clinical settings. Prevalence studies could provide a better understanding of the epidemiology of MRSA strains in various clinical settings in South Africa. Based on this information, efficient infection control procedures and appropriate antibiotic regimens for MRSA infections in both outpatient and health-care settings can be incorporated.

Alternative molecular typing techniques such as multilocus sequencing typing (MLST) and microarray-based genotyping assay for typing of MRSA strains still remain to be evaluated as well as the cost-effectiveness and robustness of these techniques. Real-time PCR directed at simultaneous PVL identification and SCC*mec* typing for MRSA may offer a more rapid and robust method in determining the molecular epidemiology of MRSA strains in the clinical setting. A combination of strategies may offer a rapid, sensitive and effective way in the control of MRSA strains in the community as well as in the health-care settings.

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APPENDIX

1. DNA Extraction reagents (Sambrook and Russell, 2001)

a) **Phenol solution** (Sigma, St. Louis, USA)
10 mM Tris HCl, pH 8.0
1 mM EDTA, pH 6.7

b) **Sodium acetate** (3M) (Sigma, St. Louis, USA)
Sodium acetate regulates the salt concentration of the DNA strands for more adhesion to each other

c) **Lysozyme** (50 $mg.ml^{-1}$) (Roche Molecular Diagnostics, Germany)
Lysozyme was used for the disruption of the Gram-positive cell walls by digestion of the polymeric cell compounds which are responsible for the cell firmness.

d) **Sodium dodecyl sulphate** (SDS) solution 20% (Promega, Madison, USA)

SDS	100 g
H_2O	500 g

Add SDS powder to distilled H_20 and bring volume to 500 ml.
Sodium dodecyl sulphate is a detergent that lysis dissolving lipid molecules and therefore causing cell disruption

e) **Proteinase K** (20 $mg.ml^{-1}$) (Finnzymes, Inqaba, Pretoria)
Proteinase K is an enzyme that denatures cell proteins during cell wall disruption

f) **TE Buffer**, pH 7.4 (Promega, Madison, USA)
10 mM of Tris-HCl, pH 7.5
1 mM EDTA, pH 8.0
TE buffer is used to disolve and re-suspend DNA

g) **Sodium Tris-HCl EDTA** (STE) buffer

0.1 M of NaCl (Promega, Madison, USA)

10 mM Tris-Cl, pH 7.5 (Promega, Madison, USA)

1 mM EDTA, pH 8.0 (Promega, Madison, USA)

2. Gram-staining

a) Crystal violet (Merck)

b) Distilled water

c) Gram's iodine (Merck)

d) Ethyl alcohol, 95% (decolouriser) (Merck)

e) Safranin (counterstain) (Merck)

3. Nutrient broth

Nutrient powder (BD Diagnostics, USA)	13 g
Distilled water	1000 ml

Autoclave at 121°C for 15 min

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