A phenotypic and genotypic characterisation of strain types, virulence factors and agr groups of colonising Staphylococcus aureus associated with bloodstream infection

by
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DECLARATION

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SUMMARY

Several studies investigating the molecular characteristics of *Staphylococcus aureus* have been conducted worldwide, however, in South Africa, most of these have focused on Methicillin-resistant *S. aureus* (MRSA). This study investigated the phenotypic and genotypic characteristics of isolates of *S. aureus* collected from the blood and nasal cavity of patients admitted to Tygerberg Hospital, South Africa. Investigations included determining the association between blood and nasal isolates, describing the molecular epidemiology of the population, determining the prevalence of various virulence factor genes among the different clones and descibing the accessory gene regulator (*agr*) functionality of *S. aureus* clones.

Pulsed-field gel electrophoresis (PFGE), performed on 208 blood and nasal isolates from 162 patients with *S. aureus* bacteraemia, showed that 93 (57.4%) of the patients were colonised with the same strain type (p =0.061). MRSA was significantly associated with endogenous bacteraemia (same strain obtained from the blood and the nose) (p = 0.042).

Molecular typing of the 208 blood and nasal isolates (43.3% MRSA) revealed that the majority of strains were ST239-t37-*agr* I (25.5%) which harboured different SCC*mec* types including SCC*mec* type III and a potentially novel type presumed to consist of *ccrC*/Class A *mec*. ST612-MRSA-IV was the second most predominant clone (10.2%). Other MRSA clones included ST5-t045 with a potentially novel variant of SCC*mec* type I consisting of *ccrA1B1* and a *ccrC*/Class B *mec*; and ST461-MRSA-IV, reported for the first time in South Africa. All 18 (8.7%) *pvl*-positive isolates were MSSA except one isolate (ST612-MRSA-IV). The identification of novel MRSA clones (ST641-MRSA-IV), MSSA STs (ST2122, ST2126), and the potentially novel SCC*mec* type and type I variant suggest the local emergence of new clones.

Twenty-one isolates (representing nine clonal complexes (CCs)) previously characterised by Multi-locus Sequence Typing (MLST) were analysed for the prevalence of 38 virulence factor genes. There was an association between different enterotoxin gene cluster (egc) gene combinations and CC5, CC22, CC30, and CC45. Both CC15 and CC97 were negative for Superantigen (SAg) genes. The intracellular adhesion locus A (*icaA*) gene was common (90.4%) and detected in all CCs (except CC30) and the enterotoxin I (*sei*) gene was significantly more widespread in MRSA isolates (77.8% in MRSA; 25.0% MSSA; $p = 0.03$).

Accessory gene regulator dysfunction was significantly higher amongst MRSA than MSSA isolates and was more commonly associated with ST36-MRSA-II, ST239-MRSA-III and ST239-MRSA- *ccr*C/Class A *mec*. Shifting of *agr* in the same host was not common. Key findings in this study relate to the likely emergence of populations at Tygerberg Hospital, as evidenced by novel STs and potentially novel SCC*mec* types.

The identification of a circulating clone within the burns unit both illustrates the potential for organisms to spread within the hospital, as well as reinforcing the value of molecular typing for infection control purposes. The association of different *agr* types, *agr* functionality, and virulence factors with typing data has shown results consistent with other studies, as well as some unusual results. However, the clinical relevance of these associations is not yet well understood, and should form the basis of further research.

OPSOMMING

Verskeie studies wat die molekulêre eienskappe van *Staphylococcus aureus* ondersoek, is al wêreldwyd uitgevoer, maar in Suid-Afrika het die meeste gefokus op Methicillinweerstandige *S. aureus* (MRSA). Hierdie studie ondersoek die fenotipiese en genotipiese eienskappe van bloed en nasale *S. aureus* isolate van pasiënte wat by Tygerberg-hospitaal opgeneem is. Ondersoeke sluit in die bepaling van die verwantskap tussen bloed en nasale isolate, die beskrywing van die molekulêre epidemiologie van die bevolking, die bepaling van die teenwoordigheid van verskeie virulensiefaktorgene tussen die verskillende klone; en die beskrywing van die "accessory gene regulator" (*agr*) funksie van *S. aureus* klone.

"Pulsed-Field Gel Electrophoresis" (PFGE), wat op 208 bloed en nasale isolate (afkomstig van 162 pasiënte met *S. aureus* bacteriemie) uitgevoer is, toon dat 93 (57.4%) van die pasiënte gekoloniseer is met dieselfde stam tipe (p = 0.061). MRSA vertoon 'n betekenisvolle verwantskap met endogeniese bakteremie (dieselfde stam teenwoordig in bloed en nasale isolate) (p = 0.042).

Molekulêre tipering van die 208 bloed en nasale isolate (43.3% MRSA) het getoon dat die meerderheid van die stamme deel van ST239-T37-*agr* I (25.5%) is en uit SCC*mec* tipe III en 'n potensiële nuwe tipe (*ccrC*/KlasA *mec*) bestaan. ST612-MRSA-IV was die tweede mees oorheersende kloon (10.2%). Ander MRSA klone het ST5-t045 ingesluit, wat 'n potensiële nuwe variant van SCC*mec* tipe I (bestaande uit *ccrA1B1* en 'n *ccrC* / Klas B *mec*); en ST461-MRSA-IV wat vir die eerste keer in Suid-Afrika gevind is. Al 18 (8.7%) PVL-positiewe isolate was MSSA behalwe een isolaat (ST612-MRSA-IV). Die identifisering van nuwe

MRSA klone (ST641-MRSA-IV), MSSA STs (ST2122, ST2126), en die potensiële nuwe SCC*mec* tipes reflekteer die plaaslike ontstaan van hierdie nuwe klone.

Een-en-twintig isolate wat voorheen beskryf is deur “Multi-locus Sequence Typing” (MLST) is bestudeer vir die teenwoordigheid van 38 virulensiefaktor gene. 'n Assosiasie tussen verskillende “enterotoxin gene cluster” (EGC) geen kombinasies en CC5, CC22, CC30 en CC45 is gevind. Beide CC15 en CC97 was negatief vir die “Superantigen” (*SAg*) gene. Die “intracellular adhesion locus A” (*icaA*) geen was algemeen (90.4%) en in al die CCe waargeneem (behalwe CC30). Die “enterotoxin I” (*sei*) geen was aansienlik meer teenwoordig in MRSA isolate (77.8% in MRSA; 25,0 % MSSA; p = 0.03).

“Accessory gene regulator” disfunksie was aansienlik hoër in die MRSA groep (in vergelyking met die MSSA groep) en was geassosieer met ST36-MRSA-II, ST239-MRSA-III en ST239-MRSA-*ccrC*/Klas A *mec*. Die verskuiwing van *agr* funksionaliteit tussen isolate afkomstig van die bloed of neus was skaars.

Hoof bevindinge in hierdie studie hou verband met die moontlike ontstaan van bevolkings (nuwe STs en potensiële nuwe SCC*mec* tipes) by Tygerberghospitaal. Die identifisering van 'n sirkuleerende kloon in die brandwonde-eenheid illustreer die potensiaal van organismes om te versprei in die hospitaal, asook die waarde van molekulêre tipering vir infeksiebeheer doeleindes. Die verband tussen die verskillende *agr* tipes, *agr* funksionalitiet en die virulensiefaktore met tiperingsdata toon soortgelyke resultate as die literatuur, asook ‘n paar buitengewone resultate. Die kliniese belang van hierdie assosiasies moet in die toekoms verder ondersoek word.

DEDICATION

I would like to dedicate this to my father, my mother and my family,

for their support and encouragement.

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Abbreviations

AIP	Autoinducing peptide
ACME	Arginine catabolic mobile element
Agr	Accessory gene regulator
AICU	Adult intensive care unit
APC	Antigen presenting cells
Arc	Carbamate kinase
aroE	Shikimate dehydrogenase
Tm	Melting temperature
attBCC	Bacterial chromosomal attachment site
attL	Left chromosomal *SCCmec* junctions
attR	Right chromosomal *SCCmec* junctions
BA	Horse blood agar
BHIB	Brain heart infusion broth
Bp	Base pair
BSI	Bloodstream infection
BURP	Based upon repeat tandem patterns
C5aR	C5a receptor
CA-MRSA	Community-associated methicillin-resistant *Staphylococcus aureus*
Cap5	Capsule type 5
Cap8	Capsule type 8
CAPD	Continuous ambulatory peritoneal dialysis
CC	Clonal complex
Ccr	Chromosome recombinase complex

CDC	Centers for disease control and prevention
CHIP	Chemotaxis inhibitory protein
ClfA	Clumping factor A
ClfB	Clumping factor B
Cm	Centimeter
CNA	Collagen binding protein
Coa	Coagulase
ddH_2O	double-distilled water
DLVs	Double locus variants
dNTP	Deoxyribonucleotide triphosphate
Eap/Map	Extra cellular adherence protein/MHC class II analogous protein
EDTA	Ethylenediaminetetraacetic acid
Egc	Enterotoxin gene cluster
EMC	Extracellular matrix component
EMRSA	Epidemic methicillin-resistant *Staphylococcus aureus*
ETA	Exfoliative toxin
Eta	Exfoliative toxin A
Etb	Exfoliative toxin B
EtBr	Ethidium bromide
Fc	Fragment crystallizable region
FnBPA	Fibronectin binding protein A
FnBPB	Fibronectin binding protein B
FPR	formal peptide receptor
glpF	Glycerol kinase gene
gmk	Guanylate kinase gene

H	Hour
HA-MRSA	Health care-associated methicillin-resistant *Staphylococcus . aureus*
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
Hla	Alpha-hemolysin
Hlb	Beta-hemolysin
Hld	Delta-hemolysin
Hlg	Gamma-hemolysin
HlgA	Gamma-hemolysin A variant
HNP-1	Human neutrophil peptide-1
HNP-2	Human neutrophil peptide-2
hVISA	Vancomycin hetero-resistant *Staphylococcus aureus*
IcaA	Intracellular adhesion locus A
ICAM-1	Intracellular adhesion molecule 1
ICAM-1	intracellular adhesion molecule 1
ICU	Intensive care unit
IE	Infective endocarditis
IEC	Immune evasion cluster
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IsdA	Iron regulated surface determinant protein A
IS	Insertion sequence
IWG-SCC	International working group on the classification of staphylococcal cassette chromosome element
J region	Junkyard region also known as joining region
Kb	Kilobase

M	Molar
$MgCl_2$	Magnesium chloride
MGE	Mobile genetic element
MHC II	Major histocompatibility receptors class II
Min	Minute
Ml	Milliliter
MLST	Multi locus sequence typing
MLST-CC	Multi locus sequence type clonal complex
mM	Millimolar
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin sensitive *Staphylococcus aureus*
MSA	Mannitol salt agar
MSB	Mannitol salt broth
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
µg	Microgram
µg/ml	Microgram per milliliter
NHLS	National Health Laboratory Service
Nm	Nanometer
Nuc	Deoxynuclease enzyme
°C	Degrees Celsius
orfx	Open reading frame
P2	promoter 2
P3	promoter 3
PBP2a	Penicillin-binding protein 2 a
PC	Primer concentration

PCR	Polymerase chain reaction
PF	Pulsed-field
PFGE	Pulsed-field gel electrophoresis
PFGE-CC	Pulsed-field gel electrophoresis clonal complex
PICU	paediatric and neonatal Intensive care unit
PK	Proteinase K
PMN	polymorphonuclear neutrophil
PMNs	Polymorphonuclear cells
Pta	Phosphate acetyletransferase
PTSAgs	Pyrogenic toxin superantigens
PVL	Panton-valentine leukocidin
RF	Renal failure
Rpm	Revolutions per minute
S	Second
SAB	*Staphylococcus aureus* bacteremia
SAgs	Superantigenic toxins
SAK	Staphylokinase enzyme
SaPI1	*Staphylococcus aureus* pathogenicity island 1
SaPI2	*Staphylococcus aureus* pathogenicity island 2
SaPI3	*Staphylococcus aureus* pathogenicity island I3
Sar	Staphylococcal accessory regulator
SasG	*Staphylococcus aureus* surface protein G
SBA	sheep blood agar
SCC*Hg*	Staphylococcal cassette chromosome mercury
SCCmec	Staphylococcal cassette chromosome *mec*

SCIN	Staphylococcal complement inhibitor
sdrC	Serine-aspartate protein C
sdrD	Serine-aspartate protein D
sdrE	Serine-aspartate protein E
Sea	Enterotoxins A
Seb	Enterotoxins B
Sec	Enterotoxins C
Sed	Enterotoxins D
See	Enterotoxins E
Seg	Enterotoxins G
She	Enterotoxins H
Sei	Enterotoxins I
Sej	Enterotoxins J
Sel	Enterotoxins L
Sem	Enterotoxins M
Sen	Enterotoxins N
Seo	Enterotoxins O
SFP	Staphylococcal food poisoning
SLST	Single locus sequence typing
SLVs	Single locus variants
Spa	*Staphylococcus aureus* protein A
spa-CC	*spa* clonal complex
SSRs	short sequence repeats
SSSS	Staphylococcal scalded-skin syndrome
SSTI	Skin and soft tissue infections

ST	Sequence type
TAE	Tris-acetic acid- **Ethylenediaminetetraacetic** buffer
TBE	Tris-boric acid- **Ethylenediaminetetraacetic** buffer
Tpi	triosephosphate isomerase
tPMP	Thrombin-induced platelet microbicidal proteins
TSS	Toxic shock syndrome
TSST	Toxic shock syndrome toxin
TSST-1	Toxic shock syndrome toxin 1
u/µl	Unit per microliter
UPGMA	Unweighted Pair Group Method with Arithmetic mean
V/cm	Volts per centimetre
VISA	Vancomycin intermediate resistant *S. aureus*
VNTR	variable-number tandem repeat
X_c	cell wall attachment part of protein A
X_r	repetitive X region
yqiL	Acetyle coenzyme A acetyltransferase

CHAPTER 1 GENERAL INTRODUCTION

1.1 Background

Staphylococcus aureus, a potent Gram-positive pathogen, was discovered in 1880. *S. aureus* is the primary cause of a wide range of diseases from skin and wound infections to life-threatening conditions including necrotising pneumonia and bactaeremia (1). The mortality rate of *S. aureus* infections was high (≈ 80%) prior to the discovery of penicillin in 1940. Later in 1942, the first *S. aureus* isolates resistant to penicillin were identified and the resistance rate steadily increased to 80% in 1960. The resistance mechanism is found on a plasmid encoding a penicillinase enzyme (2). *S. aureus* infections have become more prevalent over the past seven years and it is the second most common species (14.9%) isolated from specimens collected from outpatient clinics in the United States (3).

In 1961 Jevons *et al*., isolated the first methicillin-resistant *S. aureus* (MRSA) and said "It is well known that patients with infected skin can be a dangerous source of infection in hospitals, and the finding of just such a patient infected with a celbenin (methicillin) - resistant strain in this instance adds an additional warning" (4). When *S. aureus* acquires resistance to methicillin it becomes resistant to all β-lactam antibiotics, including the penicillinase-resistant penicillins: the cephalosporins and the carbapenems (5, 6). Since 1961, MRSA has emerged as a major concern and has led to different clinical infections in the community and hospitals (7-9). The rate of community associated MRSA (CA-MRSA) (MRSA identified from blood culture within 48 hours in hospitalised patients who do not have extensive contact with health care) and health-care associated MRSA (HA-MRSA) (MRSA identified from blood culture either after 48 hours of admission or from patients with extensive health care contact) (10), bacteraemia has increased over the past decades leading

to lengthy hospital stays and elevated treatment costs (11). MRSA clones are well adapted to survive in intensive antibiotic usage environments and this has led to the evolution of strains with a decreased susceptibility to vancomycin, including intermediate vancomycin resistant (VISA) or hetero-resistant vancomycin (hVISA) *S. aureus* (12, 13)

1.2 *S. aureus* Bacteraemia

S. aureus is considered a common cause of bacteremia and may lead to increased mortality and longer hospital stay (14-16). It is the third most common cause of nosocomial bacteremia in intensive care units in the USA (17, 18). Furthermore, it has been estimated that >400,000 hospitalisations per year are related to *S. aureus* infection, leading to approximately 11,000 deaths annually (19).

The emergence of highly resistant strains with reduced susceptibility to vancomycin increases the treatment cost and adds a burden to the health care units (20). Blood stream infection sources are classified as either primary, when the source is unknown; or secondary, when the source is skin or soft tissue infection sites (including intravascular device-associated infections) (21).

S. aureus was the second most common organism isolated from nosocomial bacteremia during the time period 1995 to 1998 in the United States of which 29% were MRSA (22) and similar findings were reported in 2004 (23). In Tygerberg Hospital, South Africa, *S. aureus* bacteremia is a major concern and the prevalence of MRSA amongst blood culture isolates steadily increased from 25% to 30.1% over a period of 14 years (1985 to 2009) (24, 25).

There are many factors that predispose to MRSA BSIs including extended periods of

antibiotic use, prolonged hospital admissions, the presence of intravenous devices and the host's immune status (11, 26). *S. aureus* infection can lead to significant complications. Fowler *et al*., found that 43% (n=310) of patients who had SAB developed complications of which 282 had HA-MRSA and developed these complications within 72 hours of hospitalisation. Complications included endocarditis (39%), septic arthritis (24%), deep tissue abscesses (18%), osteomyelitis (10%), meningitis (5%), epidural abscesses (8%), septic thrombophlebitis (8%), psoas abscess (6%); and other complications (7%). Furthermore, they found that patients could have more than one complication at the same time (27).

Different findings have been reported in studies examining the association of MRSA with complications and outcome. MRSA BSIs are associated with a higher morbidity and mortality rate than methicillin-sensitive *S. aureus* (MSSA) associated BSIs (28). In the United Kingdom, the mortality rate for MRSA BSI was significantly higher than for MSSA BSI (11.8% vs 5.1%, respectively) (29). Similar results have been reported in South Africa, where the mortality rate after 14 days of infection was 33.3% (35/105) for MRSA BSI compared to 20.1% (69/344) for MSSA BSI (30). In contrast however, at Duke University Medical centre the outcome and associated complications of MRSA and MSSA bacteraemia were similar (31).

1.3 *Staphylococcus aureus* Nasal Carriage

S. aureus colonises the skin and nose of humans and animals. The more common colonisation sites on skin include the axilla and perineum (32). The nose is the most common carriage site of *S. aureus* (33). *S. aureus* has also been found to colonise the throat and intestines with a significant association with nasal carriage. Identification of the same isolates obtained from

the nasal cavity and other body sites suggests that nasal carriage may spread from the nose into other body sites (34, 35). While colonisation rarely leads to infection, it is still considered the main source and highest risk factor for nosocomial and community-acquired infections (36, 37). Approximately 6.6% of carriers are colonised with more than one strain and because of the genetic diversity of these strains it is hypothesised that the nose is an ideal site for horizontal genetic transfer between colonising strains (38).

To colonise the nose *S. aureus* needs to: reach the nose, adhere to nasal tissues or specific receptors, evade the immune system; and multiply (39). *S. aureus* nasal colonisation is therefore a result of host pathogen interactions (1). The hands are an important source of *S. aureus* nasal colonisation especially in those who indulge in rhinotillexis (nose picking) (40). Airborne transmission also plays an important role in *S. aureus* nares colonisation and respiratory infections (41). Hospitalisation also been found to predispose to nasal colonisation (42). Children who acquire MRSA nasal colonisation due to nosocomial transmission are at higher risk of developing a subsequent *S. aureus* infection than those colonised before hospital admission (43). This may be explained by another study finding where they reported that persistent carriers show strong immune responses toward specific virulence factors expressed by their colonising strains (44). Mothers are a common vector in the colonisation of their infants' nares and the level of colonisation was found to steadily decrease from 8 weeks to 6 months of age as children were weaned from the breast (45) Furthermore, family members tend to be colonised with genotypically identical strains (46).

The lack of cell wall teichoic acid significantly reduces the ability of *S. aureus* to attach to nasal epithelial cells (47). However, *S. aureus* can adhere to desquamated nasal epithelial cells by relying on clumping factor B (ClfB), serine-aspartic acid repeat proteins *SdrC* and *SdrD*; and iron regulated surface determinant protein A (IsdA) (48). Cytokeratin 10 is a

squamous cell surface component and ClfB ligand (49) and *S. aureus* surface protein G (SasG) assists in nasal colonisation by enhancing adherence to the epithelial cells (48). Microbial surface components recognising adhesive matrix molecules (MSCRAMMs) mediate *S. aureus* adherence to fibronectin, fibrinogen, and collagen related polysaccharides (50). These will be discussed in more detail later (chapter 4).

To successfully colonise the nose; *S. aureus* needs to be able to evade the host immune response in the nasal cavity. The thick peptidoglycan layer of the cell wall of *S. aureus* prevents cell wall destruction by lysozyme (one of the nasal mucosal fluid components) (51). Staphylokinase, produced by *S. aureus*, can overcome cytolysis by decreasing the negative charge of the bacterial cell wall, thereby rendering it less attractive to the cationic antimicrobial peptides (52). The staphylococcal surface protein A binds to the fragment crystallisable (Fc) region of immunoglobulin G (IgG) and IgA antibodies, thereby inactivating them (53). Neutrophil chemotaxis is another innate immune response against *S. aureus* nasal colonisation, however, the extracellular adherence protein (Eap) can reduce neutrophil endothelial attachment leading to less neutrophil recruitment and therefore diapedesis inhibition (54). Nasal fluid collected from carriers suppresses growth of exogenous *S. aureus* and provides permissible growth conditions for the isolates from carriers (55).

Historically, *S. aureus* nasal carriage among healthy people was classified into: persistent carriers (20%), intermittent carriers (30%) and noncarriers (50%) (39, 56, 57). A recent study found that persistent nasal carriers have a higher bacterial load of *S. aureus* and a greater risk of developing infection than intermittent and non-nasal carriers, while the risk difference between the last two groups was non-significant. For this reason the authors suggested a reclassification of nasal carriers to only two groups: persistent and other carriers.

Furthermore, they found that persistent and intermittent carriers represent 24% and 47% of healthy individuals, respectively. The authors also demonstrated a specific predisposition of a carrier for their original colonising strain by inoculating a mixture of strains into the nose of the persistent and intermittent carriers after de-colonising the subjects with mupirocin. Fifty-eight and 17% of the persistent and intermittent carriers respectively became re-colonised with their own resident strain, suggesting that each strain is well adapted to its specific host and highlights the strong interaction between carriers and their colonising strains (58). Similar results were obtained when a mixture of *S. aureus* (including the resident strain) was inoculated into persistent and noncarriers. Seven of 11 persistent carriers re-colonised with their original strains and prevented the colonisation with other strains. This reflects a specific acclimatisation between the host and resident strain, as each strain re-colonised its original niche (59). Re-colonisation with the original strain could be due to both environment and host factors (60). Nasal colonisation may also be influenced by the interaction between the *S. aureus* strain and the normal flora resident in the nasal cavity (61) as it has been found that certain nasal microbiota, including *S. epidermidis*, prevent *S. aureus* colonisation(62).

Certain diseases may enhance MRSA nasal colonisation and increase the risk of infection. Human immunodeficiency virus (HIV) patients are more likely to be colonised with MRSA as is evidenced by a previous study which showed that 17% of HIV-I positive outpatients were colonised with MRSA, in contrast to 6% of HIV-I uninfected individuals (63). Also, what was interesting in this study was that overall *S. aureus* colonisation was higher in HIV infected compared to HIV uninfected individuals. *S. aureus* nasal carriage is also significantly higher in type 2 diabetic patients who are on insulin or antibiotic treatment, compared to those who use oral anti-diabetes drugs or non-diabetic individuals (64). Diabetic patients are more likely to suffer from diabetic foot ulcer MRSA infection, and nasal

colonisation is hypothesised to be the source of infection (65). MRSA nasal carriage has also been found to be significantly associated with subsequent infections in dialysis patients and may be the main source of MRSA transmission in dialysis staff and their family members (66). Individuals suffering from perennial allergic rhinitis are also more prone to *S. aureus* nasal carriage which may aggravate their symptoms even further (67).

Age is also associated with nasal colonisation, with a large study in the USA, ranging from healthy individuals aged 1 to 70 (pooled in increments of 5 years), showing the highest *S. aureus* carrier rate (~45%) amongst 6-11 year olds while MRSA nasal colonisation was associated with people ≥60 years old (68). This study did not comment on MRSA nasal colonisation in children, however, another study reporting on a similar time period in the USA found that MRSA nasal colonisation in healthy children aged from 2 weeks to 21 years significantly increased over a 3 year period (0.8% in 2001 to 9.2% in 2004) (69). Korean researchers have reported similar *S. aureus* nasal carriage rates (32%) amongst children who visited an outpatient paediatric department in the Samsung Medical Centre tertiary-care Hospital; however, their MRSA rate was much higher than that reported in the USA (18.9%) (70).

A study investigating the effect of MRSA/MSSA nasal carriage on developing nosocomial SAB found that the rate of bacteraemia among MRSA carriers was 38% and 9.5% among MSSA carriers (26). Prolonged antibiotic treatment has also been shown to lead to a shift in MSSA nasal and skin carriage to MRSA nasal colonisation (71). MRSA nasal carriage in long-term care facility patients is a significant risk factor to develop subsequent clinical infection, more so than with patients colonised with MSSA (72). A systematic review of studies comparing invasive clinical infection caused by MRSA or MSSA nasal isolates showed that patients colonised with MRSA were four times more likely to develop

endogenous infection (73).

More than 80% of *S. aureus* strains isolated from blood are genetically identical to strains carried in the patient's anterior nares. (74-76). This (and similar findings) provides good evidence that the colonised nose could be the source of infection in patients with bacteraemia (i.e. endogenous infection). In the Netherlands, Wertheim *et al*., found that patients colonised with *S. aureus* had a three-fold increased risk of *S. aureus* bacteremia compared to noncarriers. However, bacteraemia in noncarriers was associated with a higher morbidity and mortality rate (76). This could be due to the immune system of the carriers adapting to the colonising strains. In support of this hypothesis the carriers induced high level of antibodies against the super antigens produced by their own strains (77). Others have reported that antibodies against toxic shock syndrome toxin-1 (TSST-1), enterotoxin A (*SEA*), clumping factor A (ClfA) and clumping factor B (ClfB) are at higher levels in persistent carriers than in noncarriers (78). However, this is not the case for other exogenous virulence factors where experimental colonisation with the low virulence strain 8325-4 does not elicit more antibody production (44). In another study conducted in the Netherlands, infection caused by exogenous MSSA strains was found to be more virulent than by the endogenous strains (76).

1.4 *S. aureus* Genotyping

Molecular epidemiological studies are required to identify the bacterial population structure and to follow the evolutionary history of newly emerging pathogenic strains. These studies also allow us to determine the impact of new antibacterial agents and vaccinations on the bacterial population structure (79). Molecular characterisation of *S. aureus* strains, new emerging clones and molecular changes in identified clones are required to plan a successful

prevention and control strategy for *S. aureus* outbreaks in different communities and environments (80). Genotyping the different strain types allow a better understanding of the movement of *S. aureus* lineages among different populations. Additionally, conserved genes within specific strain types may be used to determine strain relatedness and their putative ancestors (81, 82). Currently the most commonly used techniques for strain typing and relatedness identification include: Pulsed-Field Gel Electrophoresis (PFGE), Staphylococcal Protein A (*spa*) typing, Multi Locus Sequence Typing (MLST) and Staphylococcal cassette chromosome *mec* (SCC*mec*) typing for MRSA (83-85). Whole genome sequencing (86, 87) and microarray analysis have been used for *S. aureus* strain typing (88), however, their technical requirements and cost have limited their use.

1.4.1 PFGE

This DNA macro-restriction based technique has been reported as the most reliable method to identify outbreak-strain relatedness and is considered the gold standard to investigate outbreaks and to conduct epidemiological studies (80, 85, 89, 90). The discriminatory power of PFGE is the highest compared to other typing techniques, therefore it is often the first method used, followed by other typing methods (SCC*mec* and MLST) for representative isolates from each PFGE cluster (91).

Pulsed-field gel electrophoresis is labour intensive, with results taking 3 to 5 days to generate; and costly, requiring expensive consumables and equipment (92, 93). Furthermore, inter-laboratory comparisons are difficult to perform. Inter-gel variation can also occur due to numerous factors including differences in bacterial genome concentration, agarose

concentration variations, current voltages, gel running temperatures and buffer strengths (94).

Many attempts to standardise PFGE protocols and nomenclature have shown limited success due to the numerous factors influencing the results analysis (90, 95, 96).

In the USA, consensus has been reached to identify eight particular *S. aureus* PFGE types (designated as USA100 to USA800) (97). Some of these pulsotypes (USA 300 and USA 400) have been recognised by the Centre for Disease control and prevention (CDC) as being associated with community-associated MRSA (98).

1.4.2 *Spa* Typing

Staphylococcal Protein A is one of the *S. aureus* cell wall components which binds covalently to the peptidoglycan layer (99). Protein A contains two different domains including the NH2-terminal region which binds to the Fc region of mammalian immunoglobulin thereby inhibiting phagocytosis by polymorphonuclear leucocytes; and the C-terminal fragment that anchors protein A to the cell wall (figure 1) (100, 101). The Protein A gene (*spa*) includes two regions, the immunoglobulin G-binding (IgG) region and C-terminus (X) that consists of the polymorphic short sequence repeat region (SSRs) (also known as the repetitive X region (Xr)) and the cell wall attachment region (Xc). The polymorphic X region is flanked by well-defined conserved regions and generally includes a variable number of tandem repeats (VNTRs), which are usually 24 bp in length, although repeats of 21 to 27 bp repeat units have also been recorded (93, 100, 102). The genetic variability of the SSR region has been attributed to point mutations and/or the deletion or duplication of the consecutive repeats units (103).

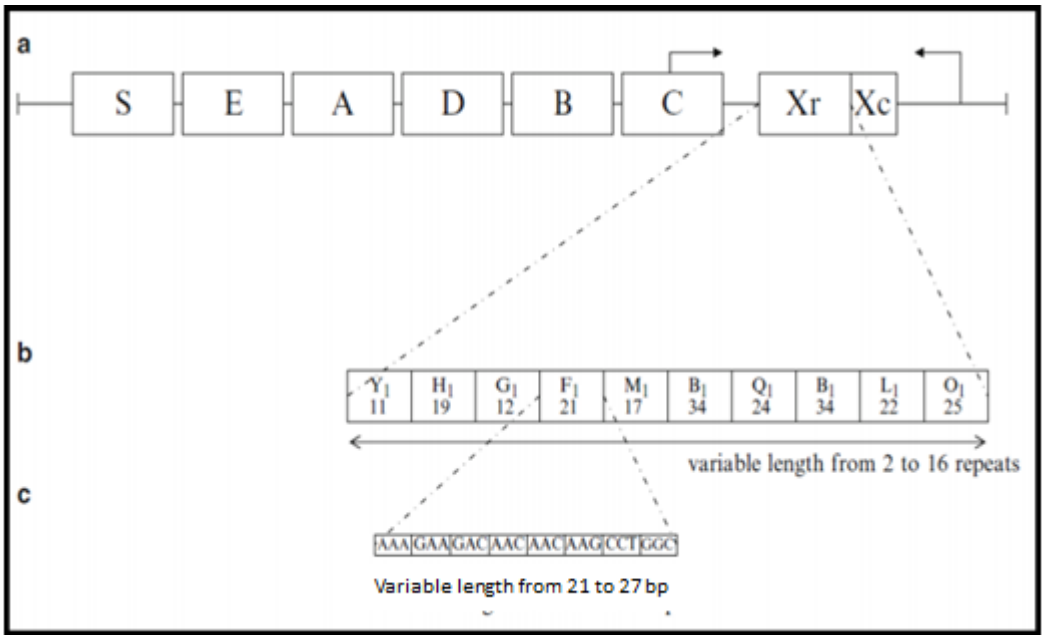

Figure 1 : Schematic structure of protein A gene

Adapted from Hallin *et al* (104). (a) Different components of the *spa* gene: S, signal sequence; A-E IgG binding protein; C-terminus region (Xr is the polymorphic short sequence repeat region and Xc is the cell wall attachment region). Arrows indicate the flanked conserved region. (b) Variable structure of the Xr region with both Kreiswirth and Ridom nomenclature. (c) Nucleotide length and sequence of one repeat.

The number of repeats in the polymorphic region was used in 1994 to discriminate between epidemic and non-epidemic MRSA (105). Sequencing of the *spa* polymorphic region was found to be a valid technique for *S. aureus* typing and can be used to identify strain types. Compared to other typing techniques, *spa* typing is rapid, easy to perform and interpret; and results and data may be shared between different laboratories (106). The discriminatory power of *spa* typing is suitable for epidemiological studies, but improved discrimination is obtained when performed in combination with PFGE, MLST and SCC*mec* typing methods (107). *spa* typing is less discriminatory than PFGE, but more discriminatory than MLST (108). *spa* typing can be used to study MRSA hospital outbreaks and the evolution of MRSA strains (109). In long term outbreaks, epidemiological studies which used a combination of

spa typing and PFGE have shown better results than using *spa* or PFGE alone in determining strain relatedness and in identifying the genetic background of the resolved clusters (107, 110).

Two common *spa* type nomenclature classification methods exist, including that proposed by Kreiswirth *et al*., where each repeat identified is represented alphanumerically (106). The second nomenclature classification method assigns repeats to numerical codes (111). Ridom StaphType software (Ridom GmbH, Würzburg, Germany) is widely used for *spa* sequence analysis in European countries. The software modules determine the nucleotide sequence, identify repeats and their sequence; and thereby assign the *spa* type. Furthermore, the software can also perform *spa* type clonal cluster analysis by using the Based Upon Repeat Tandem Patterns function (BURP). To avoid missing or including extra repeats the software can detect both leading and ending repeats of each *spa* type.

Identified *spa* types can be submitted to the online central *spa* server (http://www.spaserver.ridom.de) and novel *spa* types and repeats will receive the appropriate nomenclature and be added to the central database (111).

1.4.3 Multi Locus Sequence Typing (MLST)

Multi locus sequence typing is a commonly used technique to identify strain characteristics, the evolutionary history and bacterial population structure. Resultant data can be compared to the online MLST database (http://mlst.net) to identify the strain relatedness and population structure (79, 112).

In *S. aureus*, MLST is based on the analysis of the sequence variation of internal fragments

(402 to 516bp) of seven housekeeping genes (carbamate kinase (*arc*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glpF*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), triosephosphate isomerase (*tpi*) and acetyl coenzyme A acetyltransferase (*yqiL*)). Results are presented as an allelic profile (sequence type, ST) consisting of seven variable loci. MLST therefore provides enough discriminatory power to estimate the relatedness between different strains, excluding the random allelic profile similarity which may occur between unrelated strains (113-115). Strains with the same ST or those that have a single locus difference (single locus variants (SLVs)) in the allelic profile are closely related and subsequently grouped into one MLST clonal complex (MLST-CC). A clonal complex refers to a group of isolates having high ST similarity (5 or more of the seven loci) and thus likely evolved from a common ancestor. (113, 116).

Multi locus sequence typing has been shown to be a useful technique with good discriminatory power capable of assigning both MRSA and MSSA strains into known and novel ST (115). In the event that strains with different ST are assigned into one MLST-CC, the founder (putative ancestor) is identified as the ST which consists of the largest number of single locus variants as compared to other related STs. A sub-group of the founder is defined as when the founder's progeny (SLVs) become a prevalent ST from which their own SLVs and double locus variants (DLVs) evolve (79, 115).

Multi locus sequence typing is a useful tool to investigate the long term epidemiology and *S. aureus* population structure as well as the evolutionary history of different MRSA and MSSA clones (115, 117). However, MLST analysis requires the sequencing of seven housekeeping genes which increases the technique costs. In addition the methodology is labour intensive and time consuming (80).

1.4.4 Staphylococcal Cassette Chromosome *mec* (SCC*mec*) Typing

Staphylococcal cassette chromosome *mec* is a mobile genetic element which encodes for β-lactam antimicrobial resistance (80, 118). This mobile element also contributes to genetic transfer between staphylococcal species and provides essential support to *S. aureus* to withstand a hostile environment (119, 120). Well adapted epidemic MSSA clones can acquire the SCC*mec* element by horizontal gene transfer, thereby successfully evolving into MRSA clones. These clones are the ancestors of reduced vancomycin susceptibility (hVISA and VISA) clones (121).

Staphylococcal cassette chromosome *mec* element sizes can range from 21 to 67 kb depending on the SCC*mec* type. This transposable element integrates into the open reading frame (*orfx*) close to the origin of replication (122). Integration occurs into a specific 15 bp nucleotide sequence in the bacterial genome known as the bacterial chromosomal attachment site (*attBCC*) located downstream of the *orfx*. As a consequence of SCC*mec* element integration, the *attBCC* unique sequence will flank the SCC*mec* element at both the right (*attR*) and left (*attL*) chromosomal SCC*mec* junctions (123). The *attBCC* integration hotspot allows for easy acquisition and loss of SCC*mec* elements to and from bacterial chromosomes (124, 125).

Two conserved variable loci are located in each SCC*mec* element: (1) *mecA* and its regulatory genes (*mecI* and *mecR1*) which confer antimicrobial resistance by encoding for an altered penicillin-binding protein (PBP2a) and (2) the chromosome recombinase complex (*ccr*) that encodes for the recombinase enzyme which confers SCC*mec* element mobility (122, 126, 127). A third SCC*mec* component is the variable J regions (Junkyard regions also known as the Joining regions) which contain a variety of integrated plasmids (such as pT181,

pU110, and p1258), transposons (Tn*554*) which confer resistance to non-β-lactam antibiotics or heavy metals (128); and insertion sequences (IS*431*, IS*1272* and IS*256*) (129).

In *S. aureus*, many *mecA* class variants in the different SCC*mec* types have been identified: class A where the two genes (*mecI* and *mecR1*) are present in their entirety (considered the wild type) and classes B, C1, C2, and E where varying portions/combinations of one or both of these genes are deleted. The *ccr* gene complex in *S. aureus* also consists of different allotypes depending on nucleotide sequence variations: *ccr*1 (*ccrA1B1*), 2 (*ccrA2B2*), 3 (*ccrA3B3*), 4 (*ccrA4B4*), 5 (*ccrC*), 7 (*ccrA1B6*) and 8 (*ccrA1B3*) [(130)http://www.SCC*mec*.org/Pages/SCC_TypesEN.html]. SCC*mec* contains three J regions; J1 located in the area between the right junction (*attR*) and the *ccr* complex, J2 which occupies the area from the *ccr* genes to the *mec* complex and the third J region expanding from the *mec* complex to the left junction (*attL*) as shown in figure 2 (131, 132). The SCC*mec* element therefore consists of the following structure: J3-*mec*-J2-*ccr*-J1 (80). Sequence differences in the J regions lead to the classification of SCC*mec* type variants (133).

Currently, eleven SCC*mec* types have been identified according to the *mecA* class and *ccr* allotype combination in the mobile genetic element (126, 134) (

Table 1) (http://www.SCC*mec*.org/Pages/SCC_TypesEN.html).

The International Working Group on the Classification of Staphylococcal Cassette Chromosome Element (IWG-SCC) in December 2009 established certain rules and guidelines for SCC*mec* element nomenclature unification and identification worldwide (125). They proposed to keep the first Roman numeral nomenclature followed by the ccr gene complex allotype and *mec* gene complex class in parenthesis (Table 1). Many variants have

been identified for different SCC*mec* types based on the J region nucleotide differences. Furthermore, some SCC*mec* elements carry more than one *ccr* allotype. These are also classified by the IWG-SCC as a type variant of the original SCC*mec* type (125).

Table 1 : *ccr* allotypes and *mecA* class components of the eleven identified SCC*mec* elements. Adapted from (**http://www.SCC*mec*.org/Pages/SCC_TypesEN.html).**

SCC*mec* type	*ccr* gene complex	*mec* gene complex	IWG-SCC Nomenclature
I	1 (A1B1)	B	1B
II	2 (A2B2)	A	2A
III	3 (A3B3)	A	3A
IV	2 (A2B2)	B	2B
V	5 (C1)	C2	5C2
VI	4 (A4B4)	B	4B
VII	5 (C1)	C1	5C1
VIII	4 (A4B4)	A	4A
IX	1 (A1B1)	C2	1C2
X	7 (A1B6)	C1	7C1
XI	8 (A1B3)	E	8E

SCC*mec* type I (34.3 kb) was identified in the first MRSA isolated in the UK and has been designated as an archaic clone (122). SCC*mec* type I usually does not encode resistance to the antimicrobial agents other than the β-lactam antibiotics (122, 128).

SCC*mec* type II (53 kb) was identified in a strain obtained from Japan in 1982 (122, 130) and contains a plasmid (pUB110) which encodes for a broader spectrum of antimicrobial resistance to the aminoglycosides including kanamycin, tobramycin and bleomycin. The

element also contains a transposon (Tn*554*) which contains the *ermA* gene which encodes resistance against the macrolides, lincosamides and streptogramins (128).

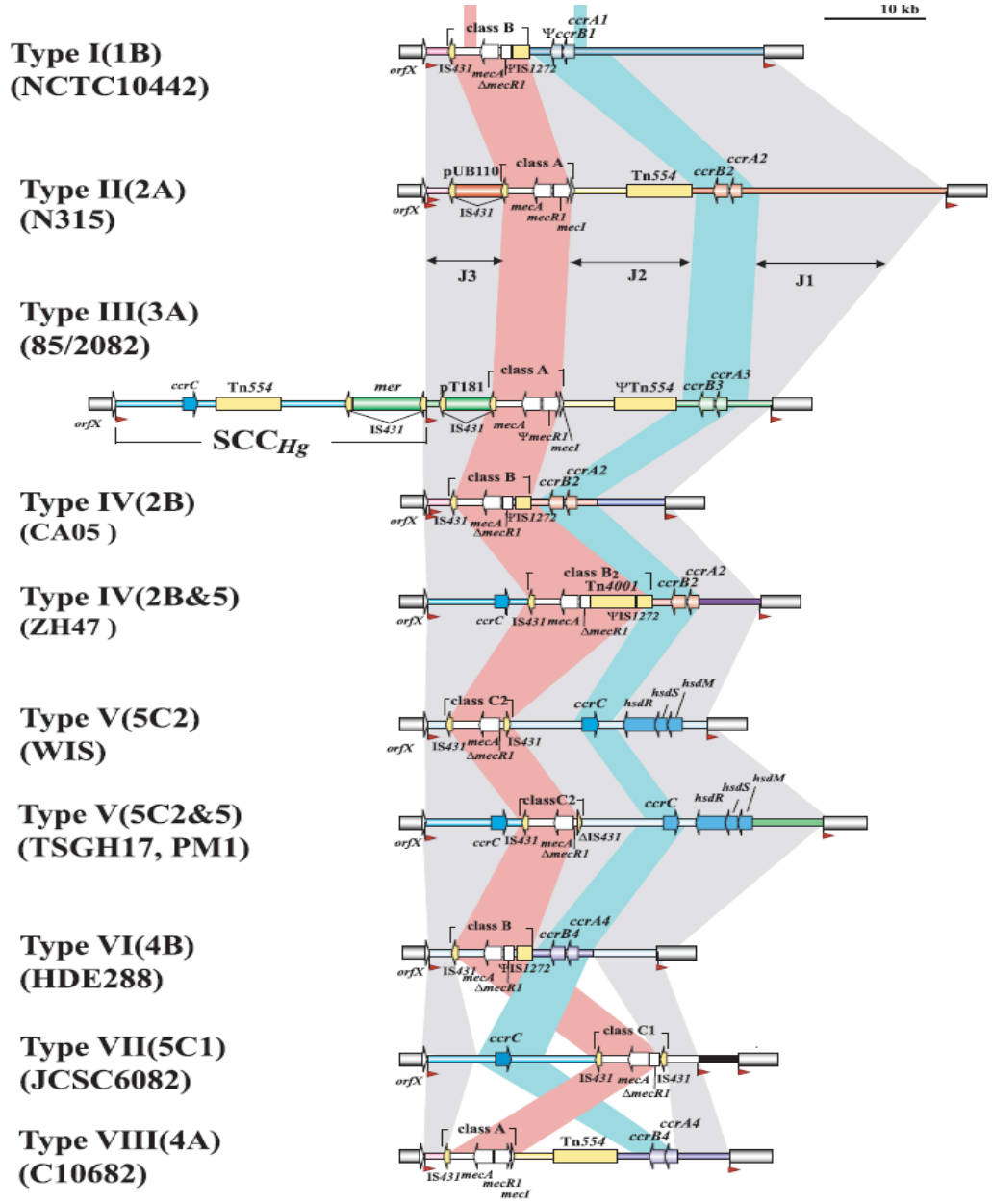

Figure 2 : Basic structure of SCC*mec* types I-VIII

Pink represents the *mec* complex, blue represents the *ccr* complex, and grey represents the J regions. Tn, transposon; IS, insertion sequence; p, plasmid; SCC*Hg*, staphylococcal cassette chromosome mercury. SCC*mec* type nomenclature represented as roman numbers and revised designation in parenthesis (*ccr* complex and *mec* complex). This figure was adapted from Ito *et al.*, 2009.

Staphylococcal cassette chromosome *mec* type III (66.9 kb) was firstly identified in a strain isolated in New Zealand in 1985 and is characterised by the presence of an additional SCC element (SCCmercury (SCC*Hg*)) which confers resistance to mercury (130). Strains harbouring SCC*mec* type 3 are multiresistant to a wide range of antimicrobial agents including the β-lactam antibiotics as well as heavy metals due to the presence of the plasmid pI258 (on SCC*Hg*) which encodes resistance to mercury, pUB110 which encodes resistance to many aminoglycosides and pT181 responsible for tetracycline resistance. As with SCC*mec* type II, Tn*554*, containing the *ermA* gene is also present, encoding resistance to the macrolides, lincosamides and streptogramins. The pseudo-transposon ΨTn*554* also present in SCC*mec* type III confers resistance to cadmium (122, 135).

In 1990 MRSA strains harbouring SCC*mec* type IV (20.9-24.3 kb, according to the variant type) spread in the community of many countries around the world (80, 126). Its small size (the smallest of the SCC*mec* elements) may facilitate its mobility between staphylococcal species and explain the worldwide spread of SCC*mec* type IV (121, 136). Currently 10 variants have been identified (IVa -IVj) (132, 135, 137-140), however most only confer resistance to the β-lactams (141).

The first MRSA strains harbouring SCC*mec* type V (28 kb) were reported in 2004 from isolates of Australian origin, but have since been identified elsewhere (80, 126). This element mainly confers resistance to the β-lactams and is predominantly associated with CA-MRSA (80, 126).

Staphylococcal cassette chromosome *mec* type VI (20.9 kb) has been identified in strains isolated in Portugal and France (80, 142-144) and confers resistance to mainly the β-lactams.

Staphylococcal cassette chromosome *mec* type VII (35.9 kb) was identified in 2008 in MRSA

strains isolated in Sweden and differs from all other SCC*mec* types in that the *ccr* complex is situated between the J2 and J3 regions (130, 145, 146). This element mainly confers resistance to the β-lactams and is predominantly associated with CA-MRSA (145).

Staphylococcal cassette chromosome *mec* type VIII (32 kb) was identified in epidemic MRSA strains in Canada in 2009. The transposon TN*554* is present, conferring additional resistance to the macrolides, lincosamides and streptogramins (130, 147).

In 2011 SCC*mec* type IX and X were identified in isolates belonging to MLST CC 398 and obtained from individuals attending a veterinary conference (148). SCC*mec* type XI was also identified in the same year in isolates belonging to MLST CC 130 (149). These new SCC*mec* types were identified in isolates obtained from individuals who had direct contact with animals and this element contains genes conferring resistance to the heavy metals copper and arsenic, which are usually present in animal isolates. This suggests that these SCC*mec* types may have originated in other mammalian species. (148, 150) (www.SCC*mec*.org).

1.5 Molecular epidemiology of MRSA

Since the first MRSA strains were identified in the UK in 1961, MRSA has spread to or emerged in many other countries and become a major public health threat (151). In the 1980s, MRSA emerged as an important nosocomial pathogen (HA-MRSA) (152), however, by the early 90s; MRSA was isolated from indigenous Australian persons who had no history of hospitalisation. This was the first report to show that MRSA could also be acquired in the community (CA-MRSA) (153).

HA-MRSA strains are most commonly associated with SCC*mec* types I, II and III which

confer multiple antibiotic resistances to these strains. This wide spectrum of antibiotic resistance provides HA-MRSA the ability to survive in hospital environments with higher antibiotic pressures (154). As mentioned above, CA-MRSA is more commonly associated with the smaller SCC*mec* types IV, V or VII elements as well as with the presence of the Panton-Valentine leukocidin (PVL) gene (80, 155).

However, the epidemiology of MRSA is constantly evolving and the distinction between HA- and CA- MRSA has become distorted. CA-MRSA strains have been identified in hospitals (156-158) and HA-MRSA clones have successfully propagated in the community (159). In Australia, the epidemic (E) MRSA-15 health-care associated clone successfully transferred from hospital settings into the community (160, 161). Clones that have been shown to survive in both community and hospital environments commonly harbour SCC*mec* type IV elements (162-165).

It has been shown that MRSA clones evolved when epidemic MSSA strains acquired the SCC*mec* element (121). MLST analysis shows us that most *S. aureus* isolates (MRSA and MSSA) group into one of the five major clusters, which include clonal complexes (CC) 5, CC8, CC22, CC30 and CC45 (117, 121, 166). Various well-described MRSA clones have been identified worldwide, including the Archaic, Berlin, Brazilian/Hungarian, Iberian, Irish, New York/Japan, Paediatric, Southern German, UK MRSA-2, UK MRSA-3, UK MRSA-15 and UK MRSA-16 (80, 167) clones. Five of these well-known lineages are considered pandemic clones: Iberian, Brazilian/Hungarian, EMRSA-15, New York/Japan and the Paediatric clones (168).

1.6 Major HA-MRSA clones

1.6.1 Clonal complex 5

The New York/Japan clone (ST5-MRSA-II) also known as USA100 belongs to MLST-CC5 and has been associated with different *spa* types, including predominantly t001 and t002, although other *spa*-types have also been associated with this clone (reviewed in more detail in reference 78) This ST5-MRSA-II clone was first identified in the USA in 1998 (167). Later on more strains of this clone were identified in Austria, Croatia, Hong-Kong, Hungary, Japan, Portugal, Taiwan and the UK (80, 169). Although this clone is identified as healthcare associated, it has spread to the community in South Korea and Sri Lanka; and is therefore considered a CA-MRSA clone in these countries (170). In Africa this pandemic clone has as of yet only been identified in Senegal (171).

The Paediatric clone (ST5-MRSA-IV) also known as USA800 also belongs to MLST-CC5. This clone is also commonly associated with *spa* types: t001and t002 , but can also consist of *spa* types t003, t010, t045, t053, t062, t105, t178, t179, t187, t214, t311, t319, t389 and t443 (80). Strains of this lineage were first reported in Portugal in 1992 (167). As a pandemic clone it has spread in different geographical regions including the USA, South America and Europe (80, 84). In Africa it has been reported in Morocco as a minor clone, but is more predominant in Senegal (171).

The Southern Germany clone (ST228-MRSA-I), also known as the Italian clone, belongs to MLST-CC5 as a double locus variant (DLV) of ST5 and has been identified with different *spa* types including t001 (predominant), t023, t041, t188 and t201 (80). It mainly occurs in European countries (84) with a high prevalence in Italy of 57% (2000 – 2007). (172). In Germany this clone occurs only in healthcare-associated settings and seems to be less

prevalent in recent years (173).

The UK EMRSA-3 (ST5-MRSA-I) clone (also known as the Geraldine clone) belongs to MLST-CC5 and has been associated with different *spa* types: predominantly t001, t002 and other *spa* types t003 and t010, t045, t053, t062, t105, t178, t179, t187, t214, t311, t319, t389, t1443 (80). It has been found mainly in Europe and South America (84). In France, it was reported as a minor clone and isolated from both community and hospital-acquired infections (143). A study including *S. aureus* isolates from nine provinces in South Africa showed ST5-MRSA-I as the third most common clone in healthcare associated settings (174). Furthermore, this clone was the second most common *S. aureus* lineage identified in a group of Cape Town hospitals (Groot Schuur, Hospital Mowbray Maternity Hospital, Red Cross War Memorial Children's Hospital, Victoria Hospital and the University of Cape Town Private Hospital) in 2011 (175).

1.6.2 Clonal complex 8

The Archaic clone (ST250-MRSA-I) was first reported in the UK in 1960 and is considered the founder of all MRSA strains (122, 167). This clone groups into MLST-CC8 as a single locus variant (SLV) of ST8 and is associated with variable *spa* types including: t008, t009 and t194. It has been reported in many countries (Australia, Canada, Denmark, Germany, Switzerland, Uganda, UK and the USA), but prevalence has declined drastically (169, 176).

The Brazilian/Hungarian clone (ST239-MRSA-III) belongs to MLST-CC8 as a SLV of ST8 and is associated with different *spa* types: t037 (predominant), t030, t234, t387 and t388 (80). It has evolved from the integration of a 557-kb DNA fragment (representing 20% of the genome) from ST30 into CC8 lineage strains (136). ST239-MRSA-III is presumed to be the

first reported pandemic MRSA clone spread worldwide (169) and was first identified in Brazil and Hungary in 1992 and 1993 respectively (167). It has subsequently been identified in many other countries (80, 169). The predominance of ST239-MRSA-III in Hungary has been replaced by both ST228-MRSA-I (Southern Germany clone) and ST5-MRSA-II (New York/Japan clone) within a 10 year period of time (from 1994 – 2004) (177). It was also the predominant clone in a Portuguese hospital until it was replaced by ST22-MRSA-IV between 1996 and 2005 (163). In 1980 it became the most dominant clone in Ireland (132), as it was imported from Iraq with injured soldiers (178). This clone is usually isolated from Turkey, Iran, Saudi Arabia, Hong Kong, China, Taiwan and Singapore (80, 169). In Malaysia, it was found that 92.5% of 389 MRSA isolates were ST239-MRSA-III; and it is therefore considered the predominant clone in that country (179). In Africa, a study including isolates from five countries (Morocco, Senegal, Niger, Cameroon and Madagascar) found ST239-MRSA-III to be the dominant clone in Morocco (representing 95% of all isolates from this country) and Niger (171). Furthermore, Harris *et al*., studied the micro-evolutionary events of a global collection of ST239-MRSA-III clones and showed that this clone was easily transmitted across continents, between hospitals and hospital wards. (87). In South Africa, two studies identified the Brazilian/Hungarian as the most dominant clone in healthcare associated isolates obtained from different infection sites and bacteremia (174, 175). In KwaZulu-Natal province it was the second most prevalent clone obtained from health care facilities between 2001 and 2003 (180). This clone was also shown to be able to spread from hospitals into the community in South Korea, Hong Kong, Taiwan and Vietnam (170).

The Iberian clone (ST247-MRSA-I) belongs to MLST-CC8 as a DLV of ST8 with different *spa* types; predominantly t008 and t051 and less commonly t052, t054, and t200 (80). The prevalence of this clone is decreasing in Portugal and in Spain and has been replaced by

ST36-MRSA-II (162).

The Irish clone (ST8-MRSA-II) belongs to MLST-CC8 and has been reported with different *spa* types t008 (predominant), t024, t064, t190, t206, t211 (80). The first clone was identified in Ireland in 1990 (181). It has subsequently been reported in Australia, Canada, Ireland, the UK and the USA (80).

The UK EMRSA-2 (ST8-MRSA-IV) clone also known as USA500 or the Lyon clone belongs to MLST-CC8. It is associated with various *spa* types including t008 (predominant), t024, t064, t190, t206 and t211 (80). This clone is commonly isolated in France (143), but has been reported in Canada, Europe, Australia, and the USA (84).

ST612-MRSA-IV has clustered in MLST-CC8 and evolved as a DLV of ST8 (http://www.mlst.net). This clone has been reported in limited geographic regions (173) and is predominantly found in South Africa and Australia where it is associated with different *spa* types including: t64 (predominant), t008, t951, t1257, t1443, t1555, t1774, t1779, t1930, t1952, t1971 and 2196 (174, 175, 182).

1.6.3 Clonal complex 22

The UK EMRSA-15 (ST22-MRSA-IV) clone belongs to MLST-CC22. It has been associated with the following *spa* types: t032 (predominant), t005, t022, t223, t309, t310, t417 and t420 (80). This clone is more prevalent in Europe, Australia, Canada and Indonesia (84). It was the predominant clone in the UK and represented 85% of all blood isolates included in a recent study, replacing the predominant UK MRSA-16 clone which showed a decrease in prevalence over the same time period (21.4% in 2001 to 9% in 2007) (183). ST22-MRSA-IV

strains harbouring the *pvl* gene have been reported in numerous geographical regions (173), including South Africa, where the prevalence was 1.9% (174).

1.6.4 Clonal complex 30

UK MRSA-16 (ST36-MRSA-II), also known as USA200, clusters in MLST-CC30 as a SLV of ST30 and has been associated with different *spa* types including t018 (predominant), t253, t418 and t419 (80). It was first reported in the UK in 1993 and subsequently followed by other reports from the USA, Australia, Canada and South Africa (84, 167). Recently, this clone has been reported less frequently (173). However, a study conducted in Spain which included *S. aureus* isolates obtained between 1998 and 2002 showed that ST36-MRSA-II was still more common than the Iberian (ST247-MRSA-I) clone (184). In Africa this clone was not found in Morocco, Senegal, Niger, Cameroon or Madagascar (171). However, in South Africa it was reported as the most predominant clone in six of the country's provinces (174). This was substantiated by another study including *S. aureus* isolates collected from Cape Town hospitals, but excluding Tygerberg Hospital (175).

1.6.5 Clonal complex 45

The Berlin clone (ST45-MRSA-IV) also known as the USA600 clone is grouped in MLST-CC45 with variable *spa* types (t004, t015, t026 and t031). This clone was first reported in Spain in 1989 and is one of the more commonly isolated strains in Germany and the predominant strain in Belgium (167). It has been found in isolates collected from the UK, the Netherlands (185), Switzerland (186), Croatia (187) and Australia (80).

1.6.6 Major CA-MRSA Clones

The USA400 clone (ST1-MRSA-IV) is grouped as MLST-CC1. It has been associated with different *spa* types including t127 (predominant), t128, t174, t176, t386 and t558 (80). Strains of this clone are mostly found in European countries, Canada and the USA (80, 167). In Australia ST1-MRSA-IV (nearly always PVL negative) was found to be the most dominant CA-MRSA clone throughout the country in 2008 (188).

The USA300 clone (ST8-MRSA-IV) is grouped into MLST-CC8 and has been associated with different *spa* types, such as t008 (predominant), t024, t064, t190, t206 and t211 (80). ST8-MSSA (the presumptive ancestor of the USA300 clone) evolved to USA300 after acquisition of a SCC*mec* type IV element, PVL and arginine catabolic mobile element (ACME) virulence factor genes (189). The presence of the *pvl* gene and ACME in the ST8-MRSA-IV lineage is considered a genetic trait of the USA300 clone (190, 191). In 1999, the first outbreak of USA300 was reported in football players from Pennsylvania and prisoners with SSTI from Mississippi (189). Recent studies showed ST8-MRSA-IV is the most prevalent CA-MRSA clone in the USA, Canada, Samoa, Columbia, Ecuador and Venezuela and that it is continually spreading, becoming a major global concern (192, 193). In the USA, this clone has exhibited an ability to adapt and to spread rapidly into the community and hospitals, leading to a higher rate of healthcare associated bloodstream infections (194).

The Southwest Pacific clone (SWP) (ST30-MRSA-IV also known as USA100) is grouped into MLST-CC30 and has been associated with different *spa* types including t012 (predominant), t018, t019, t021, t038, t268, t275, t318, t338 and t391 (80). The globally disseminated ST30-MSSA clone is the predictive ancestor of this SWP clone (167). Currently, it is one of the most commonly isolated clones in Australia, Asia, South America,

Europe and Middle East (80, 195). This clone is more susceptible to antibiotics compared to the other CA-MRSA lineages (196, 197).

The USA 1000 clone (ST59-MRSA-IV/V) belongs to MLST-CC59 and has been associated with different *spa* types: t216 (predominant), t199, t347 and t444 (80). USA 1000 is one of the most commonly identified clones in Asian countries including China and Taiwan where this clone spread from the community into hospitals (170, 195). In Taiwan, ST59 carries a specific SCC*mec* element containing two different *ccr*C genes (*ccr*C2-C8) and is classified as SCC*mec* V (also known as the Taiwan clone) (146, 198). This clone has also been identified in Australia (182).

The European (ST80-MRSA-IV) clone belongs to MLST-CC80 and has been identified with several *spa* types including t044 (predominant), t131, t376, t416, t436, tt455 and t1109 (80). It was mostly identified in European countries, North Africa, the Middle East and Australia (80, 167, 186, 195), however the prevalence is on the decrease in European countries (173, 196). This clone is still highly prevalent as a hospital associated and CA-MRSA in North Africa and the Middle East and it seems to spread from these regions into European countries via migration (199).

1.7 MSSA Lineages

It has been hypothesised that MRSA originated from the repeated horizontal transmission of the SCC*mec* element into common MSSA lineages: CC5, CC8, CC22, CC30 and CC45 (121). Genetic relatedness between the MSSA and MRSA clones harbouring SCC*mec* type IV support the hypothesis of recent acquisition of the element into the reliable genetic

background of MSSA lineages (200). The genetic background of 67% of MSSA isolates obtained from Portuguese hospitals and the community were related to one of the five major MRSA clones in that region (168). In Belgian hospitals, 45% of MSSA isolates shared genetic background with the most common MRSA clones (200). Another study including *S. aureus* blood culture isolates obtained from patients at a Dutch hospital found that 50% of MSSA isolates had a genetic background common to the major MRSA clones in that setting (201). It has been shown that MSSA lineages are more divergent than MRSA clones. Currently, many MSSA lineages have been reported in different geographic regions and distributed across 15 ST in 8 MLST CC (202-207).

MSSA strains similar to common MRSA lineages were found to be more receptive to *mecA* acquisition, integration and expression than MSSA stains belonging to other lineages. The authors hypothesised that the genetic environment of MSSA strains belonging to uncommon major clones is not convenient for *mecA* gene integration and expression (208).

1.8 *S. aureus* Virulence Factors

Comparison of the genome of different *S. aureus* strains reveals that more than 97% of the genome is conserved throughout the species. The remaining 3% is the core variable (CV) region which gives rise to the different *S. aureus* lineages, and consists of the various mobile genetic elements (MGEs) which encode for virulence factors and antibiotic resistance genes; and allow *S. aureus* to adapt to differing environments (88, 209, 210).

Staphylococcus aureus is a common human pathogen causing a wide variety of diseases which can be categorised into: (i) superficial skin lesions such as impetigo and furunculosis;

(ii) deep seated infections such as pneumonia and bacteremia; (iii) toxaemia such as toxic shock syndrome (TSS) as well as food poisoning (211-213). *S. aureus* produces an abundance of virulence factors that contribute in different ways to infection processes and allow it to adapt to different environments (50). *S. aureus* possess two main virulence factors groups: (i) cell wall attached virulence factors known as Microbial Surface Component Recognising Adhesive Matrix Molecules (MSCRAMMs) including fibronectin-binding protein A (FnBPA) and B (FnBPB), collagen-binding protein (CNA), clumping factor A (ClfA) and B (ClfB); and (ii) the extracellular secreted toxins including alpha hemolysins (*Hla*), enterotoxins, lipase and protease (214). These two virulence factor groups assist *S. aureus* in reaching and colonising various sites of the body, evading the innate immune system, and causing infection (215).

1.8.1 Virulence factor mobilisation

Recent microarray analysis showed that the *S. aureus* genome is structured from a core genome (~ 75%), a core variable genome (CV) (~ 10%) and a mobile genetic element (MGE) (~ 15%) (88). Different types of MGEs have been identified including plasmids, pathogenicity islands, and genomic islands. Vertical transmission of MGEs from parent to daughter cells leads to a strong association of specific MGEs with bacterial lineages (216). Horizontal transmission is another route of MGE mobilisation, leading to the spread of these elements between different lineages (216, 217). However, there is evidence that certain lineages may have a limited ability to acquire specific MGEs such as SCC*mec*. (208). MGEs carry a variety of virulence factor and antibiotic resistance genes, therefore increasing the ability of *S. aureus* to adapt to new environments (210). It has been reported that when *S.*

aureus acquires virulence factor genes by horizontal transmission, it leads to the emergence of more virulent strains (218).

Some gene clusters such as the innate immune evasion cluster (IEC), are introduced into *S. aureus* by phages. The IEC consists of Staphylococcal complement inhibitor (SCIN), chemotaxis inhibitory protein (CHIP), staphylokinase (SAK), and enterotoxin A (SEA) or enterotoxin P (SEP) virulence factor genes (210, 219). The genetic structure of the IEC and the variety of phages carrying this cluster has resulted in a wide distribution of this IEC throughout different *S. aureus* populations. Based on the combination of innate immune evasion genes located on the IEC, this cluster can be classified into 7 types (A-G) (219). The enterotoxin gene cluster (egc) is another MGE which is horizontally transferred by phages into *S. aureus* and consists of enterotoxin G (seg), enterotoxin I (sei), enterotoxin M (sem), enterotoxin N (sen) and enterotoxin O (seo). (86, 216). The egc may be absent in some clonal complexes (CC1, CC7, CC8, and 15) and very prevalent in other clonal complexes (CC5, 22, 25, 30, and 45). The prevalence of egc in nasal isolates has been shown to be significantly higher than in invasive blood isolates suggesting that this cluster may enhance the nasal carriage ability of these strains (220). Genes encoding enterotoxin D (SED) and enterotoxin J (SEJ) were located in the same plasmid (221).

1.8.2 Cell-Wall Attached Virulence Proteins

Colonisation is a very important first step for *S. aureus* to develop infection. The MSCRAMMs mediate the adherence between *S. aureus* and the extracellular components of the host cells. Of these, FnBPA and FnBPB, encoded by two highly related genes, play an important role in assisting *S. aureus* binding to any surface coated with fibronectin (222).

FnBPs bind to endothelial fibronectin protein thereby allowing *S. aureus* to attach to the target cells (223). Collagen binding protien is responsible for *S. aureus* attachment to collagen mediating colonisation of bone and cartilage (219), leading to infections such as arthritis (224). Clumping factor A is more likely to be the main adhesion factor up to the early exponential growth phase, while ClfB, plays a minor role in adhesion. This could be due to the higher tendency of ClfA to bind to fibrinogen and/or due to its abundance in the bacterial cell wall. The two clumping factors could potentiate each other to assist *S. aureus* attachment to thrombi , which may lead to endocarditis (225). These factors, can also work independently to induce platelet aggregation (226).

The serine-aspartate proteins (SdrC, SdrD and SdrE) are surface proteins and members of the MSCRAMM family encoded by the *sdrC*, *sdrD* and *sdrE* genes respectively, and located in the sdr locus. Previous studies have found that all *S. aureus* isolates have a sdr locus and at least two of the 3 sdr genes(227). The adhesion of *S. aureus* to squamous cells was found to be activated by SdrC and SdrD (228). Peacock and colleagues detected sdrC in all tested isolates and demonstrated a significant association between the presence of the *sdrE* gene and virulence (223). A strong association was found between MSSA and *sdrC*-positive, *sdrD*-negative and *sdrE* -negative sdr gene profiles as well as a significant association between MRSA and the *sdrD* gene. The presence of both the *sdrD* and *sdrE* genes was also found to be associated with an increased potential for *S. aureus* to infect bones (229).

The extracellular adherence protein (Eap), also known as the major histocompatibility complex class II analogous protein (Map), is a member of the surface proteins that contribute to *S. aureus* pathogenesis by allowing attachment to variable host ligands (230). Extracellular adherence protein geneis highly conserved in *S. aureus* and therefore detection of this gene is a promising tool for molecular identification of this bacterium (231). It has the ability to bind

to the host extracellular matrix and plasma proteins as well as to bind back to *S. aureus*, thereby enhancing the pathogen's attachment to host tissues (232). As a result of the broad range of ligands Eap can attach to, it contributes to and enhances *S. aureus*' ability to be internalised in eukaryotes (233). The immune modulatory effect of Eap results from its interaction with intracellular adhesion molecule 1 (ICAM-1) receptors of antigen presenting cells (APC) and T cells, thereby inhibiting neutrophil chemotaxis and modulating the inflammatory response (232, 234). The presence of the intracellular adhesion locus A (*ica*) gene enhances the ability of *S. aureus* to form biofilms (235). A combination of different virulence factors, including the *ica* gene, have been found to be associated with more virulent strains (223).

1.8.3 Superantigenic Toxins (SAgs)

S. aureus produces many types of exotoxin that can induce disease or toxicosis such as toxic shock syndrome (TSS). The most commonly reported superantigens are: TSS toxin 1 (TSST-1), enterotoxin A (*SEA*) to enterotoxin E (SEE), enterotoxin G (SEG) to enterotoxin R (SER) and enterotoxin U (SEU) (236).

Superantigenic toxins encoded by genes located on phages include: (i) staphylococcal phage Φ3, containing either *sea*, *sep*, or *sea-sek-seq*; (ii) pathogenicity islands, including *S. aureus* pathogenicity island I1 (SaPI1) which carries *seb-sek-seq*, *S. aureus* pathogenicity island I2 (SaPI2) which carries tst-sec3-sel and *S. aureus* pathogenicity island I3 (SaPI3) which carriers sec-sel; (iii) a genomic island (vSAβ) containing the egc gene cluster which includes seg-sei-sem-sen-seo and occasionally seu; and finally; (iv) other SAgs genes located on

plasmids (*sed-sej-ser*) or on the SCC*mec* transposable element (*seh*) (216).

Both TSST-1 and SEB can down-regulate other exoproteins and auto-regulate their production, thereby inhibiting the inflammatory response (237). These SAgs are also known as pyrogenic toxin superantigens (PTSAgs), which are able to bind directly to the major histocompatibility receptor class II (MHC II) on the antigen-presenting cells (APC), thereby introducing these toxins to the T Cells (238). The subsequent activation of host macrophages and T cells results in a high production of monokines and lymphokines (239).

S. aureus can multiply on food and produce enterotoxins, thereby leading to staphylococcal food poisoning (SFP), which is symptomatically characterised by vomiting and occasional diarrhoea, when consumed. SFP is a self-limiting disease, with resolution within 24 to 48 hours (239). Exfoliative toxins A (ETA) and B (ETB) can both cause impetigo and staphylococcal scalded skin syndrome (SSSS) that is characterised by skin erythema as a result of intra-epidermal desquamation (212, 240).

1.8.4 Immune Avoidance Virulence Factors

The ability of *S. aureus* to evade the host immune system, by inhibiting the innate immune system and compromising humoral and cell-mediated immunity via several mechanisms, leads to an increase in its pathogenicity and its ability to cause repeated infection (234). The immune modulatory genes staphylokinase (*sak*), chemotaxis inhibitory protein (*chp*), staphylococcal complement inhibitor protein (*scn*) and enterotoxin A (*sea*) or enterotoxin p (*sep*) are located in the immune evasion cluster (IEC) that is carried by the active βC-Φs bacteriophage (219).

Chemotaxis inhibitory protein (CHIP) binds to the formal peptide receptor (FPR) and the C5a receptor (C5aR). This binding inhibits the migration of neutrophils to the infection sites (241). CHIP is introduced with other components of IEC into *S. aureus* by β-haemolysin-converting bacteriophages (219). Staphylococcal complement inhibitory (SCIN) virulence factors stabilise and deactivate complement as well as inhibit *S. aureus* phagocytosis and killing by neutrophils (242). Production of CHIP and SCIN during the early growth phase may be responsible for *S. aureus* survival during the first stage of colonisation and/or invasion (243).

The alpha-defensins are peptides produced by the innate immune system, including the poly morphonuclear cells (PMNs), which produce neutrophil peptides-1 (HNP-1) and 2 (HNP-2) to protect the mucosal surface and skin from bacterial infections (244). Staphylokinase (*SAK*) combines with HNP-1 and HNP-2 in specific sites, thereby allowing *S. aureus* to evade destruction by the host's innate immune system and to subsequently colonise the body and mucosal surfaces. The *SAK*s predispose to super-infections by other Gram-positive and Gram-negative bacteria that are sensitive to alpha-defensins, including *Escherichia coli* and *S. epidermidis* (245). The gene encoding *SAK* is carried in a bacteriophage, transduced into *S. aureus* and up-regulated by the accessory gene regulator locus (*agr*) (246).

The majority of the *S. aureus* strain microcapsules belong to serotype 5, 8 or 336 (247, 248). However, type 5 (more common in MRSA) and 8 are responsible for 75% of human infections (240). The capsule increases bacterial resistance to phagocytosis and opsonisation (248, 249) by hiding the antibodies′ target sites and disrupting the binding of complement (c3b) to receptors on neutrophils (250, 251). The capsule's hydrophilic structure and low antigenicity also protect it from phagocytosis (252). Capsule 5 and 8 expressions have been

shown to enhance *S. aureus* virulence in an animal model (253, 254).

1.8.5 Hemolysins

S. aureus produces soluble cytotoxic molecules that induce inflammatory reactions and have a cytolytic effect. Alpha-hemolysin (*Hla*), encoded by the *hla* gene has haemolytic, dermonecrotic and neurotoxic effects (239). Hla binds to specific receptors on the target cell membrane. This integration leads to pore formation across the host cell membrane, followed by membrane destruction and cell lysis (255, 256). Hla plays an important role in brain abscess formation (257) and is also an important factor in biofilm formation. *S. aureus hla*$^{-}$ mutants are able to colonise the host surface, but are unable to form multicellular macrocolonies (258). It has also been reported that *hla* expression significantly enhances the mortality rate of mice in a murine model of pneumonia (259). Ohlsen *et al*., found that subinhibitory concentrations of β-lactam antibiotics activate *hla* expression and increase the production of alpha-toxin, while clindamycin suppresses *hla* gene expression completely (257). Some MRSA isolates produce 30 times more Hla when grown in methicillin enriched media than when cultured in control media (260). Alpha-toxin is inhibited in *S. aureus* during TSS, while in contrast, gamma and delta hemolysins are still produced (261).

Beta-hemolysin (Hlb) is produced by many *S. aureus* strains isolated from animals. In a murine model of pneumonia, expression of Hlb increased the extent of lung injury through its ability to enhance neutrophil inflammation and extravascular protein leakage (262).

1.8.6 Panton-valentine leukocidin

Panton-valentine leukocidin is more commonly associated with CA-MRSA strains (263). It is encoded by the *lukPV* operon which was introduced and integrated into the *S. aureus* genome by phage transduction (209). Vandenesch *et al*., found an association between CA-MRSA SCC*mec* type IV and PVL production (264). This association was confirmed by Shukla and co-workers (265). Another study indicated that less than 5% of SCC*mec* types I-III harbour PVL, while 40-90% of MRSA SCC*mec* type IV do (266). However, the association between SCC*mec* type IV and PVL production is still under debate. The active form of PVL consists of two polypeptide molecules, PVL-F and PVL-S (85). The PVL-S molecules are responsible for the initial attachment to the polymorphonuclear neutrophil (PMN) membrane and along with PVL-F, create the pore forming unit which leads to cell necrosis (267) and apoptosis (due to its effect on the host cell's mitochondria). PVL is a cytolytic, pro-inflammatory and pore forming exotoxin, which, due to these effects, can be used against the host immune system (268). It plays an important role in *S. aureus* SSTI and has a high affinity to collagen types I and IV; and laminin [Extracellular Matrix Component (EMC)] which is abundant in the lungs, leading to biofilm formation (269) and necrotising pneumonia (270, 271). PVL positive *S. aureus* strains were isolated from 97% of abscesses (272). Production of many secreted toxins, including haemolysin and protease, is down-regulated when PVL is expressed (271), which is a similar effect to when TSST-1 or SEB are produced (237).

Staphylococcis aureus strains causing sepsis were found to harbour more than one SAg encoding gene. The presence of the *sea* gene was associated with the severity of infection, while *egc* gene clusters were negatively correlated with pathogenicity (273). A study investigating the relationship between *agr* group type and human disease showed that *agr* does not have an influence on the site of infection caused by *S. aureus* (274). Investigation of

the presence of enterotoxin genes in *S. aureus* isolates obtained from nasal carriers and bacteraemic patients revealed that strains isolated from blood were significantly more likely to harbour the *sea, seb, sec, and seh* enterotoxin genes than those isolated from the nose (275).

The acquisition of antibiotic resistance in *S. aureus* has been suggested to influence virulence factor expression, due to the fitness cost associated with expression of resistance (127, 276). Furthermore, HA-MRSA strains containing a SCC*mec* type II element express fewer virulence factors than CA-MRSA containing SCC*mec* type IV, suggesting that antibiotic resistance is negatively associated with gene expression (277). The majority of the studies investigating the prevalence of these virulence factor genes were conducted on MRSA strains, therefore more studies need to be done on MSSA strains for a proper comparison. In a study including MRSA and MSSA isolates obtained from children (≤14 years), the prevalence of the *pvl* gene was significantly higher in MRSA compared to MSSA strains, while the presence of the *seb* gene was significantly higher amongst MSSA than MRSA isolates (278). Comparing the virulence gene profiles of three groups of isolates including CA-MRSA (USA 400), clinical MSSA and nasal carriage MSSA; the CA-MRSA virulence factor profile showed significantly higher percentages of the *sea*, *sec*, *seg*, *seh*, *sek*, *sel*, *pvl* and *clfA* genes, compared to the other two groups of isolates (279). In another study, MRSA harboured the *seg* and *sei* genes more frequently than MSSA strains (280).

South African MSSA isolates showed a distinct PFGE genotype compared to MSSA isolates from the U.S.A. The presence of the *sdrD*, *sea* and *pvl* virulence factor genes was significantly higher in the South African MSSA isolates compared to U.S.A. MSSA isolates (281). A collection of *S. aureus* bloodstream isolates from North America and Europe also showed a significant difference between the virulence gene profiles obtained from the 2

regions, thereby suggesting that *S. aureus* strains from different geographical regions vary in their virulence factor gene profiles (282).

1.9 Quorum Sensing

Bacteria were thought to be primitive single-cell organisms which could survive and multiply independently without any interaction with other members of the same species. However, quorum sensing is a form of intra-species (and sometimes inter-species) communication which enables the bacteria to detect population density and to alter and synchronise gene expression based on population density. The ability of bacteria to overcome the host immune response (thus facilitating infection) depends on the bacterial population density, otherwise known as the infective dose (283). Bacterial propagation is essential to survival and should be carefully controlled (284). For bacteria to colonise a specific body site, they need to attach to host cells and then multiply until reaching a critical mass, after which nutrients and space become limited. To survive, the bacteria must then invade other body sites, which would require suppression of genes that encode for adhesion at the current site and expression of genes that contribute to invasiveness (285). During the early exponential phase of growth, *S. aureus* produces surface proteins including the adhesins, protein A, fibronectin-binding protein and collagen. These factors are later down-regulated at the end of the exponential phase of growth. *S. aureus* will then up-regulate the extracellular proteins, including the toxins and hemolysins. This regulation of virulence factors assists *S. aureus* in colonising the skin of different body sites in the first stage of infection, and to then invade the host tissue via extracellular virulence factors as soon as it reaches a critical mass. The *agr* locus is responsible for this virulence factor regulation via a quorum sensing mechanism (286, 287).

The expression of the *agr* locus is controlled by two different promoters, P2 and P3, which function together as a typical autoactivation circuit (288) (Figure 3) . The P2 promoter consists of the *agr B,D,C* and *A* genes, which via RNAII, encode for the signal transducer (*AgrC*), the response regulator (*AgrA*), a propeptide that is processed to an autoinducing peptide (AIP) (*AgrD*); and the integral membrane protein that is responsible for the maturation and/or secretion of AIP (*AgrB*) (289, 290).

The Staphylococcal accessory protein A , encoded by the staphylococcal accessory protein regulator (*sar*) and *AgrA*, binds to a specific region between the P2 and P3 promoters (291). This binding up-regulates transcription of the *agr* locus via the P2 and P3 promoters, leading to increased RNAII and RNAIII production (292). When the *agr* locus is activated by RNAII, AgrD will be modified to AIP and secreted out of the cell by the AgrB assistant (290). Autoinducing peptide then binds to its specific receptors on AgrC leading to AgrC phosphorylation (293) and the transfer of the phosphate group from AgrC to AgrA, resulting in AgrA activation (294). RNAIII up-regulates the production of extracellular toxins and down-regulates cell surface proteins (295).

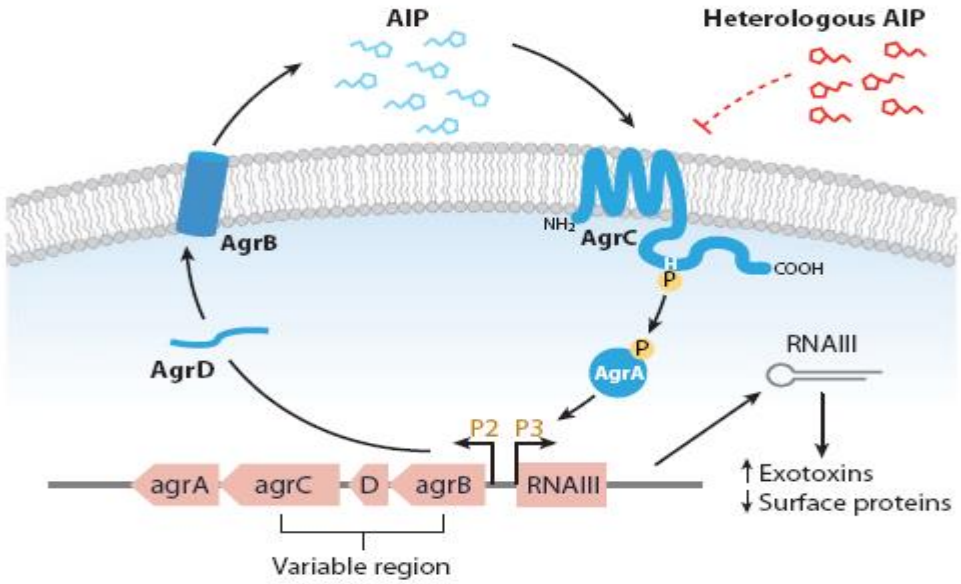

Figure 3 : *agr* locus structure and autoactivation. (281)

AIP: autoinducing peptide; Heterologous AIP: produced by different *agr* type; P2: promoter 2 which activate *agr B*, *D*, *C* and *A* genes via RNAII; P3: promoter 3 which provoke exotoxin production and supress surface protein expression via RNAIII.

According to the sequence differences in the *agr* B, D and C regions, the *agr* locus can be classified into four *agr* groups (I to IV) (290, 296). Autoinducing peptide produced from *S. aureus* strains that have the same *agr* group type are able to activate each other's *agr* responses, while AIP from strains belonging to different *agr* groups may inhibit the *agr* response in strains from other *agr* group types (290, 297, 298).

Accessory gene regulator group I has been shown to be associated with invasive infections especially bacteraemia, while *agr* group III is associated with non-invasive infections (299). Another study found a possible association between *agr* group IV strains and generalised exfoliative syndrome; and *agr* group I and II strains with suppurative infections and

endocarditis (300). Most strains that cause TSS have *agr* group III (290); and VISA strains often consist of *agr* group types I or II (U.S.A. and Japan (type II), Taiwan (type I) and Europe (type I and II)) (301).

Accessory gene regulator function may control virulence factor gene expression as is evidenced by attenuation of *agr* dysfunctional strains in different animal models, including those with skin abscesses (302, 303), osteomyelitis (304), septic arthritis (305), and endocarditis (286). Accessory gene regulator mutant strains have also been found to be less virulent than wild-type strains. Sequencing of the *agr* locus of these *agr* dysfunctional strains revealed some strains had either an insertion or deletion of an adenine in *AgrA*, B, or C. However, a few of the strains were wild-type for the *agr* sequence, suggesting that different factors may affect *agr* functionality (306). Another study showed that the acquisition of the *mecA* gene by HA-MRSA causes cell wall changes which can reduced the *agr* quorum sensing activity (307).

In CA-MRSA strains (SCC*mec* types IV and V), the *agr* locus is essentially functional. Thus, these strains are highly virulent (308) and transmissible between different hosts (309). HA-MRSA strains (SCC*mec* types I-III) are essentially *agr* dysfunctional and mostly spread in hospitals (306, 310). A study investigating the association between *agr* dysfunction and reduced vancomycin susceptibility with CA-MRSA (SCC*mec* IV/IVa) and HA-MRSA (SCC*mec* I-III) reported a significant association between carrying SCC*mec* type I-III and impaired *agr* functionality as well as with a reduced vancomycin susceptibility (310). Similar results were found in South Korea amongst bacteraemia isolates, where *agr* dysfunction was significantly higher in ST239-MRSA-III and ST5-MRSA-II than in ST72-MRSA-IV (311).

In a hospital environment, the prevalence of *agr* dysfunctional MRSA isolates ranges from 15

to 60%, whereas the prevalence of *agr* dysfunctional isolates outside the hospital environment is rare (~ 4%) and usually associated with individuals with a recent hospitalisation event (309, 312-315). Although *agr* dysfunctional isolates' virulence is attenuated, some studies show that the impaired *agr* functionality may confer advantages to these strains in health care environments under antibiotic selection pressures (309, 316). Exposure to sub-lethal concentrations of certain antibiotics may lead to the selection of *agr* negative strains which thereby increase their fitness advantage (317). MRSA *agr* dysfunctional isolates have been found to be more resistant to thrombin-induced platelet microbicidal proteins (tPMP) and lead to a more persistent bacteraemia than *agr* functional isolates (312). Clinical studies have reported that *agr*-dysfunctional isolates are associated with reduced susceptibility to vancomycin, heteroresistance to vancomycin (hVISA); and increased trends toward VISA (311, 316, 318, 319). The effect of *agr* dysfunction on mortality rates of bacteraemic patients remains unclear with some studies reporting a higher mortality rate in severely ill patients (320), with others reporting no association (310).

Even though the virulence of *agr* dysfunctional strains is impaired, they are still able to colonise the nostrils of previously hospitalised patients and are transmissible between family members (313). The similar percentage of *agr* dysfunctional strains reported among carriers who developed bacteremia and uninfected carriers, suggests that *agr* function is not essential for infection (321).

Colonisation is a risk factor for *S. aureus* infection and nostrils are the most common body site of colonisation. Colonised individuals are more susceptible than noncarriers to subsequent infections, including bacteraemia. Hospitalisation, immune compromise (HIV and diabetes) and extremes of age have been reported as predisposing factors for MRSA colonisation. Although *S. aureus* nasal colonisation as a source of endogenous bacteremia has

been studied in other countries, it has not been investigated in South Africa.

The aim of this study is to investigate the phenotypic and genotypic characteristics of isolates of *S. aureus* collected from the blood and nasal cavity of patients admitted to Tygerberg Hospital, South Africa. Using PFGE, the following chapter will determine the association between blood and nasal isolates of our patient cohort. In Chapter 3 we focus on the genotypic characteristics of our isolates using different genotyping methods. In chapter 4 we describe the prevalence of virulence factor genes amongst selected representative isolates and finally (Chapter 5) we determine the *agr* functionality of isolates in the cohort.

CHAPTER 2 USING PFGE: CORRELATING BLOOD AND NASAL *STAPHYLOCOCCUS AUREUS* ISOLATES IN PATIENTS ADMITTED TO AN ACADEMIC HOSPITAL IN SOUTH AFRICA

2.1 Introduction

As discussed in section 1.3, the nose can be considered as the main colonisation site as *S. aureus* has high adherence ability to nasal epithelial cells in the anterior part of the nostrils (322-324). The nasal secretions contain different innate immune components including lysozyme and antimicrobial peptides which protect the nose from colonisation by different microbes (325).

Colonising *S. aureus* strains are biologically well adapted to the persistent carrier's nasal environment. As a result, following nasal decolonisation, the majority of persistent carriers are recolonised with their original specific colonising strain(s) (59, 326).

In the Netherlands, a recent study on healthy volunteers found that *S. aureus* colonised the nose of approximately 71% of the healthy population (326). This consisted of persistent carriers (24%) where ≥80% of the nasal swabs (usually obtained at 3-4 weeks intervals) were positive for *S. aureus* during a six month period, and intermittent carriers (47%), when the isolation rate was <80% during the same period (326). In this study the authors suggested reclassifying carriers into "persistent" and "others", as the intermittent and noncarriers had similar antistaphylococcal antibody profiles and responded in a similar way to an artificial nasal inoculation with a *S. aureus* mixture after prior decolonization. Hospitalisations, length of hospital stay and antibiotic treatment have been shown to be risk factors for nasal colonisation (42, 327).

S. aureus infection in carriers has been found to be higher than in noncarriers (16, 39, 328). In patients on continuous ambulatory peritoneal dialysis (CAPD), nasal carriers were found to be significantly more vulnerable to develop catheter exit-site infections than noncarriers. The similar phage type and antibiotic profile of 85% of colonising and infecting isolates supports the endogenous route of infection in colonised CAPD patients (329). Similar results were obtained and demonstrated that 93% of nasal and infecting strains had the same phage type in patients on haemodialysis (330). Von Eiff and co-workers performed a study in Germany applying two approaches to investigate the correlation of blood and nasal *S. aureus* isolates. First, nasal swabs were collected immediately after the blood culture of patients admitted to the hospital revealed a positive *S. aureus* growth. In a second study, they screened 1278 patients' nostrils over a five-year period and followed up each case to identify those who developed *S. aureus* bacteraemia (SAB). According to Pulsed-Field Gel Electrophoresis (PFGE) data, the correlation between the blood and nasal isolates in the first and second study was 82.2% and 85.7% respectively (331). In another study, *S. aureus* carriers had been found to be three times more likely to develop bacteraemia than non-carriers (16). In addition, there is evidence that decolonisation of nasal carriage reduces the incidence of *S. aureus* infection (332, 333). Taken together, this indicates that the nose is a primary colonising site from where *S. aureus* can spread to other body sites and cause infections.

Nasal methicillin resistant *S. aureus* (MRSA) colonisation at admission time or acquisition during hospital stay is well known as a predisposing factor for subsequent clinical infection (334). Colonisation with MRSA compared to methicillin sensitive *S. aureus* (MSSA) leads to a four times higher risk of developing *S. aureus* infection (73).

Although many studies have evaluated the correlation of *S. aureus* nasal colonisation as a risk factor for subsequent infection, the correlation of blood and nasal isolates has not been studied thoroughly in developing countries. The patient population, geographical area, differences in infection control practices, disease profiles (such as the prevalence of immune suppression caused by HIV) and also possible *S. aureus* strain type variation between developed countries and South Africa may lead to different results. The aim of this study was to determine the correlation between the nasal colonising and invasive blood isolates of patients with *S. aureus* bacteraemia at Tygerberg Academic hospital using molecular typing methods.

2.2 Methods

2.2.1 Study design and setting

This was a prospective descriptive study designed to illuminate the main characteristics of the strains isolated from patients with SAB at Tygerberg Academic hospital, and to determine the correlation between blood and nasal isolates using molecular strain typing methods. Tygerberg Hospital is a 1384 bed tertiary-care academic hospital that provides a comprehensive clinical service. Bed numbers for the intensive care units (ICU) are 101, consisting of 83 adult ICU (AICU) beds and 18 paediatric ICU (PICU) beds. At the time of the study some wards were temporarily allocated to patients from a secondary level district hospital (Khayelitsha District Hospital, 230 beds) and are referred to as Khayelitsha wards.

The laboratory work for this study was conducted in the research laboratory of the Division of Medical Microbiology of the Faculty of Medicine and Health Sciences at Tygerberg Hospital.

2.2.2 Study population

All patients from Tygerberg Hospital with SAB as confirmed by blood culture were included in the study. Nasal swabs were collected on these patients to determine *S. aureus* carriage status.

2.2.3 Inclusion criteria

Cases/Patients were recruited over a period of 24 months from January 2010 to January 2012.

Every patient with a positive blood culture from which a pure growth of *S. aureus* was isolated, who consented to be part of the study or who qualified for a waiver of consent (see 'ethical considerations'), and from whom it was possible to obtain nasal swabs to determine colonisation, was included in the study.

If more than one *S. aureus* bacteraemic episode occurred in a patient, an isolate from the second episode was only included if the patient's doctor confirmed clinical improvement after the first episode or if a negative blood culture had been documented between the first and second bacteraemic episodes. When more than one isolate from the same patient in one episode had identical PFGE band patterns, only one isolate was included as a case.

2.2.4 Exclusion criteria

Cases where the blood culture yielded mixed growth of *S. aureus* with another bacterial species or mixed growth with different *S. aureus* strains were excluded. Cases (including their blood culture isolates) where nasal swabs could not be taken because the patient demised, was transferred to another hospital or did not give consent, were also excluded from the study.

2.2.5 Data collection

Limited clinical data including the patient's underlying diseases was collected and recorded by the clinical microbiologist on duty at the time when *S. aureus* was isolated from a positive blood culture as part of the routine consultation with the treating clinician. However, there

was no standardised, prospective collection of clinical data for the purposes of the study.

Due to the fact that the patient population of Tygerberg Hospital, as a referral hospital, have often had some form of exposure to health care facilities, and due to practical constraints related to acquiring in-depth information about the history of each case, we were not able to effectively classify cases of bacteraemia as 'hospital acquired' or 'community acquired'. However, in order to gain some insight as to where the patient contracted the invading strain, we determined the time interval in days between admission to hospital and blood culture collection for all cases.

As the epidemiology of staphylococci may be different in various age groups in our population, patients were stratified at the time of blood culture collection according to the following criteria: neonates 1-30 days, children >30 days-13 years and adult ≥ 13 years.

2.2.6 Blood culture isolates

Blood cultures were taken from patients at clinicians' discretion, and submitted to the NHLS microbiology laboratory at Tygerberg Hospital. Blood culture bottles were incubated in the BACTEC Fluorescent 9120 system (Becton Dickinson). Isolates were identified as *S. aureus* based on standard microbiological methods, including Gram morphology, catalase, mannitol fermentation, presence of DNAse, and if necessary the PastorexTM Staph-Plus (Biorad) test. The Vitek®2 System (Biom′erieux) was used to confirm the identification when the mannitol fermentation or DNAse was negative or weakly positive. *S. aureus* isolates from blood cultures processed by the diagnostic microbiology laboratory of the National Health Laboratory Service (NHLS) at Tygerberg Hospital were collected for further investigations in

this study.

2.2.7 Nasal swab collection

Nasal swab were collected on the same day that the patient's blood culture revealed the presense of *S. aureus*. After acquiring informed consent from patients that met the inclusion criteria, nasal swabs were taken by sterile distilled water moistened cotton-nasal swab (for adults: COPAN 155C RAYON Regular Tip, and for children: 160C RAYON Aluminium RAYON Mini Tip) (45, 69). The left and right nostrils were swabbed by inserting the moistened nasal swab into the vestibulum nasi (1.5 cm in adults and anatomically appropriate distance to reach the vestibulum nasi in children and neonates), applying pressure and rotating the swab on the internal side of the nostrils for four continuous times (55, 335). The specimens were transported in an ice box to the laboratory within 30 minutes.

2.2.8 Bacterial isolation of *S. aureus* from nasal swabs

The nasal swab was plated on 5% horse blood agar (BA) and incubated at 37˚C for 2 days, as well as on mannitol salt agar (MSA) as described previously (336), incubated at 37˚C for 2 days, followed by incubation at 25˚C for another 5 days. The tip of the swab was broken off and dropped into mannitol salt broth (MSB) and incubated with shaking at 37˚C for 7 days (59). The MSB was prepared according to the Difco manual (337) and stored at 4˚C in the dark and used within a one month period. If the colour of MSB changed from orange to yellow, the broth was subcultured on BA. More than one colony was chosen for identification when different colonies showed variable phenotypic characters. *S. aureus* identification was

based on conventional methods, as described for blood culture isolates.

2.2.9 Storage of bacterial isolates

A single colony from each pure culture obtained from the blood culture and/or the nose and identified as *S. aureus* was subcultured on BA. A cryopreservative tube (Pro-Lab Diagnostics) was inoculated with colonies from this pure culture according to the manufacturer's instructions. The inoculated cryovial tubes were stored at -80˚C for further testing.

2.2.10 Methicillin susceptibility testing

Methicillin susceptibility was determined using the disc diffusion method and interpreted according to the 2010 CLSI guidelines (338), where 0.5 McFarland of *S. aureus* suspension streaked on Mueller-Hinton agar and cefoxitin 30 µg (Mast Diagnostics) discs were used. Both MRSA and MSSA isolates were included for further study.

2.2.11 Molecular typing of isolates

Pulsed-field gel electrophoresis (PFGE), the most discriminatory *S. aureus* genotyping technique (203), was performed as previously described (97).

Briefly, a single colony of a pure culture was inoculated into brain heart infusion broth (BHIB) and incubated overnight at 37ºC. The cell suspension volume used in plug preparation was calculated by adjusting to a spectrophotometer (Amersham Biosiences) absorbance of 0.9 at 600 nm. The bacterial cell suspension was centrifuged and the

supernatant discarded. Plugs were prepared by re-suspension of the bacterial pellets in 300 µl of TE buffer (10 mM Tris HCL, 1 mM EDTA), 300 µl of 1.8x agarose (Lonza, Switzerland) and 4 µl of lysostaphin (130 µg/l; Sigma, St. Louis, USA). Solidified plugs were subsequently immersed in EC lysis buffer (6 mM Tris HCL, 1M NaCL, 100mM EDTA, 0.5% Brij-58, 0.2% sodium deoxycholate, 0.5% sodium lauroylsarcosine) and allowed to incubate overnight at 37ºC followed by four washes in TE buffer. The bacterial genome was restricted by *Sma*I enzyme (Fermentas life science) for 4 hours at 25ºC followed by electrophoresis in a 1% pulsed-field certified agarose gel (BIO-RAD). The electrophoresis was performed by using the CHEF-DR® III system (BIO-RAD) and the following parameters were used: 200 (6V/cm), temperature 14ºC, initial switch 5 seconds (s), final switch 40s and running time was 21 hours. The gel was then stained with ethidium bromide (EtBr) (Promega) solution (1µg/ml) for 30 minutes followed by destaining in fresh water for 45 min. Separation bands were visualised and the image captured as a TIFF file for analysis with GelCompar®II Version 6.0 software to identify PFGE clusters and strain similarities. Tolerance and optimisation at 1.5 and 0.5% respectively were used to set bands and an 80% similarity coefficient was used to define the pulsed-field type clusters (97). Isolates that exhibited indistinguishable PFGE band patterns were considered the same strain.

2.2.12 Definitions

We identified a ‘case’ as any patient with an episode of *S. aureus* bacteraemia that met the inclusion criteria. Endogenous SAB was defined as cases where the nasal and blood isolates had identical PFGE band patterns (as shown in Fig 2.1, N1 and B1; N5 and B5; N6 and B6), as this indicated that the patient was likely to be infected by his/her own colonising strain.

Exogenous SAB was defined as any case where there was no nasal colonisation or cases where blood and nasal isolates had different PFGE band patterns, as this indicated a possible external source of infection, and that the nasal isolate was not the cause of bacteraemia (as shown in Fig 4, N2 and B2; N3; N4 and B4). When *S. aureus* was not isolated from the nasal swab the patient was identified as a non-colonised patient. Although these patients were included in the exogenous SAB group, they were excluded when the correlation between nasal and blood culture isolates was analysed.

2.2.13 Statistical analysis

The correlation between blood culture and nasal *S. aureus* isolates was evaluated by the T-test for a single proportion. The Chi-squared test was used to estimate the significance of the correlation between the source of bacteremia and methicillin resistance. The difference in the time of admission and blood sample collection as well as the difference in the time of blood sample collection and nasal swabbing was statistically evaluated by Mann-Whitney U Test. Statistical significance was set at a p value of <0.05. Statistical analysis was performed using Statistica, version 10 (2012).

2.2.14 Ethical considerations

The study was approved by the Committee of Human Research at the Faculty of Medicine and Health Sciences, Stellenbosch University (N09/10/266) (Appendix A1). Three different consent forms were used to obtain informed consent from variable ages and groups: adults (≥18 years old) (Appendix A2), adolescents (≥13 - 17 years old) and the parents or guardians

of adolescent children and neonates (<13 years old) (Appendices A3 and A4, respectively). A waiver of consent was approved to take nasal swab from intubated, unconscious patients and children in cases where their parents or guardians were not available. Patient confidentiality was assured by allocating a case number to each isolate which also indicated the source of isolation (blood or nasal).

2.3 Results

2.3.1 Patient demographics

Of a hundred and sixty four patients with SAB, 162 met the case definition during the period of specimen collection and were therefore included in this study. Two patients were excluded because they were infected with more than one strain of *S. aureus*. In one patient one of the nasal isolates correlated with one of the blood isolates, however differed to the other blood isolate in the other, therefore making classification of endogenous or exogenous infection problematic.

The mean age of the study population was 29 years, ranging from 2 days to 87 years. Interquartile range (IQR) was 44.36 years.

The majority of patients were adults, 108 (66.7%) followed by 31 (19.1%) neonates and 23 (14.2%) children. The gender breakdown of the different age categories is shown in Table 2.

Table 2: The demographic profile of cases included in this study, concerning age and gender

Age category	Male N (%)	Female N (%)	Total N (%)
Adults	63 (38.9)	45 (27.8)	108 (66.7)
Children	11(6.8)	12 (7.4)	23 (14.2)
Neonates	17 (10.5)	14 (8.6)	31(19.1)
Total	91 (56.2)	71(43.8)	**162 (100)**

* Percentages are shown using the total number of SAB cases (162) as denominator

2.3.2 Blood and nasal isolate correlation in colonised patients.

Nasal colonization was detected in 128 (79%) of the 162 patients included in the study. Blood

and nasal isolate correlation in colonised patients was evaluated by using PFGE gel band patterns analysis (Figure 4), which showed a highly significant correlation ($p < 0.000001$) between the blood and nasal isolate pairs where 93/128 (72.7%) of the colonised patients were infected with the same strain.

2.3.3 Endogenous and exogenous bacteraemia

PFGE gel band pattern analysis results were used to classify SAB into endogenous and exogenous categories for all 162 cases based on the band pattern similarity between the blood and nasal isolate of a patient.

Identical blood and nasal isolates were found in 93 patients, establishing the colonizing strain as a likely source of infection in 57.4% of all SAB cases. From the limited clinical data the main underlying conditions among endogenous bacteraemia cases were burns (n=12), renal failure (RF) (n=9) and HIV infection (n=7).

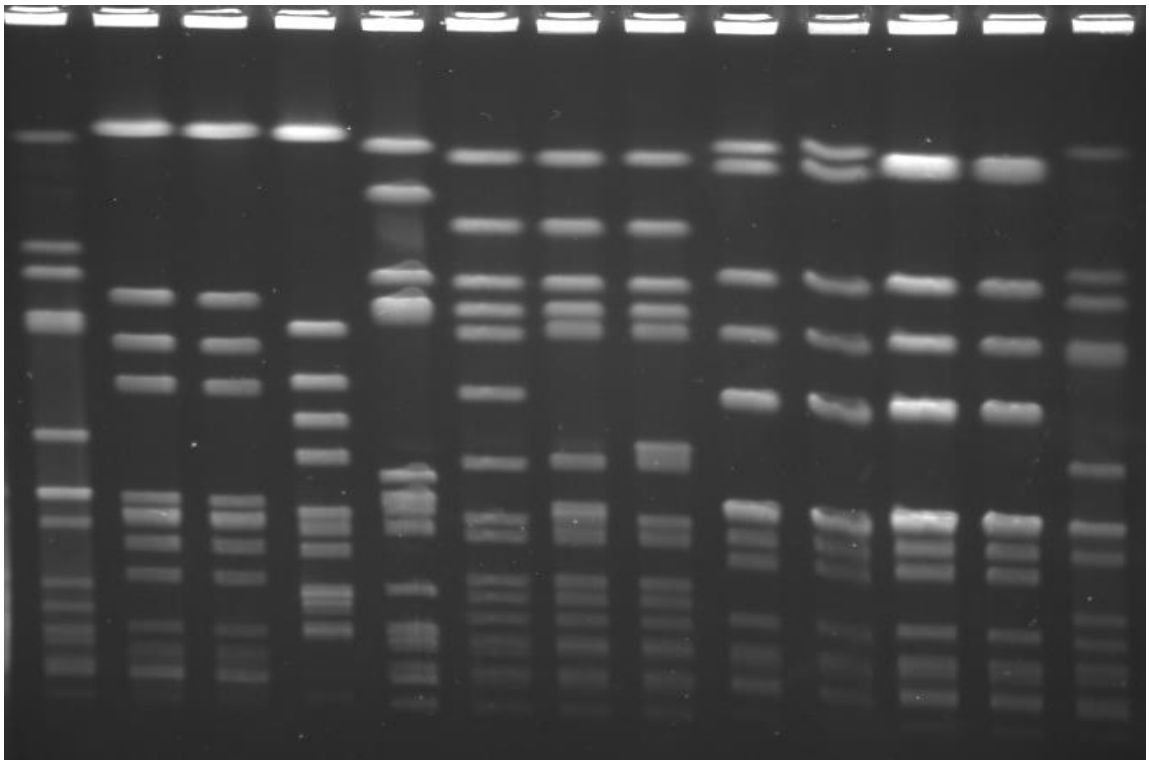

Figure 4 : SmaI PFGE band patterns results for blood and nasal isolates obtained from different patients.

*(N) Represent nasal isolates and (B) represent blood isolates. Similar colonizing and invading strains as shown in lanes N-1 and B-1 (patient 1), N-5 and B-5 (patient 5) and N-6 and B-6 (patient 6). Different blood and nasal isolates as shown in N-2 and B-2 (patient 2) and N-4 and B-4 (patient 4). Lane B-3 represents a strain from a patient that was not colonized and the first and last lanes (NCTC 8325-4) were used as markers.

The source of SAB was classified as exogenous in the other 69 (42.6%) patients: 35 (21.6%) colonized with one or more *S. aureus* strains that were not related to the blood isolate and 34 (21%) not colonised with *S. aureus* at all (Table 3). The main underlying conditions among exogenous bacteraemia cases were burns (n=15), RF (n=6), heart disease (n=5) and cancer

(n=2). Exogenous SAB was more common in medical emergency and gynaecology wards (Fig 5).

Table 3 : Correlation of blood and nasal *S. aureus* isolates in patients with SAB

Related / identical blood and nasal isolates 93 (57.4%)		**Blood isolates unrelated to nasal isolates 69 (42.6%)**		**Total**
Patients colonised with the invading strain only	Patients colonised with invading strain plus another *S. aureus* strain(s)	Patients colonised with one or more *S. aureus* strains that did not match with the blood isolates	non *S. aureus* colonised patients	
89 (55%)	4 (2.4%)	35 (21.6%)	34 (21.0%)	162

*****Percentages are shown using the total number of SAB cases (162) as denominator.

The demographics of the patients with endogenous and exogenous bacteraemia are shown in Table 54.

Table 4 : Demographic data of patients with endogenous and exogenous bacteraemia

Demographic category		Endogenous Bacteremia (n=93) N (%)	Exogenous bacteremia (n=69) N (%)	Total (n=162) N (%)
Age	Adult	64 (39.5)	44 (27.2)	108 (66.7)
	Children	11 (6.8)	12 (7.4)	23 (14.2)
	Neonates	18 (11.1)	13 (8)	31 (19.1)
Gender	Male	48 (29.6)	43 (26.6)	91 (56.2)
	Female	45 (27.8)	26 (16)	71 (43.8)

*Percentages are shown using the total number of SAB cases (162) as denominator.

When comparing endogenous infection to exogenous infection with respect to the different hospital wards, the ratio of endogenous to exogenous infection was highest in internal medicine wards compared to other wards (Figure 5).

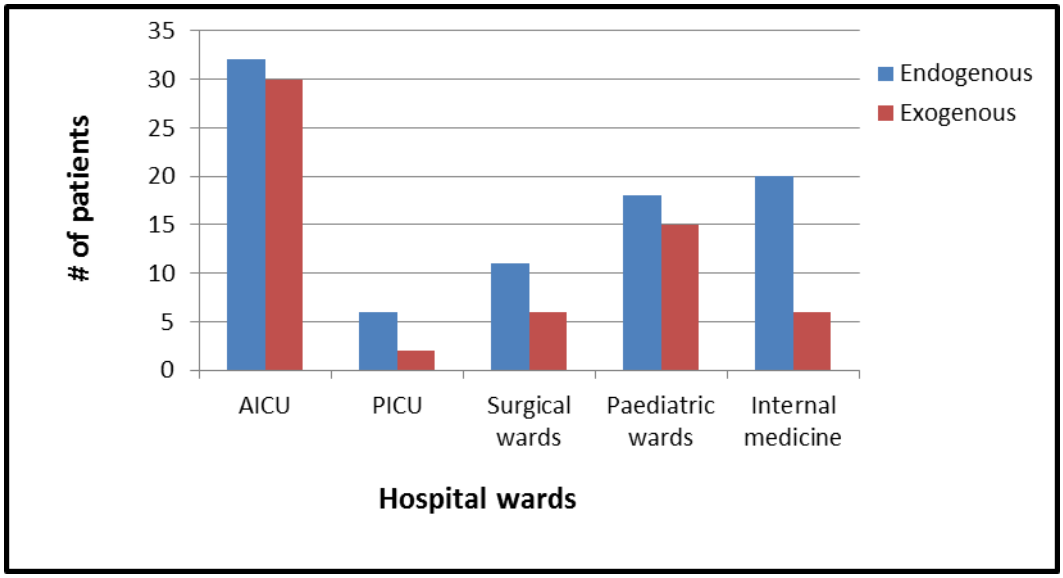

Figure 5 : Ratio of the exogenous versus endogenous SAB in Tygerberg Hospital wards with the highest number of cases.

AICU, adult intensive care unit; PICU, paediatric and neonatal Intensive care unit; paediatric wards includes neonatal patients.

2.3.4 MRSA/MSSA bacteraemia

Of the *S. aureus* strains isolated from blood, 42.6% (69/162) were MRSA. The adult surgical ICU (AICU) and paediatric wards had the highest number of MRSA cases, 39.1% (27/69) and 27.5% (19/69) respectively (Table 5). MRSA bacteraemia in neonates constituted 14.2% (23/162) of all SAB cases and 33.3% (23/69) of all MRSA bacteraemia cases, reflecting the high prevalence of MRSA bacteraemia in neonates (Table 6).

Table 5 : Numbers and percentages of endogenous versus exogenous MRSA and MSSA bacteraemia cases in different Tygerberg Hospital wards

Wards	MRSA N=69		MSSA N=93		Total N=162 N (%)
	Endogenous N (%)	Exogenous N (%)	Endogenous N (%)	Exogenous N (%)	
AICU	18 (26.1)	9 (13)	14 (15.1)	21 (22.5)	**62 (38.3)**
Paediatric wards	11 (16)	8 (11.6)	7 (7.5)	7 (7.5)	**33 (20.4)**
Internal medicine	7 (10.1)	0	13 (13.8)	6 (6.5)	**26 (16.2)**
Surgery	3 (4.3)	1 (1.4)	8 (8.6)	5 (5.4)	**17 (10.5)**
PICU	5 (7.3)	0	1 (1.1)	2 (2.2)	**8 (4.9)**
Khayelitsha	4 (5.8)	3 (4.3)	1 (1.1)	2 (2.2)	**10 (6.1)**
Gynecology	0	0	1 (1.1)	2 (2.2)	**3 (1.8)**
Medical emergency	0	0	0	3 (3.2)	**3 (1.8)**
Total	**48 (69.6)**	**21 (30.4)**	**45 (48.3)**	**48 (51.7)**	**162**

2.3.5 Endogenous and exogenous bacteraemia and MRSA/MSSA

The MRSA prevalence in cases of endogenous bacteraemia (48/69; 69.6%) was higher than the MRSA prevalence in cases of exogenous bacteraemia (21/69; 30.4%). MRSA significantly correlated with endogenous infection ($p = 0.007$). In adults, MRSA bacteraemia was more likely to be endogenous (29/40; 72.5%) than MSSA bacteraemia (35/68; 51.5%). A similar finding was present in neonates, with MRSA bacteraemia being endogenous in 16/23 cases (69.6%) compared to MSSA bacteraemia (endogenous in 2/8; 25%, of cases).

Table 6 : Correlation of MRSA/MSSA and endogenous/exogenous bacteraemia in adults, children and neonates admitted to Tygerberg Hospitals

Age category		**Endogenous bacteremia N (%)**	**Exogenous bacteremia N (%)**	**P- value**	**Total N (%)**
Adults	MRSA	**29 (18.0)**	**11 (6.8)**	**0.042**	**40 (24.8)**
	MSSA	**35 (21.6)**	**33 (20.4)**	**NS**	**68 (42.0)**
Children	MRSA	**3 (1.8)**	**3 (1.8)**	**NS**	**6 (3.6)**
	MSSA	**8 (4.9)**	**9 (5.6)**	**NS**	**17 (10.5)**
Neonates	MRSA	**16 (9.9)**	**7 (4.3)**	**0.042**	**23 (14.2)**
	MSSA	**2 (1.2)**	**6 (3.7)**	**NS**	**8 (4.9)**
Total		**93 (57.4)**	**69 (42.6)**	**NS**	**162 (100)**

*Percentages are shown using the total number of SAB cases (162) as denominator.

2.3.6 The time between hospital admission and blood sample collection

The average time between admission and collection of the blood culture for endogenous cases was 5.1 days, while for exogenous cases it was 4.75 days. This difference was not significant. The median time between the admission and blood sample collection (Figure 6) was significantly (P = 0.002) longer in patients with MRSA bacteraemia (5.8 days) than in those with MSSA bacteraemia (4.2 days).

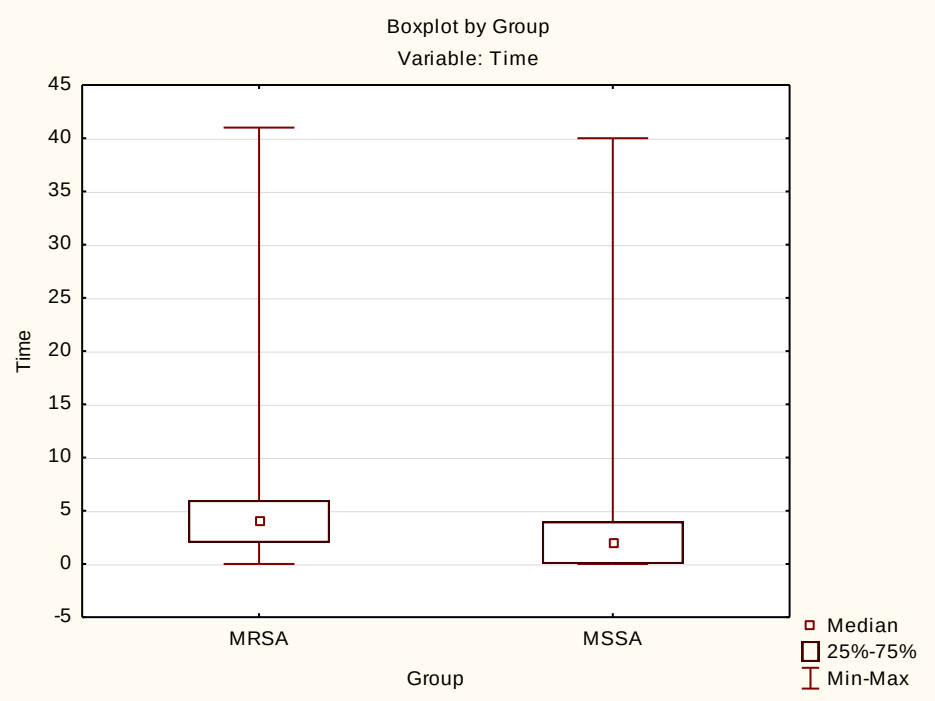

Figure 6 : Time difference between admission and blood sample collection of MRSA/MSSA bacteremia.

2.4 Discussion

2.4.1 Blood and nasal *S. aureus* correlation in colonised patients

Our results showed a 72.7% correlation between the nasal and blood *S. aureus* isolates in colonised patients. Previous studies conducted in developed countries reported that approximately 80% of blood and nasal *S. aureus* isolates in colonised patients were the same (16, 331, 339). In this study, the correlation between blood and nasal isolates was slightly lower than what has been reported in developed countries, which may reflect the impact of exogenous sources of bacteraemia in patients at Tygerberg Hospital.

Molecular typing techniques with a high discriminatory power like PFGE have in recent studies lead to improved discrimination between isolates and better determination of the source of bacteraemia (340). A study conducted in 1992 reported all nasal and blood *S. aureus* isolates in HIV patients were identical, by using the antibiotic profile of the colonising and invasive strains (341). In 1995 by using phage typing technique, Kluytmans *et al*., found 92% of the nasal and postoperative wound infection isolates were identical (342). In these studies less discriminatory techniques were used and therefore the correlation was higher compared to recent studies.

2.4.2 Endogenous and exogenous bacteraemia

We were able to establish that nasal colonisation was a possible source of infection in 57.4% of patients. In the other 42.6% of cases, the source may have been either colonisation of another body site, or, more likely, an external source.

2.4.3 MRSA bacteraemia

Tygerberg Hospital is a tertiary-academic hospital receiving patients referred from other hospitals. In this study, 42.6% (69/162) of *S. aureus* bacteraemia cases were due to MRSA. This is much higher than previous data in 1988 and 2009 where the proportion was 25% and 30.1%, respectively (24, 25). However, our MRSA prevalence is still comparable to average MRSA prevalence in South African hospitals which has been reported as between 30-60% (343). Lack of effective infection prevention and control procedures and antibiotic stewardship may be a possible cause of the high prevalence of MRSA bacteraemia cases at Tygerberg Hospital.

In the Netherlands, which effectively implemented active screening and decolonisation measures, along with a history of restricted antibiotic usage, the prevalence of MRSA among clinical *S. aureus* isolates is less than 1% (344, 345). Higher prevalence rates have been reported in different countries: Germany 19%, Belgium 19% and France 33% (345).

Results of this study also showed a significant association of MRSA bacteraemia with MRSA nasal colonisation in adult surgical ICU patients and neonates. Several studies reaffirmed the vulnerability of colonised patients admitted to ICU to develop subsequent *S. aureus* infections, including SAB. This includes the study of Pujol *et al*., where they measured the risk of patients colonised with *S. aureus* at the time of admission to the ICU to develop endogenous SAB. They found that *S. aureus* colonisation was a risk factor, but MRSA nasal carriage was a greater risk than MSSA (339). In another study, nasal carriers admitted to an ICU surgery unit were twice as likely to develop postoperative infections than were noncarriers (346). Nasal carriage of MRSA on hospital admission and during hospital stay has been reported as a predisposing factor for subsequent clinical infection in different

hospital wards including ICU and general medical surgery (334, 346).

2.4.4 MRSA/MSSA endogenous versus exogenous bacteraemia

This study clearly showed a significant association between MRSA bacteraemia and endogenous infection. Patients with MRSA bacteraemia were more likely to have an endogenous source than patients with MSSA bacteraemia (odds ratio 2.4, 95% CI 1.3-4.7). Milstone and his team found that MRSA colonisation in hospitalised children and adults was a predisposing factor for subsequent clinical infection (43). In that study, ICU admitted patients often had a serious clinical condition that compromised their immune systems and increased the risk of nasal colonisation and subsequent SAB. The high neonatal MRSA nasal carriage rate may therefore be due to mothers holding their infants close to their chest, facilitating *S. aureus* transmission, and due to the immune compromised status of the neonate. It would be interesting to test this hypothesis by examining the nasal carriage status of mothers in the maternity and/or neonatal wards.

Other studies have shown that subsequent clinical infection is four times more likely in those colonised with MRSA as opposed to MSSA colonisation (73). In our study, 69.5% of MRSA bacteraemia cases were colonised with the same strain. This confirms the need for good infection control practices including the identification and decolonisation of carriers at risk of developing infection to decrease the spread of MRSA in the hospital and preventing carriers from developing subsequent *S. aureus* clinical diseases. In our study, approximately 50% of patients with MSSA bacteraemia, developed bacteraemia with the same strain that was present in the nares.

The average time between the admission and blood culture collection was significantly longer (P = 0.002) for patients with MRSA bacteraemia than MSSA bacteraemia (5.8 and 4.2 days respectively) which may indicate hospital acquisition of MRSA. However, further studies are required to investigate when *S. aureus* nasal colonisation occurs in hospitalised patients and in particular when colonisation with MRSA occurs. Although most cases of MRSA (and MSSA) bacteraemia were endogenous, it is not clear whether the colonisation with MRSA was present on admission, or whether colonisation with MRSA developed in hospital.

The current study was limited in that it was not designed as an active surveillance study and that bacteraemia may have occurred before nasal colonisation. Due to the limited availability of clinical data, we could not determine if patients had received prior antibiotic treatment that could have eliminated *S. aureus* nasal carriage and lead to false negative results. However, this is not commonly practised in this hospital, and is felt to be an unlikely confounder. We did not investigate the colonisation of other body sites which may have caused us to miss some carriers. Lastly, Tygerberg Hospital is a tertiary referral hospital, therefore it was difficult to categorise isolates as causing either healthcare- associated or community associated infections, as some patients would have been referred in from secondary or district level health services..

This was the first study that investigated the correlation between nasal and blood *S. aureus* isolates of patients in a tertiary hospital in South Africa and we conclude that, (i) in patients with MRSA bacteraemia, approximately 70% have evidence of nasal colonisation with the same strain, while with MSSA bacteraemia, only 50% have evidence of nasal colonisation, which highlights the need for more studies to explore the feasibility of nasal decolonisation in a prevention control strategy of SAB in patients at an institution such as ours; (ii) our results also show that *S. aureus* nasal colonisation was not the only source of infection in patients

with *S. aureus* bacteraemia, 42.6% acquired the infection from sources other than the nostrils; (iii) occurrence of MRSA bacteraemia was significantly associated with MRSA nasal colonisation in adult surgical ICU patients and neonates. Studies to determine the population at risk in this specific population will also assist targeted infection control and prevention policies in a resource constrained environment such as ours, in order to lower the incidence of SAB, particularly due to MRSA.

CHAPTER 3 MOLECULAR CHARACTERISATION OF BLOOD AND NASAL *S. AUREUS* ISOLATES OBTAINED FROM PATIENTS WITH BACTERIA ADMITTED AT TYGERBERG HOSPITAL

3.1 Introduction

It has been suggested that MRSA clones evolved from MSSA lineages by horizontal acquisition of the SCC*mec* element at only one occasion; this is therefore called the single-clone theory (347). However, descriptions of both heterogenetic MRSA lineages as well as different SCC*mec* elements in isolates from the same genetic background supports the multi-clone theory where MRSA clones emerged from repeated independent introduction of different SCC*mec* elements into successfully adapted MSSA clones (117, 121, 136, 348). However, MSSA strains isolated from the hospital environment in 1960 revealed similar genetic characteristics to recently identified predominant MRSA clones. This suggests that these clones were well adapted to the hospital environment through host colonisation and invasion even before SCC*mec* element acquisition (349). Currently, there are a few MRSA clones that have disseminated worldwide, namely CC5, CC8, CC22, CC30 and CC45 (117, 121, 350).

Healthcare associated MRSA (HA-MRSA) causes nosocomial infection in individuals admitted to hospital or who are exposed to the healthcare environment (such as long-term-care facilities) (351). As discussed previously, the distinction between healthcare and CA-MRSA is becoming less clear as recent reports showed dissemination of CA-MRSA into hospitals causing serious infections (352, 353).

Limited information has been published regarding the molecular epidemiology and population structure of the MSSA lineages considered the genetic ancestors of emerging

MRSA clones. The genetic diversity of MSSA is higher than MRSA, which is not surprising given shorter evolutionary time of MRSA (≈ 50 years) compared to MSSA clones which are much older and thus more heterogeneous (202-204, 354). MSSA isolates related to the common MRSA clones have been identified in many countries (80). However, MSSA lineages other than the common MRSA clones have been reported worldwide such as CC7, CC9, CC12, CC15, CC25, CC51 and CC101, which supports the fact that MSSA strains are genetically more diverse than MRSA strains (200, 355). This might be influenced by the fact that some epidemic MSSA lineages showed more ability to acquire and maintain the SCC*mec* element than other clones (121).

3.1.1 Geographic distribution

The prevalence of *S. aureus* clones is variable in different geographical areas (84). Of the 5 common HA-MRSA clonal complexes, CC22 is prevalent worldwide; CC30 (ST36) is common in the USA and the UK while CC45 is frequently isolated in the USA and Europe (80, 356). In Asia the most commonly reported clones are CC5 (ST5), CC8 (ST239) and CC22 (ST22) (170, 179, 357). In Latin America CC5 (ST5), CC8 (ST239) and CC30 are the most frequently identified strains (358).

3.1.2 Situation in Africa and South Africa

In Africa, there are limited studies on *S. aureus* prevalence and antibiotic resistance, leading to an outdated and incomplete understanding of *S. aureus* epidemiology on the continent. In Algeria, a study that included 46 MRSA isolates from one hospital showed that 50% of the

isolates harboured SCC*mec* type VI and 43.75% SCC*mec* type V. All PVL positive strains (29.68%) harboured SCC*mec* type IV (359). A recent study including MRSA isolates from five different African countries, Morocco (Casablanca), Senegal (Dakar), Niger (Niamey), Cameroon (Yaounde) and Madagascar (Antananarivo), found that the dominant clones were ST239-MRSA-III (40%) followed by ST88-MRSA-IV (28%) and ST5-MRSA-IV (21%) (171). Another study including 228 MSSA isolates from the same African countries showed that 132 (58%) of the isolates belonged to one of five MLST clonal complexes, namely CC1 (9%), CC15 (12%), CC30 (9%), CC121 (19%) and CC152 (9%) (205). In Nigeria, MRSA isolates were found to belong to one of three MLST CCs: ST241 and ST8 (CC8), ST37 and ST39 (CC30) and ST88 (CC88), while 8 MSSA sequence types were identified: ST5, ST8, ST15, ST30, ST80, ST121, ST152 (singleton), ST508 (CC45) (207).

In South Africa, MRSA prevalence ranges from 27% to 46% across the 9 provinces (360, 361). Of 97 MRSA isolates obtained from different clinical specimens from Steve Biko Academic Hospital, in Pretoria, SCC*mec* type II was predominant (67%) followed by SCC*mec* type III (14%) (362). In this study MLST clonal complexes and ST were not investigated. In a study conducted in KwaZulu-Natal province including 241 isolates from 16 hospitals, 24 MRSA and MSSA isolates were randomly chosen for further MLST analysis. MLST analysis identified ST8-MRSA-IV (67%), ST239-MRSAIII (13%), ST8-MRSA-II (8%), ST5-MRSA-IV (8%), and ST45-MRSA-IV (1%). The MSSA clones identified were ST1, ST5, ST6, ST8, ST9, ST22, ST30, ST31, ST45, ST72, ST88, ST121, ST199, ST239 and ST243 (206). Another study included 100 MRSA isolates from 5 hospitals in Cape Town (excluding Tygerberg Hospital) and found ST239-MRSA-III, ST36-MRSA-II, ST5-MRSA-I and ST612-MRSA-IV were the most frequently identified clones (175). To investigate the prevalent MRSA clones in South Africa, 320 isolates were collected from 9 provinces and

characterized by PFGE, *spa* typing, MLST, SCC*mec* typing and PCR for PVL genes. The predominant clonal lineages were ST612-MRSA-IV (25.3%), ST36-MRSA-II (23.8%), ST239-MRSA-III (20.9%), and ST5-MRSA-I (6.6%) (174).

At Tygerberg Hospital (one of the academic hospitals in Cape Town that provides healthcare services to a large population in the Western Cape Province), two previous studies have been conducted. The first study (included S. aureus blood isolates collected during the period March 2008 to May 2009) aimed to describe the *spa* type, methicillin susceptibility and *pvl* gene prevalence of *S. aureus* strains causing bacteraemia in Tygerberg Hospital. In this study the MRSA prevalence was 30.1% (34/113). In blood culture isolates, *spa* types t037, t891, t1257, t002, t015 and t021 were identified as the predominant types among the 113 strains (25). This was followed by another study (included isolates were collected over one year from September 2009 to 2010) in which isolates from specific sites of infection were investigated by PFGE, *spa* typing, MLST analysis, SCC*mec* typing, *agr* typing and PVL detection. In this study, MRSA among *S. aureus* isolates was 15.3% (56/367). The major MRSA clonal lineages were ST612-MRSA-IV (n = 21), ST5-MRSA-I (n = 9), ST36-MRSA-II (n = 6) and ST239-MRSA-III (n = 5) (363).

In the current study we characterised blood and nasal isolates collected from patients with *S. aureus* bacteraemia at Tygerberg Hospital using molecular techniques to estimate the correlation between the colonising and invasive isolates. In this chapter we describe the molecular characteristics of nasal (colonising) and blood (invasive) *S. aureus* isolates (MRSA and MSSA) obtained from patients with bacteraemia at Tygerberg Hospital.

3.1.3 Aims of the study:

Genotypic characterisation of colonising and invasive *S. aureus* (MRSA and MSSA) strains collected from patients with *S. aureus* bacteraemia at Tygerberg Hospital.

3.1.4 Objectives

1- To determine the dominant *spa* type disseminated in Tygerberg Hospital and the dominant *spa* clonal complexes

2- To determine the SCC*mec* type of collected MRSA isolates

3- To determine the *agr* group of the isolates

4- To determine the MLST sequence types and MLST clonal complexes of a representative sample of isolates

3.2 Methods

3.2.1 Isolates

The isolates examined were those collected from 164 patients. Of these, 162 were those described in chapter 2, and the two excluded patients were included in this analysis. The two patients excluded from analysis in Chapter 2 were included for analysis in this chapter and consisted of 6 additional isolates (3 blood and 3 nasal isolates). Of 164 patient, 333 blood and nasal isolates were collected and genotyped using PFGE, *spa* typing, SCC*mec* typing, *agr* typing and were also examined for the presence of the *pvl* gene. Where a patient had both blood and nasal isolates, and these had the same PFGE pulsotype, only the isolate from blood was included in the data analysis. However, where the nasal and blood isolates had different pulsotypes, both isolates were included. Likewise, if patients had more than one isolate (from nasal swab or blood) with different pulsotypes, all isolates were included. If patients had only blood culture isolates, and no nasal carriage, the blood isolate was included. A total of 208 blood and nasal isolates were obtained from 164 patients with SAB included in the study. Of the 208 isolates, 90 (43.3%) were MRSA and 118 (56.7%) were MSSA. The numbers of isolates from each group are specified in Table 7.

A total of 208 blood and nasal isolates were obtained from 164 patients (Table 7): 93 males (average age, 32 years; range, 3 days to 87 years) and 71 females (average age, 23.7 years; range, 2 days to 86 years) with SAB. Of the 164 patients, 110 (67%) were adults, 23 (14%) children and 31 (19%) neonates.

Table 7 : source of the 208 included blood and nasal isolates.

Number of isolates	Source
92	Obtained from the blood sample of carriers where the blood and nasal isolates had the same pulsotype.
34	Obtained from the blood sample of noncarriers
35	Recovered from the blood sample of carriers where the blood and nasal isolates had different pulsotype.
35	Recovered from the nasal swab of carriers where the blood and nasal isolates had different pulsotype.
6	Additional isolates from the nasal swab of carriers where more than one strain was identified
6	Additional blood isolates obtained from 3 patients where each patient infected with 2 different isolates according to PFGE gel patterns analysis.

3.2.2 Control strains

The control strains used in this section were obtained from the ATCC. ATCC 49775 was used as a positive control for detection of pvl, and the strains used as controls for SCC*mec* and *agr* typing are listed in Table 8.

Table 8 : *S. aureus* control strains used in this study

Strain	SCCmec type	agr type
BAA1680	IV	I
BAA1681	II	II
BAA1683	-	III
BAA-1688	V	VI
BAA-38	I	-
BAA-39	III	-
BAA-42	VI	-

3.2.3 DNA extraction

The DNA was extracted by a modified version of a previously described method (364). One bead from the frozen strains was streaked on blood agar plate for 18-24 hours at 37˚C then a single colony inoculated in Brain heart infusion broth (BHI) for 18-24 hours at 37˚C. One ml of cultured BHI broth was centrifuged (sigma, model 1-15) at 13 000 rpm for 1 minute, the pellet was re-suspended in 50 µl of lysostaphin (130µg/ml; Sigma) and incubated at 37˚C for 2 hours. A volume of 50 µl of proteinase K (PK) (130 µl/ml; Sigma) was added with 150 µl of Proteinase K buffer (0.1 M Tris [PH 7.5]) and incubated at 37˚C. After one hour the suspension was incubated at 100˚C for 10 minutes in a heating block. Finally the suspension was centrifuged at 13 000rpm for 3 minutes. The extracted DNA was used as a template in the various PCR assays. DNA integrity and concentration were determined using a Nanodrop ND 1000 Spectrophotometer V3.1.0 instrument (Nanodrop Technologies, Inc., Wilmington,

DE, USA).

3.2.4 *spa* typing

3.2.4.1 PCR performance

Two sets of primers (set 1 and set 2,

Table 9) were used: Each PCR reaction contains 1.5 mM of *spa* primers, 1 µL of DNA template, and 12.5 µl of Go Taq® Colourless Master Mix (Promega). The final PCR reaction volume was 25 µl. The amplification conditions were 94˚C for 4 minute, 35 cycles (94˚C for 45 seconds, 63˚C for 45 seconds and 72˚C for 90 seconds) with a final extension at 72˚C for 10 minutes (106) . A 5ul aliquot of the final PCR reaction was visualised by electrophoresis in a 1% agarose gel containing 50µg EtBr (Sigma) per 100ml agarose. Amplification of the *spa* gene in non-typeable strains was repeated at different annealing temperatures (62, 61 and 60˚C) and the $MgCl_2$ final concentration was increased up to 2 mM.

Table 9 : Primers for different gene targets used in this study.

Genetic region	Primer No	Primer description	Primer sequence	Band size/bp	Primer concentration (mM)	Reference
Spa	1	1095 F	AGACGATCCTTCGGTGAGC	polymorphic	1.5	(106)
		1517 R	GCTTTTGCAATGTCATTTACTG			
	2	1084-F	ACAACGTAACGGCTTCATCC		1.5	(365)
		1618-R	TTAGCATCTGCATGGTTTGC			
Agr	3	agr-R	ATGCACATGGTGCACATGC	pan reverse primer	5	(366)
	4	agr1-F	GTCACAAGTACTATAAGCTGCGAT	439		
	5	agr2-F	TATTACTAATTGAAAAGTGCCATAGC	572		
	6	agr3-F	GTAATGTAATAGCTTGTATAATAATACCCAG	321		
	7	agr4-F	CGATAATGCCGTAATACCCG	657		
PVL	8	luk-PV-1	ATCATTAGGTAAAATGTCTGGACATGATCCA	433	1	(367)
		luk-PV-2	GCATCAASTGTATTGGATAGCAAAAGC			
arcC	9	arcC-Up	TTGATTCACCAGCGCGTATTGTC	~450	1	(115)
		arcC-Dn	AGGTATCTGCTTCAATCAGCG			
aroE	10	aroE-Up	ATCGGAAATCCTATTTCACATTC			
		aroE-Dn	GGTGTTGTATTAATAACGATATC			
glpF	11	glpF-Up	CTAGGAACTGCAATCTTAATCC			
		glpF-Dn	TGGTAAAATCGCATGTCCAATTC			
Gmk	12	gmk-Up	ATCGTTTTATCGGGACCATC			
		gmk-Dn	TCATTAACTACAACGTAATCGTA			
Pta	13	pta-Up	GTTAAAATCGTATTACCTGAAGG			
		pta-Dn	GACCCTTTTGTTGAAAAGCTTAA			
Tpi	14	tpi-Up	TCGTTCATTCTGAACGTCGTGAA			
		tpi-Dn	TTTGCACCTTCTAACAATTGTAC			
yqiL	15	yqiL-Up	CAGCATACAGGACACCTATTGGC			
		yqiL-Dn	CGTTGAGGAATCGATACTGGAAC			
II, J1 region	16	kdpF1	AATCATCTGCCATTGGTGATGC	284	2	(14)
		kdp R1	CGAATGAAGTGAAAGAAAGTGG			
III, J3 region	17	RIF5 F10	TTCTTAAGTACACGCTGAATCG	414	4	(368)
		RIF5 R13	GTCACAGTAATTCCATCAATGC			
SCC*mec*-I, J1 region	18	CIF2 F2	TTCGAGTTGCTGATGAAGAAGG	495		
		CIF2 R2	ATTTACCACAAGGACTACCAGC			
V, J1	19	SCCmec V J1 F	TTCTCCATTCTTGTTCATCC	377	4	(369)

region		SCCmec V J1 R	AGAGACTACTGACTTAAGTGG			
III, J1 region	20	SCCmec III J1 F	CATTTGTGAAACACAGTACG	243		
		SCCmec III J1 R	GTTATTGAGACTCCTAAAGC			
V, *ccr* complex	21	ccrC F2	GTACTCGTTACAATGTTTGG	449	8	(369)
		ccrC R2	ATAATGGCTTCATGCTTACC			
II and IV, *ccr* complex	22	ccrB2 F2	AGTTTCTCAGAATTCGAACG	311		
		ccrB2 R2	CCGATATAGAAWGGGTTAGC			
I, II, IV, and VI, J3 region	23	dcs F2	CATCCTATGATAGCTTGGTC	342	8	(368)
		dcs R1	CTAAATCATAGCCATGACCG			

Table 9 cont. Primers for different gene targets used in this study.

II and III, *mec* complex	24	mecI P2	ATCAAGACTTGCATTCAGGC	209		(42)
		mecI P3	GCGGTTTCAATTCACTTGTC			
Internal positive control	25	mecA P4	TCCAGATTACAACTTCACCAGG	162		
		mecA P7	CCACTTCATATCTTGTAACG			
ccrA	26	ccrA R1	YCCWAA YTA YTG TGGYCGTGT	296	0.5	(370)
		ccrA F1	TKY TKG TGC RTTKAT NCC T			
ccrB	27	ccrB F1	CGW YTR GCW MGW AAY ACHTC	496		
		ccrB R1	CTT TTC GWC KYT TWT CRY TCC			
mec gene complex	28	mecI-F	CCCTTTTTATACAATCTCGTT	3,635	0.5	(147)
		mecA147-R	ATGCGCTATAGATTGAAAGGAT			
ccrC2	29	ccrC2-F2	ATAAGTTAAAAGCACGACTCA	257	1	(146)
		ccrC2-R2	TTCAATCCTATTTTTCTTTGTG			

3.2.4.2 Determination of *spa* type

S. aureus spa types were identified as described previously (111). Resultant PCR reaction products were sent for sequencing and analysed using the Ridom StaphType™, version 1.4 (Ridom Gmb, Würzburg, Germany) software package to determine the different *spa* types. Based upon repeat patterns (BURP) analysis in the software was used to identify *spa* clones or clonal complexes using the following parameters: *spa* types shorter than 5 were excluded from BURP analysis and *spa* types belonging to the same group were only cloned if the cost was less than or equal to 5.

3.2.5 *agr* typing

A multiplex PCR assay to amplify the *agr* alleles of all blood and nasal isolates was performed as previously described using the following *agr* primers (

Table 9): 3 (pan reverse primer), 4, 5, 6, 7, and 8 (366). The PCR mixture contained 12.5 µl of Go Taq® Colorless Master Mix (Promega), 5 mM of each primer, 1 µl of the crude DNA, and 2.75 mM $MgCl_2$. Denaturation at 95˚C for 6 minutes was followed by 30 cycles of 95˚C for 0.45 S, 58˚C for 0.45 S and 72˚C for one minute and a final extension at 72˚C for 10 minutes. Twenty µl of amplification products were visualised in 3.5% agarose gels with EtBr and electrophoresed at 120V for 2 hours. Isolates with non typeable *agr* were repeated as a uniplex PCR with each primer pair combination.

3.2.6 SCC*mec* typing

SCC*mec* types were initially determined using a multiplex PCR as described by Milheirico (369). Primer sets 16-25 (with sequences and concentrations as shown in

Table 9) were used to amplify different genetic component in the SCC*mec* element. The PCR reaction contained 12.5 µl of Go Taq® Colorless Master Mix (Promega), 1 µl of the crude DNA, and 3 mM $MgCl_2$. Cycling conditions were 95˚C for 5 min followed by 35 cycles of 95˚C for 45 s, 49˚C for 45 s and 72˚C for 60 s and a final extension time of 72˚C for 10 min. The PCR reaction products were visualised by electrophoresis at 120V for 2 hours in 3.5% agarose gels withEtBr. The SCC*mec* type was assigned by comparing the sizes (Table 9) of the bands generated with those generated by the 6 control strains (Table 8) using a 100 bp ladder.

3.2.7 Untypeable SCC*mec* band patterns

Further characterisation of the SCC*mec* element was carried out where necessary by uniplex PCR of specific genes and subsequent DNA sequence analysis. The assay to amplify the class A *mec* gene complex (

Table 9, primer set 28) contained: 2.5 mM $MgCl_2$, 500 µM of each dNTP, 0.2 µl of QIAGEN®LongRange Taq enzyme, 1 µl of DNA (100ng) and 0.5 mM of primers, in a final volume of 25µl. PCR was performed under the following conditions; 35 cycles of 93˚C for 15 S, 45˚C for 15 S and 68˚C for 4 min following an initial denaturation at 95 C˚ for 3 min, and ended by final elongation at 68˚C for 7 min. Twenty microliters of the amplification

product was sequenced using the forward primer (Number 28, *mecI*-F,

Table 9) by the Inqaba Biotec Company.

Separate uniplex PCR assays for *ccrA*, *ccrB* and *ccrC* were performed using the following primer pairs:

- *ccrA*: primer set 26 as shown in
-
-

- Table 9 (*ccrA* R1 and *ccrA* F1 at concentration of 0.5mM).

- *ccrB*: primer set 27 as shown in
-
-

- Table 9 (*ccrB* R1 and *ccrB* F1 at concentration of 0.5mM).

- *ccrC*: primer set 21 as shown in
-
-

- Table 9 (*ccrC* R2 and *ccrC* F2 at concentration of 1mM).

Each assay was performed in a total volume of 25µl, with 12.5µl of Go Taq® Colorless Master Mix (Promega), 2.5 mM of $MgCl_2$ and commenced with denaturation at 95ºC for 5

minutes. The remainder of the assay conditions are shown on Table 10.

Table 10: PCR conditions for amplification of the *ccrA*, *ccrB* and *ccrC* genes.

Target gene	Denaturation ˚C /Time	Annealing ˚C/Time	Extension ˚C/Time	Final extension ˚C/Time	# of cycles	Reference
ccrA	95/ 60 s	50/ 60 s	72/ 120 s	72/10 min	30	(370)
ccrB	94/30 s	44/60 s	72/120 s	72/4 min	35	(370)
ccrC	95/60 s	50/45 s	72/60 s	72/ 10 min	30	(369)

Five µl of the amplification products of the above PCR assays (class A *mec* gene complex, *ccrA*, *ccrB* and *ccrC* genes) were visualised by electrophoresis on 1% or 1.5% agarose gels (diluted in 1X TAE) with EtBr (Sigma) at 120V for 45 minutes. Selected amplification products were sent to Inqaba Biotec Company for sequencing.

3.2.8 MLST

Isolates were selected from each of the major (five isolates) and intermediary (five isolates) PFGE clusters, as well as some of the minor clusters (10 isolates) in order to have a representative collection of isolates for Multi locus sequence type (MLST) analysis. The seven housekeeping genes, carbamate kinase (*arc*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glpF*), guanylate kinase (*gmk*), phosphate acetyletransferase (*pta*), rtiosephosphate isomerase (*tpi*) and acetyle coenzyme A acetyltransferase (*yqiL*) were amplified by using primer sets 9-15 (

Table 9) in a uniplex PCR (115). The thermal cycles were slightly modified and consisted of: primary denaturation at 95℃ for 5 min followed by 30 cycles of 95℃ for 45s, 56℃ for 45s and 72℃ for 1 min followed by final extension at 72℃ for 10 min. The reaction contained; 12.5 µl of Go Taq® Colorless Master Mix (Promega), 1 mM of each primer, 2.5 mM $MgCl_2$, 1 µl crude DNA with the final volume adjusted to 25 µl. Five µl of PCR product was visualised by electrophoresis at 120V for 1 hour in a 1.5% EtBr containing agarose gel (diluted in 1X TAE).

The remainder of the amplification product (20 µl) was sent for sequencing (Inqaba Biotec). Each gene sequence was aligned and compared to the standard sequence as described by the MLST web site (http://www.mlst.net). The allelic profile number for each single isolate was determined by submitting the seven gene sequences to the MLST web site where the sequence type (ST) was identified. Isolates with the same ST were considered to belong to one MLST clonal complex and therefore one isolate was selected for further analysis. The eBURST program was used to identify the MLST clonal complex of different STs, and to determine the evolutionary model of the collection of study isolate. Novel STs were confirmed by re-sequencing both the forward and reverse primers and resending the sequences to the MLST group for confirmation.

3.2.9 DNA sequencing

DNA sequencing was performed by Inqaba Biotec using ABI V3.1 Big dye kit on the ABI 3500XL genetic analyser, using the same forward primers (10mM) as used in the PCR assays

(

Table 9).

3.2.10 Definitions

Pulsed-field gel electrophoresis clusters were classified into three categories as follows: major clusters contained more than 10 different pulsotypes, intermediate from 6 to 10 different pulsotypes and minor clusters when less than 6 different pulsotypes were identified in one cluster.

Isolates obtained from the nostrils and blood or only from the blood were classified as invasive isolates. Colonising isolates were defined as any isolates obtained only from the nasal cavity. Bacteraemia episodes were categorised as endogenous and exogenous as described in the second chapter.

3.3 Results

3.3.1 PFGE

Pulsed-field gel electrophoresis of the 208 isolates showed 179 unique pulsotypes, grouped into 31 clusters and 24 singletons using a similarity cut-off of 80%. The clusters were named

alphabetically (A, B, C to AF). One major and 2 minor PFGE clusters contained isolates belonging to different *spa* clonal complexes and *agr* groups. Pulsed-field gel electrophoresis was repeated on the isolates from these 3 clusters using *ApaI* restriction enzyme. This resulted in 4 clusters each of which contained strains belonging to the same *spa*-CC and *agr* groups. One strain was resistant to *ApaI* enzyme and considered as a singleton.

Restriction with *SmaI* and *ApaI* resulted in four major clusters (2 consisting of only MRSA isolates and 2 only MSSA isolates), 4 intermediary clusters, all exclusively MSSA except for 1 cluster that contained a mixture of MSSA and MRSA isolates, and 23 minor clusters (5 MRSA, 16 MSSA and 2 mixed MSSA and MRSA). The details are shown in Table 11. The major clusters (D, S, U and AA) represented 39.4% of all isolates analysed. The dominant MRSA clone (U) represented 19.2% (40/208) of all included isolates, and 44.4% of all MRSA isolates. The intermediary clones represented 13.9% (29/208) of all study isolates (Table 11).

Of the ninety MRSA isolates, 52 (57.8%) clustered in the two major clones: S (n = 12) and U (n = 40), 2 were in the intermediary clone G and the other MRSA strains were scattered between the minor clones and 8 singletons.

The MSSA strains (n = 118) were distributed among 2 major clones: D (n = 18) and AA (n = 12), 4 intermediate clones: G (n = 5), P (n = 7), Q (n = 8) and AE (n = 7), with the rest distributed between 23 minor PFGE clones and 16 singletons.

Table 11 : Overview of PFGE clusters.

PFGE clone	category	MRSA N (%)	MSSA N (%)	Source			Isolates N (%)
				Invasive		Colonizing N (%)	
				Endogenous N (%)	Exogenous N (%)		
D	major	0	18 (15.3)	9 (9.5)	5 (6.9)	4 (9.9)	18 (8.6)
S	major	12 (13.3)	0	6 (6.3)	3 (4.2)	3 (7.3)	12 (5.7)
U	major	40 (44.4)	0	22 (23.3)	10 (13.9)	8(19.6)	40 (19.2)
AA	major	0	12 (10.2)	3 (3.2)	6 (8.3)	3 (7.3)	12 (5.8)
G	Intermediary	2 (2.2)	5 (4.2)	3 (3.2)	4 (5.6)	0	7 (3.3)

P	Intermediary	0	7 (5.9)	5 (5.4)	0	2 (4.9)	7 (3.3)
Q	Intermediary	0	8 (6.8)	3 (3.2)	4 (5.6)	1(2.4)	8 (3.8)
AE	Intermediary	0	7 (5.9)	1 (1)	4 (5.6)	2 (4.9)	7 (3.3)
A	Minor	0	4 (3.4)	0	3 (4.2)	1(2.4)	4 (1.9)
B	Minor	4 (4.5)	0	3 (3.2)	1 (1.4)	0	4 (1.9)
C	Minor	0	2 (1.7)	2 (2.1)	0	0	2 (1)
E	Minor	0	2 (1.7)	0	2 (2.7)	0	2 (1)
F	Minor	1 (1.1)	4 (3.4)	3 (3.2)	2 (2.7)	0	5 (2.4)
H	Minor	0	2 (1.7)	0	1 (1.4)	1 (2.4)	2 (1)
I	Minor	0	2 (1.7)	1 (1)	1 (1.4)	0	2 (1)
J	Minor	0	2 (1.7)	1 (1)	1 (1.4)	0	2 (1)
L	Minor	0	2 (1.7)	1 (1)	1 (1.4)	0	2 (1)
M	Minor	10 (11.1)	0	7 (7.4)	2 (2.7)	1 (2.4)	10 (4.8)
N	Minor	5 (5.6)	0	3 (3.2)	1 (1.4)	1 (2.4)	5 (2.4)
O	Minor	1 (1.1)	1 (0.8)	2 (2.1)	0	0	2 (1)
R	Minor	4 (4.5)	0	1 (1)	0	3 (7.3)	4 (1.9)
T	Minor	3 (3.3)	0	1 (1)	0	2 (4.9)	3 (1.4)
V	Minor	0	2 (1.7)	0	1 (1.4)	1 (2.4)	2 (1)
W	Minor	0	8 (6.7)	3 (3.2)	3 (4.2)	2 (4.9)	8 (3.8)
X	Minor	0	2 (1.7)	0	2 (2.7)	0	2 (1)
Y	Minor	0	2 (1.7)	1 (1)	0	1 (2.4)	2 (1)
Z	Minor	0	2 (1.7)	1 (1)	1 (1.4)	0	2 (1)
AB	Minor	0	2 (1.7)	2 (2.1)	0	0	2 (1)
AC	Minor	0	2 (1.7)	1 (1)	1 (1.4)	0	2 (1)
AD	Minor	0	2 (1.7)	1 (1)	1 (1.4)	0	2 (1)
AF	Minor	0	2 (1.7)	1 (1)	1 (1.4)	0	2 (1)
24	Singleton	8 (8.9)	16 (13.5)	8 (8.4)	11 (15.3)	5 (12.2)	24 (11.5)
Total		90	118	95	72	41	208

3.3.1.1 Source of the major PFGE clusters

The ICU was the most common source of isolates belonging to the 4 major clusters as shown in Table 12. Interestingly, 72.5% (29/40) of the isolates in cluster U were from patients in the adult ICU (n = 29).

Table 12 : Ward of origin for isolates belonging to the major PFGE clusters

PFGE cluster (n)	D (%) N =18	S (%) N=12	U (%) N = 40	AA (%) N = 12
AICU	7 (38.9)	2 (16.8)	29(72.5)	5 (41.6)
PICU	0	0	3 (7.5)	0
Internal medicine	5 (27.8)	4 (33.3)	1 (2.5)	2 (16.7)
Surgery	2 (11.1)	1 (8.3)	1 (2.5)	1 (8.3)
Paediatrics	2 (11.1)	1 (8.3)	5 (12.5)	2 (16.7)
Khayelitsha	2 (11.1)	4 (33.3)	1 (2.5)	0
Gynaecology	0	0	0	2 (16.7)

Medical emergency	0	0	0	0
Total	18 (100)	12 (100)	40 (100)	12 (100)

3.3.2 *spa* typing

The *spa* gene was detected in 206 of the isolates and only 2 MSSA isolates were nontypeable.

Using Ridom software, 68 different *spa* types were identified that segregated into 10 clones and 17 singletons. Two strains were excluded from this analysis as the number of repeats was less than 5 (Table 13). After Ridom *spa* server synchronisation, 4 MSSA strains were identified as novel *spa* types (t7330, t7335, t7343, t9499).

3.3.2.1 Distribution of different *spa* types

The most prevalent *spa* type in this study was t037, with 44 isolates (21.4%) belonging to this *spa* type. All *spa* type t037 strains were MRSA and all except for 4 isolates were assigned to the major PFGE cluster, U. The 4 other isolates included 3 strains in PFGE cluster T, and one singleton. The second most common *spa* type was t045 representing 15 (7.3%) of the isolates. These were also all MRSA and belonged to two minor PFGE clusters. The third most prevalent was *spa* t1257, represented by 12 isolates (5.8%) all of which were MRSA. Ten were assigned to the major PFGE cluster S and two assigned to the minor PFGE cluster,

R. All *spa* type t084 isolates were MSSA (n=9; 4.4%), 7 assigned to one PFGE cluster and two singletons. The frequency of the remaining *spa*-types is presented in Table 13.

Table 13 : Frequencies of all *spa* types of included strains

spa type	Number of isolates (%)	MRSA/MSSA
t37	44 (21.4)	MRSA
t045	15 (7.3)	MRSA
t1257	12 (5.8)	MRSA
t084	9 (4.4)	MSSA
t1476	8 (3.9)	MSSA
t891	8 (3.9)	MSSA
t012	6 (2.9)	MRSA (4)/MSSA (2)
t015	5 (2.4)	MSSA
t2360	5 (2.4)	MSSA
t701	5 (2.4)	MSSA
t002	4 (1.9)	MSSA
t 064	4 (1.9)	MRSA
t1443	4 (1.9)	MRSA
t032, t050, t071, t073, t148, t318, t359, t888,	Reported 3 times	MRSA and MSSA
t021, t127, t160, t174, t267, t355,	Reported twice	MRSA and MSSA
t008, t022, t026, t088, t094, t1062, t1154, t116, t133, t160, t164, t1866, t2194, t223, t2293, t2554, t2643, t2763, t279, t304, t346, t363, t3662, t3772, t4463, t4490, t465, t537, t5471, t570, t690, t7020, t7330, t7335, t7343, t7515, t8079, t8636, t8637, t939, t9499	Reported once	MRSA and MSSA

3.3.2.2 Most dominant *Spa* clonal complexes

Using the BURP algorithm of the RIDOM StaphType software, the *spa t*ypes were grouped into 10 *spa* clonal complexes and 13 singletons while 2 *spa* types with less than 5 repeats were excluded **(**Table 14).

Table 14 : *spa* typing BURP clone analysis of *S. aureus* strains at Tygerberg Hospital.

Clone	spa-CC	spa types	spa types prevalence N (%)	Strains prevalence N (%)
1	064	t008, t064, t304, t701, t1257, t1443, t1476, t2293, t2360.	9 (13)	41 (20)
2	002	t002, t045, t071, t088, t570, t1062, t1154, t7515.	8 (12)	27 (13)
3	073/015	t015, t050, t073, t116, t133, t465, t2194, t7343.	8 (12)	16 (8)
4	021	t012, t021, t037, t318, t363, t1866, t2643	7 (10)	58 (28)
5	084	t084, t094, t279, t346, t1517	5 (7)	13 (6)
6	174	t127, t174, t5471, t8637	4 (6)	6 (3)
7	022	t022, t032, t891	3 (4)	12 (6)
8	160	t160, t888, t 4490	3 (4)	6 (3)
9	267	t267, t359, t7335	3 (4)	6 (3)
10	No founder	t3662, t3772	2 (3)	2 (1)
Singletons		t164, t355, t690, t939, t2763, t4463, 7020, 9499, t148, t223, t537, t8079, 8636,	13 (19)	16 (8)
Excluded		t026, t2554	2 (3)	2(1)

Clone 1: represents *spa*-CC 064 which includes 9 (13%) of all 68 *spa* types and 41 strains (20%). The founder of this *spa*-CC was *spa* type t064 which evolved to 8 *spa* types t2293, t1443, t1257, t1476, t304, t008, t701 and t2360 (

Figure 7). The isolates belonging to *spa-CC* 064 were distributed across 7 PFGE clusters: 1 major, 2 intermediary and 4 minor.

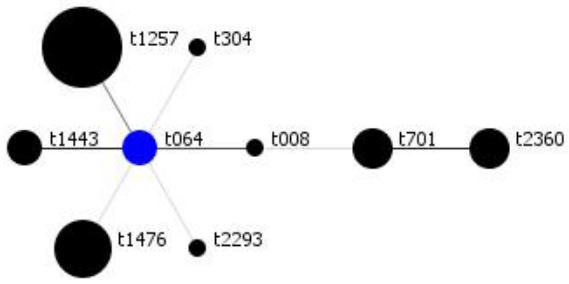

Figure 7 : The founder and the evolved *spa* types grouped in *spa*-CC 064

Clone 2: *spa*-CC 002 consists of 8 (12%) *spa* types and 27 (13%) strains. Seven *spa* types evolved from the founder (t002): *spa* type t045, t071, t088, t570, t1062, t1154 and t7515 (Figure 8). This *spa*-CC was found in 1 intermediary and 6 minor PFGE clusters.

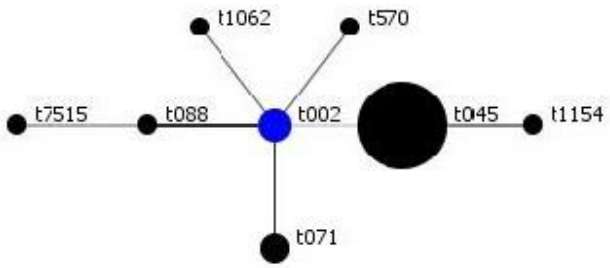

Figure 8 : The relatedness of *spa* types identified as *spa*-CC 002.

Clone 3: represents *spa*-CC 073/015, which includes 8 (12%) *spa* types and 16 (8%) of all strains. Both *spa* type t073 and t015 have an identical founder score and are closely related to the *spa* types t050, t116, t133, t465, t2194 and t7343 (Figure 9). This *spa*-CC was related to only one MSSA PFGE cluster.

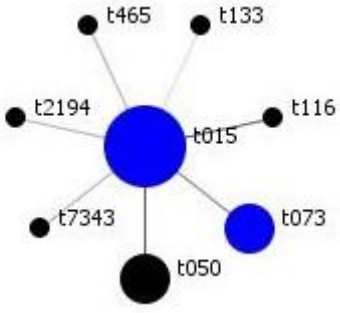

Figure 9 : The evolution of *spa* types grouped in *spa*-CC 073/015.

Clone 4: represents *spa*-CC 021, and includes 7 (10%) *spa* types and 58 (28%) of all strains. Six *spa* types, t012, t021, t037, t318, t363, t1866 and t2643 evolved from the founder *spa* type t 021 (Figure 10). PFGE further differentiates these strains into 4 (2MRSA, 2MSSA) minor and 1 (MRSA) major PFGE cluster.

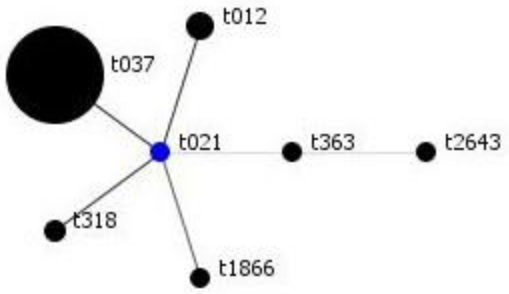

Figure 10 : The evolutionary model of *spa* types grouped in *spa*-CC 021.

Clone 5: *spa*-CC 084 represented 5 (7%) and 13 (6%) of all *spa* types and strains respectively. *spa* types t094, t279, t346 and t1517 evolved from *spa* type t084 (Figure 11). Based on PFGE, *spa*-CC 084 was represented in 1 major cluster and 2 singletons.

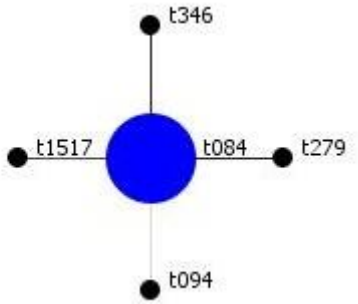

Figure 11 : The evolutionary model of *spa* types grouped in *spa*-CC 084.

In clone 6: *spa* types t127, t5471 and t8637 evolved from *spa* type t174. *spa*-CC 174 represented 4 (6%) and 6 (3%) of the *spa* types and strains respectively (Figure 12). This *spa*-CC disseminated into two minor MSSA PFGE clusters.

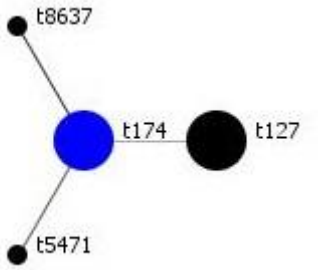

Figure 12 : The evolutionary model of *spa* types grouped in *spa*-CC 174.

3.3.3 MLST

For MLST analysis, 21 strains were selected from different PFGE clusters as follows: one strain from each of 4 major, 4 intermediary and 11 minor PFGE clusters. Furthermore, an additional strain with a different SCC*mec* type was chosen from the dominant PFGE cluster U, and an additional strain from the intermediary PFGE cluster, G. The additional strain from clone G was chosen so that the two strains from clone G consisted of one MSSA, PVL positive strain, and one MRSA, PVL negative strain. Strains selected for MLST analysis were thus selected from 19 PFGE clusters which represents 75.5% (157) of the 208 strains.

Analysing the sequence of the seven housekeeping genes of all selected strains yielded 14 different sequence types (ST): 239 (dominant, n = 40), 2122, 2126, 1, 12, 612, 6, 97, 461, 5, 8, 45, 1865 and 36. Two novel STs were identified: ST 2122 with allelic profile 1.4.1.4.232.1.1 and ST2126 with allelic profile 13.13.1.1.12.112.13. ST 2122 is a double

locus variant (DLV) of ST5 (*pta* and *yqil* alleles were different), and ST 2126 is a single locus variant of ST15, where one new *tpi* allele was identified. ST 2126 was identified in 16 (10.2%) of the isolates. MLST eBURST analysis for the all identified STs revealed 9 MLST clonal complexes (MLST-CC) as shown in Table 15.

As a result of the identification of the two new *S. aureus* STs, the MLST data base eBURST indicated that STs belonging to MLST-CC 8 should be grouped into MLST-CC 5. However, in this study we will consider MLST-CC 5 and 8 as separate clonal complexes as they have a genetic diversity and in order to keep our data compatible with previous studies (195).

Table 15 : Characteristics of the MLST-CCs.

MLST-CC	ST	spa-CC	spa types	PFGE Clone	MRSA/MSSA	Strain N	Total (%) N=157
1	1	174	t5471, t127	Z (Minor)	MSSA	2	4 (2.5)
			t174, t8637	AF (Minor)	MSSA	2	
5	2122	002	t002, t7515 t071, t088	W (Minor)	MSSA	8	20.4 (47.1)
	6	064	t2360 t701	AE (Intermediary)	MSSA	7	
	461	002	t045	N (Minor)	MRSA	5	
	5	002	t045	M (Minor)	MRSA	10	
		002	t002, t570	Y (Minor)	MSSA	2	
8	239	021	t037	U (Major)	MRSA	40	66 (42)
	612	064	t1257, t1443	S (Major)	MRSA	12	
		064	t1257, t1443 t064	R (Minor)	MRSA	4	
	8	064	t1476	Q (intermediary)	MSSA	8	
		064	t2293, t008	O (Minor)	MRSA/MSSA	2	
12	12	160	t160	AB (Minor)	MSSA	2	2 (1)
15	2126	084	t346, t279 t084, t8079 t094, t1517	AA (Major)	MSSA	12	12 (7.6)
22	22	022	t032, t8636 t891	G (Intermediary)	MRSA (2)/MSSA	7	7 (3.4)
45	45	073/015	t015, t0465 t073, t133 t050, t116 t2763, t2194 t7343	D (Major)	MSSA	18	18 (8.7)
30	36	021	t012	B (Minor)	MRSA	4	8 (3.8)
	1865	021	t318, t012	A (Minor)	MSSA	4	
97	97	267	t7330, t7335 t359, t267	P (Intermediary)	MSSA	7	7 (3.4)

3.3.4 SCC*mec* typing

SCC*mec* typing was performed on all 90 MRSA isolates; all were *mecA* positive, confirming methicillin resistance. Comparison with the multiplex PCR banding patterns of the control strains allowed for designation of the SCC*mec* type for 38 of the isolates as follows: type II 5.6% (n = 5), type III 11.1% (n = 10), type IV 23.3% (n = 21), type V 2.2% (n = 2). Examples of these are shown in Figure 13. In addition, 6 isolates (6.7%) were non-typeable.

The banding pattern of the remaining 46 isolates did not correspond to any of the controls used, with two unrecognised patterns being identified, as discussed below, and illustrated in the flowchart (Figure 18).

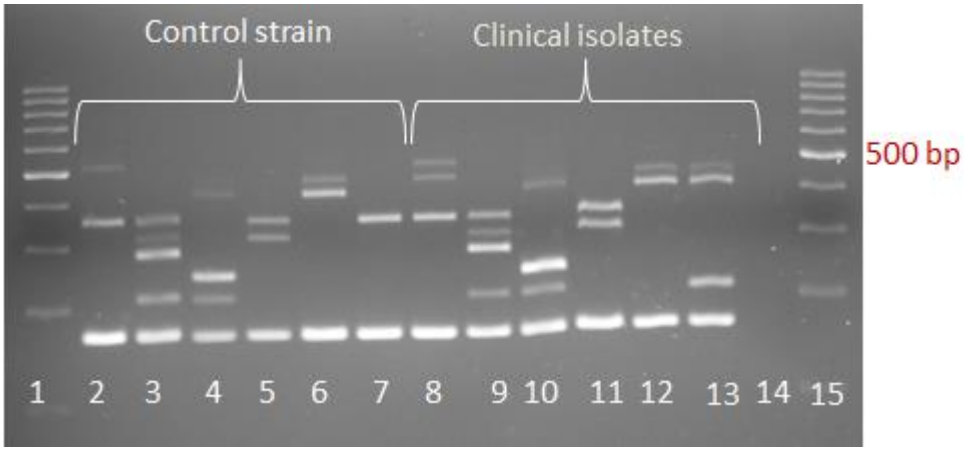

Figure 13 : Determination of SCC*mec* types of included clinical isolates.

lane 1, 100 bp ladder; Lane 2, SCC*mec* type I (BAA 38); lane 3, SCC*mec* type II (ATCC 1681); lane 4, SCC*mec* III (BAA 39); lane 5, SCC*mec* IV (ATCC 1680); lane 6, SCC*mec* V (ATCC 1688); lane 7, SCC*mec* VI (BAA 42); lane 8, Untypeable SCC*mec* type (*ccrA1B1*, *ccrC*/Class B *mec*); lane 9, SCC*mec* type II; lane 10, SCC*mec* type III; lane 11, SCC*mec* type IV; lane 12, SCC*mec* type V; lane 13, Untypeable SCC*mec* type (*ccrC*/ Class A *mec*); lane 14, negative control; lane 15, 100 bp ladder.

3.3.4.1 Untypeable SCC*mec* type (*ccrA1B1*, *ccrC*/Class B *mec*)

Fifteen of the 90 MRSA isolates had a similar PCR band patterns to the SCC*mec* type I control strain. This is illustrated in Figure 13, lane 8. In this lane, the bands present for these two isolates most likely correspond to *mecA* (162 bp), J3 region (342 bp), J1 region (495 bp) and one additional band with the same size as *ccrC* (449 bp). Uniplex PCR assays for *ccrA1*, *ccrB1* and *ccrC* were performed on two clinical isolates with this SCC*mec* pattern (TKN-8 and TKB-16) to confirm the presence and combination of *ccrA1B1* and *ccrC*.

The *ccrA1*, *ccrB1* and *ccrC* were amplified (Figures 14-16), sequenced and analysed using the BLAST website (http://blast.ncbi.nlm.nih.gov). The results showed a sequence similarity of 99% for *ccrA1*, 99% for *ccrB1* and 97% for *ccrC* (accession numbers: gi|408436493|emb|HE579073.1|, gi|32435727|gb|AY254742.1| and gi|254803085|gb|FJ931046.1| respectively). These results thus suggest the presence of both *ccrA1B1* and *ccrC* in one SCC*mec* element, and support the tentative SCC*mec* designation as *ccrA1B1*, *ccrC*/Class B *mec*. This designation will be used for these isolates for the remainder of the thesis.

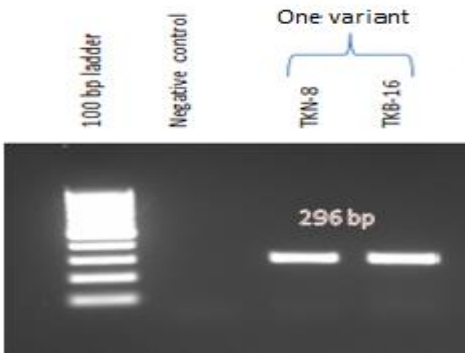

Figure 14 : Uniplex PCR gel of *ccrA* amplification of untypeable SCC*mec* type (*ccrA1B1, ccrC*/ Class B *mec)* strains (TKN-8, TKB-16).

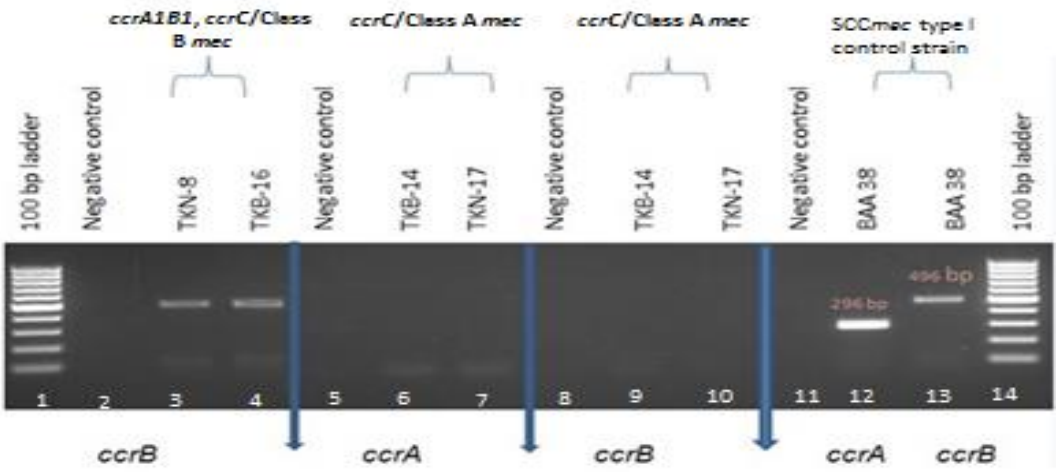

Figure 15 : Uniplex PCR assays for *ccrA* and *ccrB*.
Lanes 2-4, *ccrB* of SCC*mec* I variant (TKN-8, TKB-16); lanes 5-7, *ccrA* of untypeable SCC*mec* (TKB-14, TKN-17); lanes 8-10, *ccrB* of untypeable SCC*mec* (TKB-14, TKN-17); lane 11, negative control; lane 12, *ccrA* (BAA 38); lane 13, *ccrB* (BAA 38).

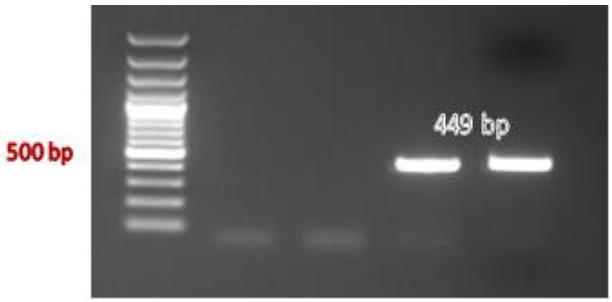

Figure 16 : Amplification of *ccrC*.
Lane 1, 100 bp ladder; lane 2, negative control; lane 3, SCC*mec* type I positive control strain (BAA 38); lane 4, TKN-8; lane 5, TKB-16.

3.3.4.2 Untypeable SCC*mec* type (*ccrC*/Class A *mec*)

Thirty one isolates appeared to have banding pattern suggestive of a combination of *ccrC* and class A *mec*. As seen in Figure 13 (lane 13) the multiplex PCR generated fragment sizes of 162 bp (*mecA*), 209 bp (*mecI*), 414 bp (J3 region) and 449 bp (*ccrC*). This combination of

class A *mec* gene and type 5 (C) *ccr* gene complexes has not been reported.

To confirm this combination, two clinical isolates (TKB-14, TKN-17) with this band pattern were analysed further, using uniplex PCR assays for *ccr*A1, *ccrA*2, and *ccrC*, as well as class A *mec*. Neither the *ccrA* nor *ccrB* genes were amplified (Figure 15). However, the *ccrC* gene was amplified and sequenced (Figure 16). The nucleotide sequence was found to be 99% homologous to the *ccrC* gene (accession number gi|269939526|emb|FN433596.1|).

The class A *mec* gene complex was also amplified (shown in Figure 17), sequenced and compared to the database available in the NCBI gene database and was identified as class A *mec* gene complex with 99% similarity (accession number, FJ670542).

Based on these results, this second SCC*mec* type, identified in 31 of the isolates, appears to consist of a combination of *ccrC* (*ccr* 5) and the class A *mec* gene complex. For the rest of the thesis, this SCC*mec* type will be designated *ccrC* / class A *mec*.

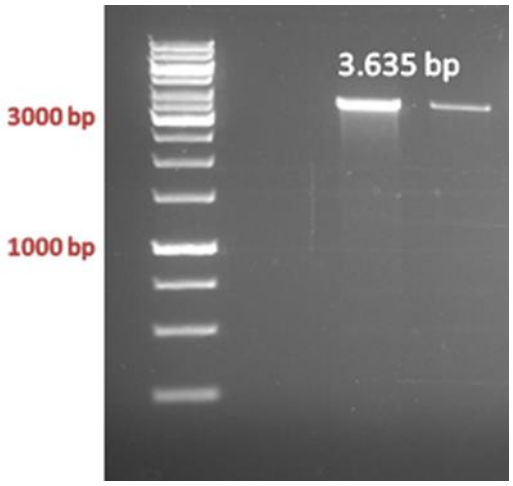

Figure 17 : *mec* gene complex amplification.

Lane 1, 1 kb ladder; lane 2, negative control; lane 3, TKB-14; lane 4, TKN-17.

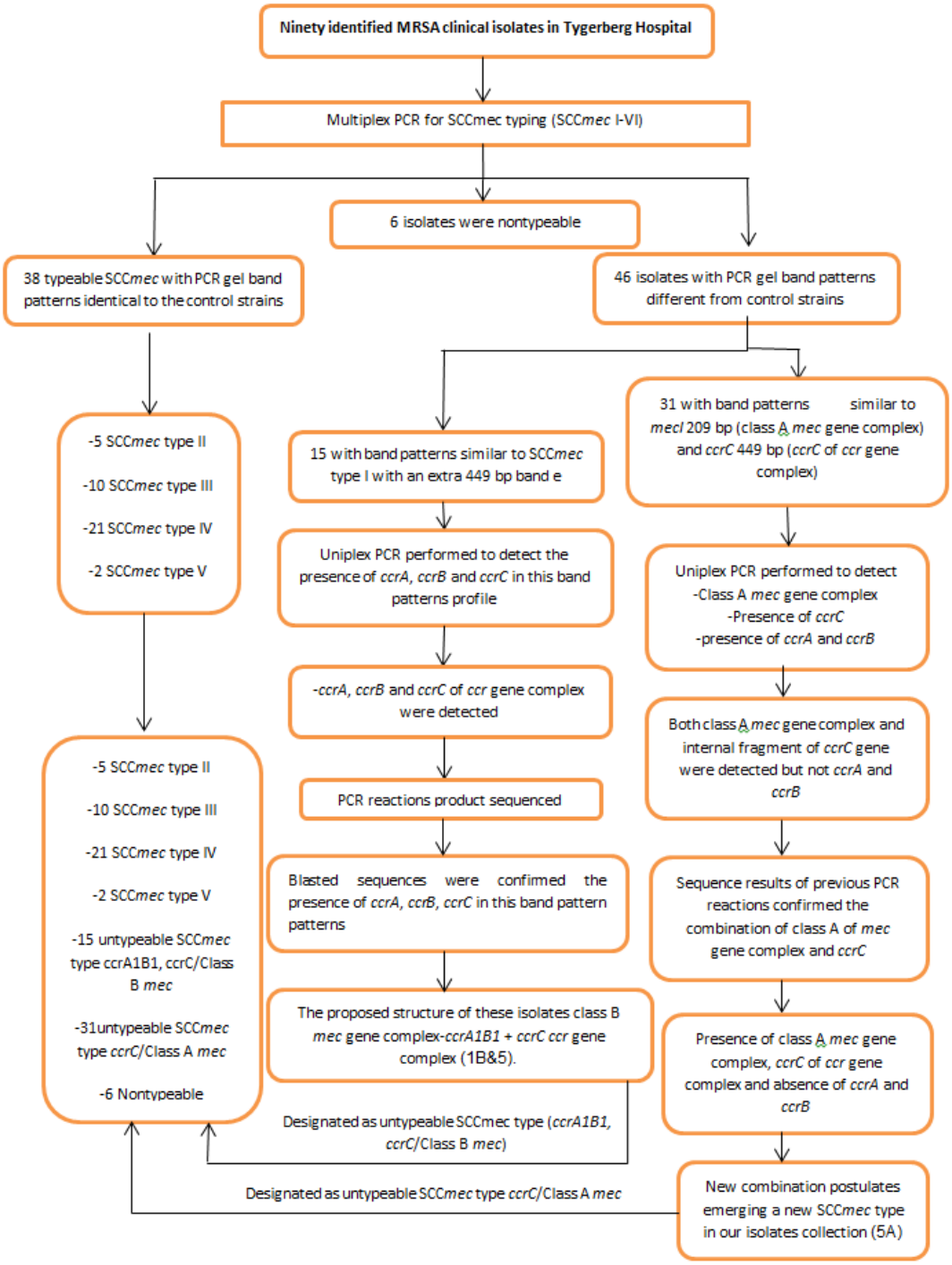

Figure 18 : Flow-diagram describing the molecular structure of SCC*mec* element of isolates showing different PCR gel band patterns compared to control strains.

The association of SCC*mec* type, *spa*-CC and MLST CC is shown in Table 16. Isolates with SCC*mec* type *ccrA1B1*, *ccrC*/Class B *mec* were grouped in the M and N minor PFGE clusters (similarity between these two clusters is 78.1%). All 31 of the isolates with the SCC*mec* type *ccrC*/class A *mec* were assigned to cluster U, and furthermore, 20 of the 31 isolates containing this SCC*mec* type were obtained from the burns unit, with the remainder arising from surgical wards (n = 4), the high care unit (n = 3), neonatal internal medicine (n = 3) and Khayelitsha wards (n = 1).

Table 16 : Genotypic characteristics of SCC*mec* types identified in this study.

SCC*mec* type	PFGE clone	*spa-CC*	spa type	MLST-CC	ST	Number of isolates
ccrA1B1, *ccrC*/Class B *mec*	M (minor)	002	t045	5	5	10
	N (minor)	002	t045	5	461	5
II	S	021	t021	-	-	1
	B (minor)	021	t021	30	36	4
III	T (minor)	021	t037	8	239	3
	U (major)	021	t037	8	239	7
IV	G (intermediary)	022	t8636	22	22	1
		022	t891	22	22	1
	R (minor)	064	t1443, t1257, t064	8	612	4
	S (major)	064	t1257, t1443	8	612	10
	F (minor)	022	t032	-	-	1
	S	022	t022	22	22	1
	S	022	t032	-	-	1
	S	064	t064	-	-	1
	S	064	t064	-	-	1
V	O (minor)	064	t008	8	8	1
	S	064	t1443	-	-	1
ccrC/Class A *mec*	U (major)	021	t037	8	239	31
Non-typeable	S (major)	064	t1257	8	612	2
	U (major)	021	t037	8	239	2
	S	064	t064	-	-	1
	S	021	t021	-	-	1

During the study period in 2010, five strains harboured SCC*mec* type III (3 assigned to the PFGE clone T and 2 obtained from PFGE clone U) and 6 strains from PFGE clone U were identified as untypeable SCC*mec* type *ccrC*/Class A *mec*. However, in 2011 SCC*mec* type III was reported in 5 strains and untypeable SCC*mec* type *ccrC*/Class A *mec* identified in 25 strains (Table 17).

Table 17 : Prevalence of identified SCC*mec* types among 2010 and 2011.

SCCmec type	1st half 2010	2nd half 2010	1st half 2011	2nd half 2011	Total
II	1	2	1	1	5
III	1	4	4	1	10
IV	3	5	7	6	21
V	1	0	0	1	2
ccrA1B1, *ccrC* / class B *mec*	6	5	4	0	15
ccrC/ class A *mec*	5	2	7	17	31
Total	17	18	23	26	84

3.3.5 *agr* group

The *agr* gene was detected in 204 of 208 included isolates, and could not be amplified in 4 strains which were reported as nontypeable. The prevalence of agr groups was as follows: agr type I, 67.3% (n = 145); agr type II, 21.6% (n = 45); agr type III, 9.1% (n = 19). Agr type IV was not identified during the study period. The *agr* typing results, along with *agr* functionality is discussed in more detail in Chapter 5.

3.3.6 PVL prevalence

In our collection 8.7% (n = 18) of the isolates were positive for the *pvl* gene. All PVL positive strains were MSSA (14.4%, 17/118) except for one, identified as MRSA SCC*mec* type IV, *spa* type t1257, *spa*-CC 064, ST 612 and MLST-CC 5. The majority of PVL positive strains 44.4% (n = 8) were *spa* type t891, followed by *spa* type t318 (3, 16.7%), *spa*-CC 021, ST 1865 (local emerging spa-type), MLST-CC 30 (Table 18).

Table 18 : Prevalence and genotypic characteristics of PVL positive strains

PFGE-CC	Spa type	Spa-CC	ST	MLST-CC	MRSA/MSSA	Number of isolates
A	t318	021	1865	30	MSSA	3
F	t891	022	-	-	MSSA	3
G	t891	022	22	22	MSSA	5
J	t355	S	-	-	MSSA	2
S	t1257	064	612	8	MRSA SCC*mec* IV	1
Z	t5471	174	-	-	MSSA	1
AF	t174, t8637	174	1	1	MSSA	2
Singleton	t1866	021	-	-	MSSA	1

Table 19: Genotypic characters of PFGE clusters of which strains selected for MLST analysis.

PFGE clone	spa type	spa-CC	ST	MLST-CC	MRSA\MSSA (SCCmec type)	agr	PVL	Designation (clone)
A (minor)	t318, t012	021	1865	30	MSSA	III	+ve	-
B (minor)	t012	021	36	30	MRSA (II)	III	-ve	ST36-MRSA-II UK EMRSA-16
D (major)	t015, t465, t073, t133, t050, t2194, t7343, t116	073/015	45	45	MSSA	I	-ve	ST45-MSSA
	t2763	S						
G (intermediary)	t032	022	22	22	MRSA (IV)	I	-ve	ST22-MRSA-IV UK-EMRSA-15
	t8636	Singleton						
	t891	022	22	22	MSSA	I	+ve	ST22-MSSA
M (minor)	t045	002	5	5	MRSA	II	-ve	ST5-MRSA-ccrA1B1,ccrC/Class B mec
N (minor)	t045	002	461	5	MRSA	II	-ve	ST461-MRSA-ccrA1B1,ccrC/Class B mec
O (minor)	t008	064	8	8	MRSA (V)	I	-ve	ST8-MRSA-V
	t2293	064	8	8	MSSA			
P (intermediary)	t7335, t267 t359,	267	97	97	MSSA	I	-ve	ST97-MSSA
	t7330	S						
Q (intermediate)	t1476	064	8	8	MSSA	I	-ve	ST8-MSSA
R (minor)	t1443, t064 t1257,	064	612	8	MRSA (IV)	I	-ve	ST612-MRSA-IV

PFGE clone	spa type	spa-CC	ST	MLST-CC	MRSA\MSSA (SCCmec type)	agr	PVL	Designation (clone)
S (major)	t1257, t 1443	064	612	8	MRSA (IV)	I	+ve*	ST612-MRSA-IV
U (major)	t037	021	239	8	MRSA (III)	I	-ve	ST239-MRSA-III Brazilian\Hungarian
					Untypeable	I	-ve	ST239-MRSA-ccrC/Class A mec
W (minor)	t002, t7815, t071,t088	002	2122	5	MSSA	II	-ve	ST2122-MSSA
Y (minor)	t002, t570	002	5	5	MSSA	I	-ve	ST5-MSSA
Z (minor)	t5471, t127	174	1	1	MSSA	I	-ve**	ST1-MSSA
AA (major)	t346, t279, t084, t1517	084	2126	15	MSSA	II	-ve	ST2126-MSSA
	t8079	S						
AB(minor)	t160	160	12	12	MSSA	II	-ve	ST12-MSSA
AE (intermediary)	t2360, t701, t2360,	064	6	5	MSSA	I	-ve	ST6-MSSA
AF (minor)	t174, t8637	174	1	1	MSSA	III	-ve	ST1-MSSA

-ve*: one strain (spa type t1257) of this PFGE cluster was PVL positive.

-ve**: one strain (spa type t 5471) was PVL positive.

3.3.7 Characteristics and prevalence of MRSA clones

In this study, 8 MRSA clones were identified, four of which, ST239 (ST239-MRSA-III and ST239-MRSA-ccrC/Class A *mec*), ST612-MRSA-IV, ST5-MRSA- *ccrA1B1*, *ccrC*/Class B *mec* and ST36-MRSA-II (UK MRSA-16), represented the most dominant strains. The other clones, ST22-MRSA-IV (UK MRSA-15), ST461-MRSA-*ccrA1B1*, *ccrC*/Class B *mec* and ST8-MRSA-V, accounted for the minority of strains. Of note is the fact that this is, as far as the author is aware, only the second time ST461 has been identified as methicillin resistant. Clones were designated based on the strain ST and the SCC*mec* type as shown in Table 19.

The prevalence of the MRSA lineages is shown in Figure 19.

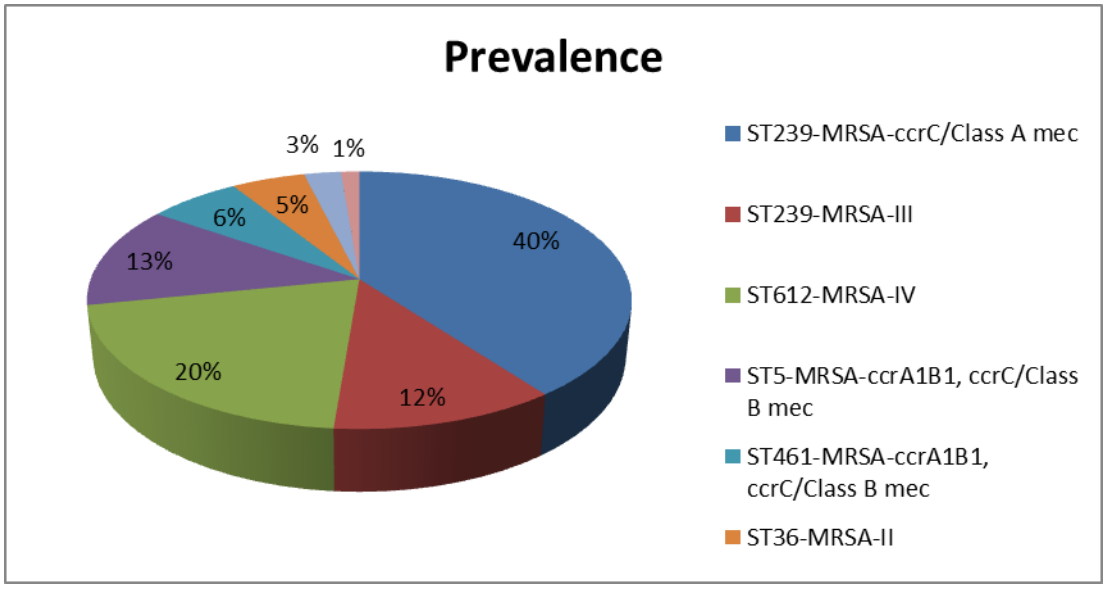

Figure 19 : Prevalence of MRSA clones.

3.3.8 Association between genetic background association and endogenous/exogenous infection

The isolates selected for MLST analysis represented 156 isolates, and the association between genetic background and endogenous or exogenous infection was analysed using the data from these 156 isolates.

To investigate the association between CCs and invasion; isolates belonging to any CC obtained from the blood and nasal cavity or only from the blood were classified as invasive and CCs obtained only from the nasal cavity were classified as colonising. The association of MLST CCs with invasive (both exogenous and endogenous) and colonising strains is shown in Table 20.

Table 20 : Distribution of MLST-CC among invasive/colonising isolates and invasive endogenous/exogenous groups.

MLST-CC	Invasive N (%)		Colonising N (%)	Total N (%)
	Exogenous	Endogenous		
1	2 (4.7)	2 (2.5)	0	4 (2.6)
5	8 (18.6)	18 (22.2)	6 (19)	32 (20.5)
8	16 (37.2)	34 (42)	16 (50)	66 (42.3)
12	0	2 (2.5)	0	2 (1.3)
15	5 (11.6)	4 (5)	3 (9.3)	12 (7.7)
22	4 (9.3)	3 (3.7)	0	7 (4.5)
30	4 (9.3)	3 (3.7)	1 (3)	8 (5.1)
45	4 (9.3)	10 (12.3)	4 (12.5)	18 (11.5)
97	0	5 (6.1)	2 (6.2)	7 (4.5)
Total	43 (100)	81	32 (100)	156

Isolates belonging to three CCs (CC1, CC12, and CC22) were invasive, as they were obtained from the blood or blood and nostrils only. Two CCs, CC12 and CC97, only caused endogenous bacteraemia. Interestingly, CC97 is rarely identified as a human pathogen and it is more typically isolated from animals.

3.4 Discussion

3.4.1 MRSA transmission

MRSA strains segregated into two major and one intermediate PFGE clusters, with the other strains disseminated between minor clusters and sporadic isolates. Each of the PFGE clusters included strains obtained from different hospital wards suggesting the possibility of MRSA strain transmission between different hospital wards. Isolates belonging to PFGE cluster U were commonly isolated from patients in the burns unit, and interestingly there were more isolates from this cluster in the second half of 2011(17/31, 54.8%) than in 2010 (7/31, 22.6) or the first half of 2011 (7/31, 22.6). This may represent local transmission within the ward, and will help inform infection control practices. However, the fact that a number of isolates from this cluster were also identified in patients in other wards could either be due to patient movement from the burns unit to other wards, or staff carrying the organism to other wards. While we did not examine isolates from other hospitals, it is certainly possible, and even likely, that patient transfer between hospitals is another route of transmission of MRSA.

Isolates from other PFGE clusters were obtained from a variety of different wards in the hospital, and there was no clear link found between any of the other PFGE clusters and a specific ward or region of the hospital.

3.4.2 MRSA and MSSA clones and genetic diversity:

Internationally, it has been reported that hospital acquired MRSA infection is mainly caused by a few MRSA clones: Iberian (ST247-MRSA-I), Brazilian/Hungarian (ST239-MRSA-III), Berlin (ST45-MRSA-IV), New York/Japan (ST5-MRSA-II), Paediatric (ST5-MRSA-IV),

EMRSA-15 (ST22-MRSA-IV), and EMRSA-16 (ST36-MRSA-II) (348). In the isolates included in this study, the common clones that were identified included: ST36-MRSA-II, ST22-MRSA-IV, ST239-MRSA-III and ST239-MRSA-*ccrC*/Class A *mec*. The MRSA clones that occurred more infrequently were ST5-MRSA-*ccrA1B1*, *ccrC*/Class B *mec*, ST461-MRSA- *ccrA1B1*, *ccrC*/Class B *mec* (according to our knowledge, this was the second time this was identified as a MRSA clone), ST8-MRSA-V, and ST612-MRSA-IV.

In this study ST239 (CC8) was the most prevalent ST. It was identified with two different SCC*mec* types; ST239-MRSA-III and ST239-MRSA-*ccrC*/Class A *mec*, both of which were *spa* type t37. This clone was reported as the most dominant in Asian countries except Japan and South Korea (371). Recently, 92.5% of the prevalent MRSA clones in Malaysia were shown to be ST239-MRSA-III (179). Similar results were reported in Morocco and Niger where this clone was predominant in both countries (171). In Tygerberg Hospital, a previous study conducted in 2009 used *spa* typing and found that *spa* type t37 was the most dominant *spa* type. Considering all *spa* type t37 in the current collection were ST239 one can suggest that ST239 was the most dominant in the 2009 study as well (25). In South Africa, the first study reporting the prevalence of ST239-MRSA-III was conducted in KwaZulu-Natal province and included 61 MRSA isolates collected between March 2001 and August 2003 (372). This study reported 16.4% (10/61) of the included MRSA isolates as ST239-MRSA-III. It was reported as a minor clone in a study conducted in Cape Town where 100 MRSA isolates (blood = 7) from city hospitals (except Tygerberg) were analysed (175). It was also the second most dominant clone in a study where 38.4% of the isolates were obtained from patients with bacteraemia (174). In KwaZulu-Natal province it was the second predominant clone obtained from health care facilities between 2001 and 2003 (206). The dominance of this clone (ST239-MRSA-III) in different provinces in South Africa is similar to that reported

in Asia (152, 371) and may relate to the migration of Asians due to forced labour in the country's past.

This clone (ST239-MRSA-III and ST239-MRSA-*ccrC*/Class A *mec*) is the most prevalent in Tygerberg Hospital and this may cause concern for two reasons. Firstly, due to the previously described ability of this clone to spread between hospitals and wards in one hospital (87), and secondly, due to a recent study showing that this clone has spread from hospitals into the community (170).

In agreement with other studies conducted in South Africa, this study reported ST612-MRSA-IV (a member of CC8 and a double locus variant of ST8) as a spreading clone in Tygerberg Hospital (174, 175). To our knowledge this clone has been reported only in South Africa and Australia (169). Spread of this lineage in limited geographic regions (South Africa and Australia) is supported by other studies that reported certain endemic clones may emerge locally in some geographic areas (373, 374). It has been suggested that the small size of the SCC*mec* element type IV compared to other elements enhances its mobility between staphylococci and explains the worldwide spread of this element (121, 136). We have identified the first strain of this clone that harbours the *pvl* gene in South Africa. The finding that ST612-MRSA-IV isolates were distributed across two PFGE clones, with different *spa* types, as well as one strain acquiring *pvl*, raises the possibility of the emergence of this local *S. aureus* lineage over a sufficient period of time to the point where it has become more genetically diverse. It is known that the *pvl* gene is introduced into *S. aureus* by a prophage, and we presume that this clone acquired this gene locally through horizontal transfer.

ST5-MRSA-I (UK EMRSA-3) is a pandemic clone spread worldwide (80, 169). This study reported this ST with a potentially novel variant of SCC*mec* type I, ST5-MRSA-*ccrA1B1*,

*ccrC/*Class B *mec*-t045, as the third most prevalent clone in Tygerberg Hospital. Previously, ST5 was identified in one study from Cape Town as the second dominant clone (175). Other studies also reported the prevalence of this clone in isolates collected from the nine South African Hospital provinces (174, 175). Other ST5-MRSA (III and IV) clones have been identified in KwaZulu-Natal province in South Africa (206, 372). Nübel *et al.*, suggested that the spread of ST5 between different areas is limited and that this clone may evolve via local horizontal SCC*mec* element transmission in different geographical areas (373). Therefore, it is possible to suggest the local emergence of this clone through horizontal introduction of SCC*mec* type *ccrA1B1, ccrC*/Class B *mec* element into hospital MSSA strains, followed by spread within the hospital from patient to patient. However, it is also possible it was imported from other hospitals.

We identified another common international clone ST36-MRSA-IV (UK EMRSA-16) in our collection. The first report of this clone came from the UK in 1993 (84). Subsequent reports showed the spread of this clone in USA, Australia, Canada and recently South Africa (84, 167). This clone has been identified as a hospital clone in many South African hospitals, in multiple provinces (169, 174, 175, 361, 375). A study of isolates from Spanish hospitals between 1998 and 2002 showed the prevalence of EMRSA-16 which was recently replaced by ST247-MRSA-1 (184). The spread of EMRSA-16 from the UK into South Africa may have been facilitated, at least in part, by the historic and social links between the two countries.

ST461-MRSA-*ccrA1B1, ccrC/*Class B *mec* (SLV of ST5 and a member of CC5) clone was identified in this study. According to our knowledge this is only the second time that this ST has been identified as MRSA [http://www.mlst.net]. Considering the limited prevalence of this clone in South Africa, which has only been reported in Tygerberg Hospital, and that

independent horizontal introduction of SCC*mec* elements into *S. aureus* occurs locally (373), we suggest the possible local emergence of this clone. The limited genetic variation revealed by PFGE (all isolates were representative of one PFGE cluster) and *spa*-typing (all strains were *spa* type t045), suggest the recent local emergence of ST461-MRSA- *ccrA1B1, ccrC/*Class B *mec spa* t045.

ST22-MRSA-IV (UK EMRSA-15) *spa* type t8636 was reported in this study as one of the minor STs. It is commonly identified in Europe, Australia, Canada and Indonesia (84), and was recently introduced into Malaysian hospitals (179). It was first identified in the UK in 1993 (167). In contrast to our results indicating a low prevalence of this clone in Tygerberg Hospital, recent studies showed increasing prevalence of this clone in Europe and replacement of ST36-MRSA-II in the UK (169, 183). On the other hand, our results are in agreement with previous studies which reported a low prevalence of this clone in South Africa (174, 175). This clone has also not been reported in other African countries including Nigeria (207), Morocco, Senegal, Niger, Cameroon and Madagascar (171). The low prevalence of ST22-MRSA-IV in South Africa as well as the absence in other African countries may reflect only recent spread in Africa or, possibly, a lack of adaptation to the environment.

Finally ST8-MRSA-V *spa* type t008 was reported in our study as a representative of a minor PFGE clone. It is an infrequently identified clone and has been identified in Australia (169) and Nigeria. In Nigeria it was identified with two different *spa* types (t064 and t451) (376). This is the first study reporting this clone in South Africa.

3.4.3 Commonly identified MSSA STs

The prevalent MSSA STs were: ST1865-MSSA, ST45-MSSA, ST8-MSSA, ST22-MSSA, ST97-MSSA, ST1-MSSA, ST5-MSSA, ST2122-MSSA, ST2126-MSSA, ST12-MSSA and ST6-MSSA. Our results showed more MSSA STs than MRSA STs and supported the suggestion that the MSSA genetic background is more diverse (203, 204). The presence of certain MSSA STs that correspond to MRSA clones in the same study population and time period raises the possibility of the local emergence of MRSA clones after horizontal acquisition of SCC*mec* elements locally, as has been previously hypothesized (121, 200).

ST45-MSSA *spa*-CC 073/015 was identified in this study as the most predominant MSSA clone. It has been identified as a worldwide spreading clone (80, 169). In this study all ST45 strains were only identified as methicillin susceptible, in contrast to a previous study in KwaZulu-Natal where ST45 isolates were identified as both methicillin susceptible and resistant (206). The identified MSSA STs of CC5 are: ST2122-MSSA *spa*-CC 002 (novel), ST6-MSSA *spa*-CC 064, and ST5-MSSA- *spa*-CC 002. Similar results were reported in a previous study conducted in KwaZulu-Natal showing a prevalence of both MSSA ST5 and ST6 (206). ST5 has been identified only in Cameroon, Madagascar, Niger and Senegal (205), and Nigeria (376). ST8-MSSA *spa*-CC 064 is assigned to CC8, and has also been identified in KwaZulu-Natal province (206), and Nigeria (376). Two MSSA STs of CC8 have been identified in some African countries; ST8 in Senegal, Niger and Morocco; and ST1404 identified in Madagascar (205). ST22-MSSA *spa*-CC 022 (*spa* type t891) PVL positive, was identified in this study as a member of CC22. This clone has been identified worldwide as both MRSA and MSSA (80), and has been reported in KwaZulu-Natal province (206). This clone grouped together with ST22-MRSA-IV in one PFGE clone, again raising the possibility of local SCC*mec* element transfer.

3.4.4 Infrequently identified MSSA STs

In this study, MSSA ST2126 *spa*-CC084 and ST97 *spa*-CC267 were identified as members of CC15 and CC97 respectively, and all members of these two CCs were MSSA; this is in agreement with previous reports that showed members of these CCs are mainly MSSA strains (168, 169). MSSA strains belonging to CC15 have been identified in Belgium, England, Germany and Portugal (80). In Africa two MSSA STs of CC15 have been identified; ST15 in Senegal, Niger, Cameroon, Madagascar and Morocco; while ST1403 has only been described in Senegal and Cameroon (205). ST97 (a member of CC97) is commonly isolated from animals and rarely identified in humans (169). This study is the first report of ST97 in South Africa.. ST1 *spa*-CC174 PVL positive was prevalent in KwaZulu-Natal province (206). The genetic background of this ST has been described as providing a convenient environment for community associated SCC*mec* element integration (80).

Two strains were identified as ST12-MSSA *spa*-CC022 and belonged to CC12. This is a worldwide clone which has been identified in Germany, Belgium, Brazil, England, Portugal and the Netherlands (80), however, it has not been identified in South Africa. Finally, ST1865-MSSA *spa*-CC021 PVL positive was identified in this study. According to our knowledge this clone has not been identified in other countries as well as other hospitals in South Africa.

3.4.5 SCC*mec* element

Our preliminary data showing the presence of *ccrA1B1*, *ccrC* and Class B *mec* in the same SCC*mec* element suggests that a new SCC*mec* I variant is prevalent in isolates from our

patient population. According to the International Working Group on the Classification of Staphylococcal Cassette Chromosome Element (IWG-SCC) when two *ccr* genes are integrated in one SCC*mec* element it should be considered as a variant of the previously identified element and designated as (1B&5). Two STs (ST5 and ST461) were found carrying this SCCmec element, both belong to CC5.

A possibly novel SCC*mec* element, *ccrC*/Class A *mec* (5A), was carried by strains of ST239 *spa* type t37. This combination indicates the potential prevalence of a novel SCC*mec* type in Tygerberg Hospital. In Nigeria, the same combination was identified in one MRSA strain belonging to ST8 (CC8) (207). During the study period the prevalence of the untypeable SCC*mec ccrC*/Class A *mec* was higher in 2011 than 2010. In addition, the prevalence of SCC*mec ccrC*/Class A *mec* in 2011 was higher than SCC*mec* type III suggesting the possible replacement of SCC*mec* type III with the potentially novel SCC*mec ccrC*/Class A *mec*. These findings may indicate the emergence of a new MRSA clone in Africa that has evolved from the integration of a new SCC*mec* type into CC8 strains.

To avoid misclassification, the IWG-SCC recommends sequencing of the whole SCC*mec* element to identify any putative new SCC*mec* types, as available multiplex PCRs only represent limited parts of the element and are not necessarily indicative of the entire nucleotide sequence of the element (130). Therefore, further studies, involving sequencing of the entire SCC*mec* element, are required to confirm these preliminary results. The presence of novel SCC*mec* elements and the emergence of a new MRSA lineage (ST461-MRSA-*ccrA1B1*, *ccrC*/Class B *mec*) in Tygerberg Hospital supports the local evolution of MRSA via several independent horizontal transmission events of the SCC*mec* element into local MSSA isolates. This has been suggested as the multi-clone theory in previous studies (121, 348, 373).

In this study 3 PFGE clusters (F, G and O) contained both MRSA and MSSA. It has been presumed that the presence of MRSA and MSSA isolates in the same PFGE cluster may be due to local transfer of the SCC*mec* element into MSSA or deletion of *mecA* from prevalent MRSA isolates (377). Another finding reported in this study was that of different SCC*mec* types grouping in one PFGE cluster (SCC*mec* type III and untypeable SCC*mec ccrC*/Class A *mec* in PFGE cluster U). In agreement with this result, a study investigating MRSA genotypic characteristics in KwaZulu-Natal reported 4 different SCC*mec* types grouped in one PFGE cluster (372). The presence of multiple SCC*mec* types in the same PFGE cluster raises the possibility of particular mechanisms of adaptation in certain MRSA lineages via excision of the SCC*mec* element followed by replacement with another SCC*mec* element.

3.5 Conclusion

This is the first study to investigate the molecular characteristics of nasal and blood *S. aureus* (MRSA and MSSA) isolates at Tygerberg Hospital. The results showed the majority of MRSA strains were ST239 followed by ST612-MRSA-IV and ST5. Untypeable SCC*mec* types were identified in both ST239 and ST5 and the preliminary results suggest a new SCC*mec* type (*ccrC*/Class A *mec*) and new variant of SCC*mec* type I (*ccrA1B1*, *ccrC*/Class *B mec*). However, the nucleotide sequence of the entire SCC*mec* element should be determined to confirm these findings. This is the first study to report ST612-MRSA-IV as being PVL-positive in South Africa and worldwide. It is also the first study reporting the prevalence of ST461-MRSA-IV in South Africa and the second report worldwide. Novel MSSA STs were identified (ST2122 (CC5) and ST2126 (CC15)), and MSSA STs were more diverse than MRSA clones.

CHAPTER 4 PREVALENCE AND ASSOCIATION OF VIRULENCE FACTOR GENES IN REPRESENTATIVE STRAINS OF *STAPHYLOCOCCUS AUREUS* ISOLATES AT TYGERBERG HOSPITAL

4.1 Introduction: Prevalence and association of virulence factor genes with *S. aureus* genetic structure

The association between *S. aureus* genetic background and certain virulence factors remains unclear (378). Microarray analysis showed that the variation in the genetic component of different lineages of invasive and colonising isolates does not influence variation in pathogenesis (88). Another study which included different genotypes of *S. aureus* isolates obtained from the nasal cavity, blood and other body sites suggested that the pathogenesis of isolates was not associated with genotypes and that in the same lineage some strains were more virulent than others (379). Although there is no clear association between virulence and any specific genetic lineage, invasive disease has been associated with *S. aureus* strains harbouring different combinations of seven virulence factor genes including: exfoliative toxin A (*eta*), fibronectin binding protein A (*fnbA*), collagen binding protein (*cna*), serine-aspartate repeat protein E (*sdrE*), staphylococcal enterotoxin J (*sej*), gamma hemolysin (*hlg*), and intracellular adhesins A (*icaA*) (380). This study did control for the different frequencies of virulence genes in different lineages.

A number of associations between specific virulence factors and genetic background have been reported (21). For example, one study reported an association between ST8-MRSA-IV and Panton-Valentine leukocidin (*pvl*), enterotoxin Q (*seq*) and enterotoxin K (*sek*) virulence factor genes, while ST30 was associated with the presence of the *pvl* gene, and ST36 was associated with the presence of enterotoxin A (*sea*) and toxic shock syndrome toxin (*tst*)

genes (218). The presence of *seb* and *sec* virulence factor genes has been associated more frequently with *spa*-CC002 compared to other *spa*-CCs (008, 037, 0084 and 1039 and *spa* types t037 and t1151 (275).

The association of specific virulence factors with MRSA or MSSA is variable between studies conducted in different areas. A study conducted in Spain reported that MRSA was associated with the presence of *sep*, while *tst* was associated with MSSA (381). In the Czech Republic, a study comparing the prevalence of enterotoxin, exfoliatins, *pvl* and *tst* between MRSA and MSSA showed that *seg* and *sei* were significantly associated with MRSA strains (280). In Columbia, MSSA was associated with *seb* and *sed* while *pvl* was more common in MRSA (278). This chapter aimed to describe the prevalence of virulence factor genes among the representative nasal and blood isolates selected for MLST analysis as described inCHAPTER 3.

4.2 Methods

4.2.1 Bacterial isolates

For detection of *pvl*, all 208 isolates were chosen, however, the presence of the other virulence factor genes was investigated in 21 representative isolates (Table 21) that were previously analysed by PFGE, *spa* typing, MLST, SCC*mec* typing and *agr* typing (CHAPTER 2 and CHAPTER 3). These 21 were chosen to represent the range of lineages present in the study, as well as to include both MRSA and MSSA. The decision to use only a representative sample of isolates for these genes was made primarily due to practical considerations; in addition, it was felt that since PVL has arguably been more widely studied than many of the other virulence factors, analysis of PVL in all isolates may be more valuable for this specific virulence factor. The control strains used for virulence factor detection were as shown in table 22.

Table 21 Characteristics of selected representative isolates.

#	Genetic lineage	PFGE clone	MLST-CC	*spa* type	*agr*	PVL
1	ST1865-MSSA	A (minor)	30	t318	III	+ve
2	ST36-MRSA-II	B (minor)	30	t012	III	-ve
3	ST45-MSSA	D (major)	45	t015	I	-ve
4	ST22-MRSA-IV	G (intermediary)	22	t8636	I	-ve
5	ST22-MSSA	G (intermediary)	22	t891	I	+ve
6	ST5-MRSA-I	M (minor)	5	t045	II	-ve
7	ST461-MRSA-I	N (minor)	5	t045	II	-ve
8	ST8-MRSA-V	O (minor)	8	t008	I	-ve
9	ST97-MSSA	P (intermediary)	97	t267	I	-ve
10	ST8-MSSA	Q (intermediary)	8	t1476	I	-ve
11	ST612-MRSA-IV	R (minor)	8	t1257	I	-ve
12	ST612-MRSA-IV	S (major)	8	t1443	I	+ve*
13	ST239-MRSA-III	U (major)	8	t037	I	-ve
14	ST239-MRSA-*ccrC*/class A	U (major)	8	t037	I	-ve
15	ST2122-MSSA	W (minor)	5	t071	II	-ve
16	ST5-MS	Y (minor)	5	t570	I	-ve
17	ST1-MSSA	Z (minor)	1	t5471	I	-ve/+ve**
18	ST2126-MSSA	AA (major)	15	t084	II	-ve
19	ST12-MSSA	AB (minor)	12	t160	II	-ve
20	ST6-MSSA	AE (intermediary)	5	t701	I	-ve
21	ST1-MSSA	AF (minor)	1	t174	III	-ve

*Only for *spa* type t1257 **Only for *spa* type t5471

Table 22 : *S. aureus* control strains used for the detection of virulence factor genes

Strain	Target gene(s)	Reference
BAA-1681	*Eta*	(363)
BAA-38	*seb, chp*	(363)
NCTC 8325-4	*cap5, sak, hld, hla, hlb*	(363)
	icaA, hlg	(281)
MW2	*cap8*	(5)
	fnbA, fnbB	(363)
	hlgA	This study
Newman	*sdrC, sdrD, clfA, clfB*	(281)
	sdrE	(382)
	Eap	(231)
ATCC 19095	*seh, seg, sel*	(383)
	sen, seo, sem	(363)
ATCC 23235	*coa, nuc, sej, scn*	(363)
	Sed	(384)
ATCC 700699	*sea, sec, sei, see, tst*	(363)
ATCC 25923	*Can*	(363)
ATCC 47795	*Pvl*	http://www.atcc.org

4.2.2 Virulence factors

Thirty-eight virulence factor genes were investigated including the adhesins (*clfA*, *clfB*, *fnbA*, *fnbB*, *sdrC*, *sdrD*, *sdrE*, *cna*, *icaA* and *eap* (*map*)), the superantigenic toxins (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, *sel*, *sem*, *sen*, *seo*, *eta and tst*), the immune avoidance genes (*chp*, *sak*, *scn*, *cap5*, and *cap8*), the hemolysins (*hld*, *hlg*, *hlgv*, *hla* and *hlb*) and toxins (*pvl*, *coa* and *nuc*) (Table 23).

4.2.3 Detection of Virulence factor genes by PCR

4.2.3.1 Panton-Valentine leukocidin (PVL)

All 208 unique non-repeated isolates included in chapter 3 were screened for the presence of *pvl*. Uniplex PCR was set up as following: primary denaturation was at 95℃ for 4 min followed by 30 cycles of 95℃ for 30 s, 60℃ for 30 s and 72℃ for 1 min. The final extension was at 72℃ for 10 min. The final PCR reaction volume was 25 µl and included 12.5 µl of Go Taq® Colorless Master Mix (Promega), 1 mM of primers (Table 24) and 1 µl of template (367).

4.2.3.2 Other virulence factors

The virulence factor genes were amplified by uniplex PCRs except four sets of genes which were amplified by multiplex PCRs: (*coa*, *nuc* and *sed*), (*seg* and *sej*), (*sea* and *sec*) and (*hld* and *hlg*).

Table 23 : Virulence factor genes investigated in this study

Group	Virulence factor	Abbreviation	gene
Adhesins	Clumping factor A	ClfA	*clfA*
	Clumping factor B	ClfB	*clfB*
	Fibronectin binding protein A	FnBPA	*fnbA*
	Fibronectin binding protein B	FnBPB	*fnbB*
	Collagen binding protein	CNA	*cna*
	Extra cellular adherence protein/MHC class II analogous protein	Eap/Map	*eap/map*
	Serine-a*spar*tate protein C	*sdrC*	*sdrC*
	Serine-a*spar*tate protein D	*sdrD*	*sdrD*
	Serine-a*spar*tate protein E	*sdrE*	*sdrE*
	Intracellular adhesion locus A	IcaA	*icaA*
Superantigenic toxins	Enterotoxins A, B, C, D, E, G, H, I, J, L, M, N and O	*Sea*, Seb, Sec, Sed, See Seg, Seh, Sei, Sej, Sel, Sem, Sen, Seo (in order)	*Sea, seb, sed, seg, seh, sei, sej, sel, sem, sen, seo.* (in order)
	Toxic shock syndrome toxin-1	TSST-1	*tst*
	Exfoliative toxin	ETA	*eta*
Immune avoidance	Chemotaxis inhibitory protein	CHIP	*chp*
	Staphylokinase	*SAK*	*sak*
	Staphylococcal complement inhibitor	SCIN	*scn*
	Capsule type 5	Cap5	*cap5*
	Capsule type 8	Cap8	*cap8*
Hemolysins	Alpha-hemolysin, Beta-hemolysin, Delta-hemolysin, Gama-hemolysin and Gama-hemolysin variant	Hla, Hlb, Hld, Hlg, HlgA	*hla, hlb, hld, hlg, hlgA*
Other virulence factors	Paton-Valentine leukocidin	PVL	*pvl*
	Coagulase	Coa	*coa*
	Nuclease	Nuc	*nuc*

The primers used for each reaction, along with the primer concentration and annealing temperature for each reaction are shown in Table 24. All uniplex and multiplex PCRs started with an initial denaturation at 95°C for 5min followed by 30 cycles of 95°C for 0.45s, annealing temperatures (as shown in Table 24) for 0.45 s and extension at 72°C for 1 min; with a final extension at 72°Cfor 10 min. PCR reactions consisted of 12.5 µl of Go Taq® Colourless Master Mix (Promega, USA), primers (Inqaba Biotech or Whitehead Scientific, South Africa), 1 µl of crude DNA and brought to a final volume of 25µl with ddH2O. Amplified genes were separated by gel electrophoresis and visualised on a 1.5% and 3.5% agarose gel containing EtBr for the uniplex and multiplex PCR assays respectively.

Table 24: Primers used in the study

Gene	Primer description	Primer sequence	Product size/bp	Primer concen-tration (µm)	T_m	Reference
pvl	*luk-PV-1*	ATCATTAGGTAAAATGTCTGGACATGATCCA	433	1	60°C	(367)
	luk-PV-2	GCATCAASTGTATTGGATAGCAAAAGC				
sea	*SEA*-F1170	TAA GGA GGT GGT GCC TAT GG	180	1	56°C	(383)
	SEA-R1349	CAT CGA AAC CAG CCA AAG TT				
seb	Forward	GACATGATGCCTGCACCAGGAGA	355	1	67°C	(281)
	Reverse	AACAAATCGTTAAAAACGGCGACACAG				
sec	SEC-F467	ACC AGA CCC TAT GCC AGA TG	371	1	56°C	(383)
	SEC-R578	TCC CAT TAT CAA AGT GGT TTC C				
sed	SED-F578	TCA ATT CAA AAG AAA TGG CTC A	339	2	56°C	(383)
	SED-R916	TTT TTC CGC GCT GTA TTT TT				
see	Forward	TGCCCTAACGTTGACAACAAGTCCA	532	1	60°C	(281)
	Reverse	TCCGTGTAAATAATGCCTTGCCTGAA				
seg	SEG-F322	CCA CCT GTT GAA GGA AGA GG	432	1	56°C	(383)
	SEG-R753	TGC AGA ACC ATC AAA CTC GT				
seh	SEH-F260	TCA CAT CAT ATG CGA AAG CAG	463	1	58°C	(383)
	SEH-R722	TCG GAC AAT ATT TTT CTG ATC TTT				
Sei	SEI-F71	CTC AAG GTG ATA TTG GTG TAG G	300	1	66°C	(383)
	SEI-R637	CAG GCA GTC CAT CTC CTG TA				
sej	SEJ-F349	GGT TTT CAA TGT TCT GGT GGT	306	1	56°C	(383)
	SEJ-R654	AAC CAA CGG TTC TTT TGA GG				
Sel	SEL-F158	CAC CAG AAT CAC ACC GCT TA	240	1	66°C	(383)
	SEL-R397	CTG TTT GAT GCT TGC CAT TG				
sem	mpSEM-1	CTATTAATCTTTGGGTTAATGGAGAAC	300	1	52°C	(274)
	mpSEM-2	TTCAGTTTCGACAGTTTTGTTGTCAT				
sen	mpSEN-1	ATGAGATTGTTCTACATAGCTGCAAT	680	1	54°C	(274)
	mpSEN-2	AACTCTGCTCCCACTGAAC				

Table 25 Cont. Primers used in the study

seo	mpSEO-1	AGTTTGTGTAAGAAGTCAAGTGTAGA	180	1	54°C	(274)
	mpSEO-2	ATCTTTAAATTCAGCAGATATTCCATCTAAC				
Tst	TST-F	AGCCCTGCTTTTACAAAAGGGGAAAA	306	1	60°C	(281)
	TST-R	CCAATAACCACCCGTTTTATCGCTTG				
hla	*HLA*-1	CTGATTACTATCCAAGAAATTCGATTG	209	1	57°C	(274)
	HLA-2	CTTTCCAGCCTACTTTTTTATCAGT				
hlb	HLB-1	GTGCACTTACTGACAATAGTGC	309	1	65°C	(274)
	HLB-2	GTTGATGAGTAGCTACCTTCAGT				
hld	HLD-1	AAGAATTTTTATCTTAATTAAGGAAGGAGTG	111	1	56°C	(274)
	HLD-2	TTAGTGAATTTGTTCACTGTGTCGA				
hlg	HLG-F	TTGGCTGGGGAGTTGAAGCACA	306	1		(281)
	HLG-R	CGCCTGCCCAGTAGAAGCCATT				
hlg-2	mpHLG2-1	GACATAGAGTCCATAATGCATTYGT	390	1	59°C	(274)
	mpHLG2-2	ATAGTCATTAGGATTAGGTTTCACAAAG				
eta	ETA- F	CGCTGCGGACATTCCTACATGG	676	1	68°C	(281)
	ETA-R	TACATGCCCGCCACTTGCTTGT				
clfA	Forward 368	GTAGGTACGTTAATCGGTT	1,584	1	56°C	(380)
	Reverse 1951	CTCATCAGGTTGTTCAGG				
clfB	Forward	TGGCGGCAAATTTTACAGTGACAGA	404	1	55°C	(281)
	Reverse	AGAAATGTTCGCGCCATTTGGTTT				
fnbA	Forward	GGCCAAAATAGCGGTAACC	228	1	55°C	(385)
	Reverse	GTGAATATGTGGCACACTG				
fnbB	Forward	GGAGCGGCCTCAGTATTCTT	201	1	57°C	(386)
	Reverse	AGTTGATGTCGCGCTGTATG				
sdrC	Forward	CGCATGGCAGTGAATACTGTTGCAGC	731	1	66°C	(281)
	Reverse	GAAGTATCAGGGGTGAAACTATCCACAAATTG				
sdrD	Forward	CCACTGGAAATAAAGTTGAAGTTTCAACTGCC	467	1	64°C	(281)
	Reverse	CCTGATTTAACTTTGTCATCAACTGTAATTTGTG				
sdrE	Forward	GCAGCAGCGCATGACGGTAAAG	894	1.5	65°C	(281)
	Reverse	GTCGCCACCGCCAGTGTCATTA				

Table 26 Cont. Primers used in the study

coa	COA-F2591	CCG CTT CAA CTT CAG CCT AC	204	0.5	56°C	(383)
	COA-R2794	TTA GGT GCT ACA GGG GCA AT				
nun	NUC-F166	AGT TCA GCA AAT GCA TCA CA	400	2	56°C	(383)
	NUC-R565	TAG CCA AGC CTT GAC GAA CT				
cna	CNA-F	TTCACAAGCTTGGTATCAAGAGCATGG	452	1	62°C	(281)
	CNA-R	GAGTGCCTTCCCAAACCTTTTGAGC				
chp	*Chp*-1	TTTACTTTTGAACCGTTTCCTAC	346	1	54°C	(387)
	Chp-2	CGTCCTGAATTCTTAGTATGCATATTCATTAG				
scin	*Scn*-1	AGCACAAGCTTGCCAACATCG	238	1	42°C	(387)
	Scn-2	TTAATATTTACTTTTTAGTGC				
sak	*Sak*-1	AAGGCGATGACGCGAGTTAT	203	0.5	58°C	(219)
	Sak-2	GCGCTTGGATCTAATTCAAC				
eap	eap-CON1	TACTAACGAAGCATCTGCC	230	1	53°C	(231)
	eap-CON2	TTAAATCGATATCACTAATACCTC				
cap5	Cap5 K1	GTCAAAGATTATGTGATGCTACTGAG	361	0.5	59°C	(388)
	Cap5 K2	ACTTCGAATATAAACTTGAATCAATGTTATACAG				
cap8	Cap 8K1	GCCTTATGTTAGGTGATAAACC	173	0.5	58°C	(388)
	Cap 8K2	GGAAAAACACTATCATAGCAGG				
ica	ICA-F	TCAGACACTTGCTGGCGCAGTC	936	1	65°C	(281)
	ICA-R	TCACGATTCTCTCCCTCTCTGCCATT				

4.2.4 Statistical methods

In this study 21 representative isolates were used to investigate the prevalence of virulence factor genes among different *S. aureus* genetic lineages. Owing to the small number of isolates included, statistical analysis was done by comparing isolates belonging to one genetic structure to other isolates belonging to all other genetic lineages. The Fisher exact and two-tailed tests were used to analyse all results, however, the distribution of virulence factor genes among the 3 identified *agr* groups was analysed by the Chi-squared test. Statistical analysis was performed using Statistica, version 10 (2012).

4.3 Results

4.3.1 Virulence factor gene prevalence

All the included isolates harboured the *clfA*, *fnbA* and *fnbB* genes, and all but one isolate (ST97-MSSA) contained the *clfB* gene. Among the superantigenic virulence factor genes the most prevalent was *sea* (76%), followed by *sei* and *sel* (47%), *sem*, *sen*, *seo* and *seg* (42%), *seb* (14%), *sec*, (19%), *sed* (4.8%), *see* (38%), *seh*, *sej* and *eta* (9.5%) and *tst* (14%). The prevalence of immune avoidance genes among the clones was *chp* (42%), *sak* (81%), *scn* (85%), *cap5* (52%) and *cap8* (48%). The presence of hemolysin toxin virulence factor genes was *hld* (100%), *hlg* (90%), *hlgA* (76%) *hla* (90%) and *hlb* (4.8%). Amongst the other toxin genes analysed, *coa* and *nuc* were present in all the clones (

Table 27). The *pvl* gene was detected in 18 (8.7%) of the 208 isolates (1 MRSA and 17 MSSA).

Table 27 : Prevalence of virulence factor genes among included isolates

Group	Virulence factor genes	Prevalence (%) N = 21
Adhesins	*clfA*	21 (100)
	clfB	20 (95)
	fnbA	21 (100)
	fnbB	21 (100)
	cna	11 (52)
	Eap	20 (95)
	sdrC	11 (52)
	sdrD	12 (57)
	sdrE	15 (71)
	icaA	19 (90)
Super antigenic toxins	*Sea*	16 (76)
	Seb	3 (14)
	Sec	4 (19)

	Sed	1 (4.8)
	See	8 (38)
	Seg	9 (42)
	seh	2 (9.5)
	Sei	10 (47)
	Sej	2 (9.5)
	Sel	10 (47)
	Sem	9 (42)
	Sen	9 (42)
	Seo	9 (42)
	Eta	2 (9.5)
	Tst	3 (14)

Table 28 cont. Prevalence of virulence factor genes among included isolates

Immune avoidance	*Chp*	9 (42)
	Sak	17 (81)
	Scn	18 (85)
	cap5	11 (52)
	cap8	10 (47)
Haemolysins	*Hld*	21 (100)
	Hlg	19 (90)
	Hlgv	16 (76)
	Hla	19 (90)
	Hlb	1 (4.8)
Other virulence factors	*Nuc*	21 (100)
	Coa	21 (100)

4.3.2 Innate immune evasion cluster:

Of the 21 included isolates, 18 harboured four different types of innate immune evasion clusters (IEC): *sea*, *sak*, *scn*, *chp* (type A); *sea*, *sak*, *scn* (type D); *chp*, *scn* (type C) and *sak*, *scn* (type E). In 3 cases no IEC genes were amplified (Table 29).

Table 29 : Distribution of IEC among identified MLST clonal complexes.

IEC type (N)	Gene combination	Clones (N)
A (8)	*sea*, *sak*, *scn*, *chp* (8)	CC5 (1), CC8 (2), CC22 (2), CC30 (2), CC45 (1)
D (8)	*sea*, *sak*, *scn* (8)	CC1 (2), CC5 (1), CC8 (4), CC12 (1)
C (1)	*chp*, *scn* (1)	CC15
E (1)	*sak*, *scn* (1)	CC97
	No IEC gene (3)	CC5 (3)

4.3.3 Enterotoxin gene cluster (egc):

The distribution of the egc genes among the included isolates showed genetically diverse gene combinations. However, four (80%) of the CC5 isolates harboured the *sei*, *seg*, *sem*, *sen* and *seo* genes. All CC8 isolates contained only the *sei* gene, while CC22, CC30 and CC45 isolates contained the *seg*, *sem*, *sen*, *seo* genes. Six isolates lacked any egc genes (Table 30).

Table 30 : Distribution of egc genes among identified MLST clonal complexes

***egc* gene combination (N)**	**Clones (N)**
sei, *seg*, *sem*, *sen*, *seo* (4)	CC5 (4)
seg, *sem*, *sen*, *seo* (5)	CC22 (2), CC30 (2), CC45 (1)
sei (6)	CC8 (6)
No *egc* gene (6)	CC1 (2), CC5 (1), CC12 (1), CC15 (1), CC97 (1)

From this data, we can see that the distribution of virulence factor genes amongst the included *S. aureus* clonal complexes was variable; however significant differences were identified in the distribution of certain virulence factor genes in some lineages compared to other CCs (Table 31). All isolates belonging to CC5 were positive for egc genes except one isolate (ST6). The *sed* and *sej* genes were amplified in one isolate belonging to CC5, whereas only the *sei* gene was present in all isolates belonging to CC8.

Comparing the virulence factor gene profiles of MRSA (90 isolates) versus MSSA (118 isolates) revealed significant differences in the presence of the *pvl* gene [MSSA 14.4% and MRSA 1.1% ($p = 0.0007$)]. In contrast, among 21 selected isolates, *sei* gene presence was significantly associated with MRSA [MSSA 25%, MRSA 77. 8% ($p = 0.0299$)].

Table 31 : Selected virulence factors showing significant differences in distribution of MLST-CCs

Virulence factor genes	**MLST CCs (total number)** **No of positive (%)**		***P* value**
sdrC	CC5 (5) 5 (100)	Other CCs (16) 6 (37.5)	0.0350
sdrC	CC8 (6) 6 (100)	Other CCs (15) 5 (33.3)	0.0123
icaA	CC30 (2) 0	Other CCs (19) 19 (100%)	0.00476
Sec	CC1 (2) 2 (100)	Other CCs (19) 2 (10.53)	0.0285
Seg	CC8 (6) 0 (0)	Other CCs (15) 9 (60)	0.0185
She	CC1 (2) 2 (100)	Other CCs (19) 0	0.00476
Sei	CC8 (6) 6 (100)	Other CCs (15) 4 (20.67)	0.00387
Sem	CC8 (6) 0	Other CCs (15) 9 (60)	0.0185
Sen	CC8 (6) 0	Other CCs (15) 9 (60)	0.0185
Seo	CC8 (6) 0	Other CCs (15) 9 (60)	0.0185
Sak	CC5 (5) 2 (40)	Other CCs (16) 15 (93.75)	0.0275
Scn	CC5 (5) 2 (40)	Other CCS (16) 16 (100)	0.00752

Hla	CC5 (5) 3 (60)	Other CCs (16) 16 (100)	0.0476
hlgA	CC22 (2) 0	Other CCs (19) 16 (84.21)	0.0476
Pvl	CC1 (2) 2 (100)	Other CCs (19) 3 (15.79)	0.0476

The distribution of virulence factor genes among *spa*-CCs was variable and significant differences were found in some *spa*-CC compared to others (

Table 32).

Table 32 : Selected virulence factors which have significance difference between one of *spa-CCs* compared to others

Virulence factor genes	***Spa*- CCs (total number)** **No of positive (%)**		***P* value**
Can	*spa*-CC002 (4) 0 (0.00)	Other *spa*-CCs (17) 11 (64.7)	0.035
Sak	*spa*-CC002 (4) 1 (25)	Other *spa*-CCs (17) 16 (94.12)	0.011
Scn	*spa*-CC 002 (4) 1 (25)	Other *spa*-CCs (17) 17 (100)	0.003
Hla	*spa*-CC 002 (4) 2 (50)	Other *spa*-CCs (17) 17 (100)	0.028
cap5	*spa*-CC 021 (4) 0 (0.00)	Other *spa*-CCs (17) 11 (64.7)	0.035
cap8	*spa*-CC 021 (4) 4 (100)	Other *spa*-CCs (17) 6 (35.29)	0.035
sdrC	*spa*-CC064 (5) 5 (100)	Other *spa*-CCs (16) 6 (37.5)	0.035
sdrD	*spa*-CC 021 (4) 0 (0.00)	Other *spa*-CCs (17) 12 (70.59)	0.021
sdrD	*spa*-CC 064 (5) 5 (100)	Other *spa*-CCs (16) 7 (43.75)	0.045
sdrE	*spa*-CC 021 (4) 4 (100)	Other *spa*-CCs (17) 11 (64.7)	0.002
Sea	*spa*-CC002 (4) 1 (25)	Other *spa*-CCs (17) 15 (88.24)	0.0275
Seg	*spa*-CC 002 (4) 4 (100)	Other *spa*-CCs (17) 5 (29.14)	0.021

Seg	*spa*-CC 064 (5) 0 (0.00)	Other *spa*-CC (16) 9 (56.25)	0.045
Sei	*spa*-CC 002 (4) 4 (100)	Other *spa*-CCs (17) 6 (35.29)	0.035
Sel	*spa*-CC 002 (4) 4 (100)	Other *spa*-CCs (17) 6 (35.94)	0.035
Sem	*spa*-CC 002 (4) 4 (100)	Other *spa*-CC (17) 5 (29.41)	0.021
Sem	*spa*-CC 064 (5) 0 (0.00)	Other *spa*-CC (16) 9 (56.25)	0.045
Sen	*spa*-CC 002 (4) 4 (100)	Other *spa*-CC (17) 5 (29.41)	0.021
Sen	*spa*-CC064 (5) 0 (0.00)	Other *spa*-CCs (16) 9 (56.25)	0.045
Seo	*spa*-CC 002 (4) 4 (100)	Other *spa*-CCs (17) 5 (29.41)	0.021
Seo	*spa*-CC064 (5) 0 (0.00)	Other *spa*-CCs (16) 9 (56.25)	0.045
icaA	*spa*-CC 021 (4) 2 (50)	Other *spa*-CCs (17) 17 (100)	0.028

4.4 Discussion

4.4.1 Prevalence of virulence factor genes in different lineages:

The adhesion virulence factor genes were most prevalent amongst the 21 representative isolates. Three CCs; 12, 30 and 45 were negative for the *sdr* locus, which is in contrast to a previous study that found that all isolates were positive for at least one gene of this locus (227). However, this study did not specify whether these were MSSA or MRSA, or what the genetic lineage was. All CCs except CC30 were positive for the *icaA* gene. The high prevalence of the *icaA* gene has previously been reported in community acquired MSSA and MRSA isolates (279). A study comparing the presence of the *icaA* gene in MSSA isolates from the USA and South Africa found that all isolates from both countries were positive (281). Another study which included invasive and nasal isolates found that 51% of CC30 isolates harboured the *icaA, fnbB, hlg* and *cna* genes (380). This is in contrast to our results, where all the CC30 isolates were positive for *fnbB, hlg* and collagen binding protein (*cna*) genes.

The enterotoxin genes *sed*, *sej* and *ser* are located on the same plasmid (221). This study showed the presence of the *sed* and *sej* genes in CC5 (new ST2122) and the *sej* gene in ST8. These genes are usually identified in isolates belonging to CC8 (389). While previous studies have reported a high association of the *tst* gene with isolates belonging to CC30 (88, 380, 389), this study only detected the *tst* gene in 3 isolates; CC30 (n = 1) and CC5 (n = 2). In accordance with a previous study the *seh* gene was only identified in CC1 isolates, confirming the restriction of this gene to CC1 (218).

Superantigenic toxin genes were absent in both CC15 and CC97. The absence of these genes in CC15 is in agreement with a previous study where the authors hypothesised that the restriction modification system may interfere with the acquisition of all SAg genes carried by MGEs by horizontal transfer (389). Isolates belonging to CC97 are rarely isolated from humans and mostly occurs in animals, thus the literature regarding virulence factors associated with CC97 is limited (169, 390). In this study, isolates from CC97 showed an absence of SAg genes, similar to findings from isolates belonging to CC15.

4.4.2 Association with the Immune Evasion Cluster

The majority of isolates (except 3 of the CC5 isolates) were positive for one or more of the IEC genes. IEC types varied despite similar genetic backgrounds. Of the six isolates from CC8, four contained IEC type D, and two contained type A. Similarly, IEC type A was present in 1 CC isolate, and IEC type D in another CC isolate. IEC type A was found in isolates belonging to 5 different CCs, and IEC type D in isolates from 4 CCs. Taken together, these findings suggest that this MGE transferred into the *S. aureus* population regardless of the genetic background structure. A similar hypothesis was presented in a previous study

where the same IEC type was identified in isolates obtained from different PFGE clones and *agr* groups and different IEC types were identified in related isolates (219, 391).

4.4.3 Association with the Enterotoxin gene cluster

The *seg*, *sei*, *sem*, *sen* and *seo* genes which make up the egc are introduced into *S. aureus* by phages (392-394). We showed an association between genes of the egc and CC5, CC22, CC30 and CC45. The egc genes were absent in other CCs (CC1, CC12, CC15, CC97, and ST6 of CC5) and CC8 was only positive for *sei*. These results reaffirm results from previous studies which have shown that the egc genes are associated with specific genetic lineages, with a low prevalence in CC1, CC12, CC15, CC8 and CC395; and a high prevalence in CC5, CC22, CC30 and CC45 (220, 389). Other studies have found a high prevalence of the *seg* and *sei* genes among colonising (57.1%) and invasive (53%) isolates (395) and that the combination of *sei*, *seg*, *sem*, *sen* and *seo* was restricted to isolates belonging to CC5 and CC30 (218). Our study showed the same findings for most isolates belonging to CC5, apart from the one ST6 isolate which did not have these genes present. Our CC30 isolates contained another egc gene combination, namely: *seg*, *sem*, *sen* and *seo*. The high prevalence of egc genes amongst the representative isolates in this study is in line with previous reports that isolates containing these genes are more successful in invasion and colonisation (395).

spa typing is more discriminative than MLST and isolates belonging to one *spa* CC are genetically more related to each other than isolates belonging to one MLST CC. The association between certain virulence factors and specific *spa* CCs or MLST CCs showed that isolates with these virulence factors may have evolved from one ancestor.

4.4.4 Association with *spa* type

Based on *spa* genetic background, the *sea* gene showed a low prevalence in *spa*-CC002 as has been previously reported (275). The egc gene combination *seg, sei, sem, sen, seo* was present in all isolates belonging to *spa*-CC002 and was absent in all *spa*-CC064 isolates. This finding may due to the genetic structure of *spa*-CC002 being more receptive to egc MGE acquisition than *spa*-CC064. In agreement with a previous study, no SAg genes were detected in *spa*-CC084 (389).

4.4.5 MRSA/MSSA

The virulence factor profiles of MRSA and MSSA vary across different studies conducted in diverse geographical regions. Our results showed the prevalence of the *sei* gene among MRSA was significantly higher than in MSSA isolates. Another study found both the *seg* and *sei* genes to be more prevalent in MRSA than MSSA (280). Additionally, in Colombia, a study compared the prevalence of SAgs and *pvl* between MRSA and MSSA isolates and found that the presence of the *seb* and *sed* genes were significantly higher in MSSA isolates, while the presence of the *pvl* gene was significantly more prevalent in MRSA isolates (278). In a study which included *S. aureus* isolates from the United States and South Africa, among the USA isolates the *sdrC, eap, fnbB, tst, sea, sed, seg, sej, sdrD, sdrE, eta, etb* and *see* genes were associated with MRSA, while MSSA isolates from South Africa were more likely than MSSA isolates from the United States to carry *sdrD, sea*, and *pvl* genes (281). Similar results have been reported in Beijing, China, where all *pvl* positive isolates were MSSA (396). These results are different from those reported in Shanghai, China, where the emergence and spread

of pvl positive MRSA has been documented (396, 397).

According to some studies and the MLST website (www.mlst.net), ST6 is categorised as a member of CC5 (169). In this study, the ST6 isolate differed from other isolates belonging to CC5 in that it showed the presence of the *cap8* gene and had no IEC genes. Therefore, due to the genetic diversity of the virulence factor profile of ST6 in our population, we suggest that it be considered as a separate CC. However, further studies on a much larger selection of ST6 isolates would be needed.

4.4.6 PVL prevalence and association with studied clones

In this study, the prevalence of the *pvl* gene among MRSA isolates was very low, with only one MRSA isolate being *pvl* gene positive. This is in accordance with previous studies conducted in different provinces in South Africa all of which showed a low prevalence of the *pvl* gene among MRSA isolates (174, 175, 362, 372). These studies reported that all MRSA *pvl* gene positive isolates were SCC*mec* type IV. However, in this study, for the first time MRSA clone ST612-MRSA-IV was identified as a *pvl* gene positive lineage. The prevalence of the *pvl* gene (8.7%) was lower than a previous study conducted at Tygerberg Hospital that found 15.9% of included blood isolates were *pvl* gene positive; all were MSSA isolates. Our study showed similar results where MSSA *pvl* positive *spa* type t891 was the most prevalent among the included isolates (25). In Nigeria, of 51 *S. aureus* isolates 17 were *pvl* gene positive, 1 of which was MRSA (207). Another study reported a high prevalence of *pvl* gene (40%) among MSSA isolates in Nigeria (376). The *pvl* gene was present in 57% of MSSA isolates collected from Cameroon, Madagascar, Morocco, Niger and Senegal (205). The prevalence of the *pvl* gene in MRSA isolates in Algeria was 29.7% and all *pvl* gene-positive

isolates were SCC*mec* IV (359). From the limited data available, the high prevalence of the *pvl* gene among MSSA isolates in west and sub-Sahara Africa countries is a common finding while reports from North Africa show a higher presence of this gene in both MSSA and MRSA isolates. This is a major concern as PVL-positive MSSA can cause severe infection and is a potential reservoir which may lead to the emergence of MRSA PVL-positive clones in Africa.

4.5 Study Limitations

Due to the large number of virulence factors analysed, only 21 representative isolates were chosen. This small sample size was one of the study limitations as it was difficult to determine statistically significant associations. Furthermore, the isolates for this study were restricted to blood and/or nasal isolates and this may not representative of all *S. aureus* isolates in this geographical area.

4.6 Conclusion

Despite these limitations, we have shown that the virulence factor genetic profile is variable among different genetic backgrounds of *S. aureus*. The acquisition of MGEs carrying SAg genes is uncommon in isolates from CC15 and CC97. Interestingly, the association of the *icaA* gene with CC30 was reported in another study; however this study showed the two CC30 isolates were negative for the presence of this gene. The presence of IEC genes in different genetic lineages indicates that the acquisition of this gene cluster is not restricted to specific lineages while conversely egc genes were restricted to specific *S. aureus* CCs. The *pvl* gene was highly prevalent in MSSA isolates which may evolve to PVL positive MRSA following SCC*mec* element acquisition. This is the first study to report ST612-MRSA-IV as being PVL-positive in South Africa and worldwide. A larger selection of isolates needs to be tested to confirm some of these findings, and a prospective study capturing clinical information may be helpful to elucidate the potential clinical relevance of these findings.

CHAPTER 5 PREVALENCE AND GENOTYPIC CHARACTERISTICS OF *AGR* DYSFUNCTIONAL STAPHYLOCOCCUS AUREUS BLOOD AND NASAL ISOLATES OBTAINED FROM PATIENTS WITH BACTERAEMIA AT TYGERBERG HOSPITAL

5.1 Introduction

As described in section 1.9, the accessory gene regulator (*agr*) locus is a global regulatory gene which controls the expression of the most important cell wall associated and secreted virulence factors in *S. aureus* (398). It is also considered to be the main quorum sensing system coordinating intra-species communication in *S. aureus* (399).

The *agr* locus consists of four genes (*agrB*, *agrD*, *agrC* and *agrA*) in operon RNAII under the control of promoter P2 and one delta-haemolysin gene (*hld*) in operon RNAIII (213, 400, 401) under the control of promoter P3. Activation of the *agr* locus occurs when the RNAII operon expresses the auto-inducing peptide (AIP) which then accumulates outside bacterial cells until it reaches a critical level which activates *AgrC* and *AgrA* transcription. This results in the activation of the RNAIII operon, with a resultant up-regulation of extracellular virulence factor and delta-haemolysin production (chapter 1, figure 3) (402). Thus, the *agr* functionality may be evaluated by analysing delta-haemolysin production, but not beta-haemolysin which is only weakly regulated by *agr* (403); or alpha-haemolysin which is controlled by the staphylococcal accessory regulator gene (*sar*) via the *agr* global regulatory system and directly by *AgrA* (402, 404). In several studies, the synergistic relation between delta and beta-haemolysin and the inhibitory interaction of alpha and beta-haemolysins to lyse sheep red blood cells have been used as a phenotypic assay to detect *agr* functionality (310-312, 318, 320, 403, 405, 406).

The prevalence of *agr* dysfunction among isolates obtained from hospital environments ranges from 15 to 60%,(309, 312-315), and these less virulent *agr* dysfunctional strains may be commonly isolated from chronic infections sites such as the lungs of cystic fibrosis patients (407). The association of *agr* dysfunction with increased mortality in patients with bacteraemia is still unclear; Schweizer *et al*., found the mortality rate among bacteraemic patients infected with *agr* dysfunctional isolates to be significantly higher (18%) than patients infected with *agr* functional isolates (12%) (320). In contrast, another study found similar mortality rates in bacteraemic patients infected with *agr* functional and dysfunctional isolates (310).

It has been reported that *mecA* gene acquisition and expression causes cell wall changes which attenuate *agr* function (307). *S. aureus* strains carrying SCC*mec* type IV and V (CA-MRSA) are generally *agr* functional and highly virulent (308), while strains carrying SCC*mec* types I-III are usually *agr* dysfunctional and commonly isolated from the healthcare environment (306, 310). In South Korean hospitals, *agr* dysfunction was significantly higher among ST239-MRSA-III and ST5-MRSA-II than in ST72-MRSA-IV (311).

A study comparing the *agr* function of isolates obtained from both blood and nasal cultures showed that in the majority of cases, there was no difference in the *agr* function between the colonising and invasive isolates (321). In the same study they showed that development of bacteraemia was not related to whether isolates were *agr* functional or dysfunctional.

Although *S. aureus* strains with defective *agr* are attenuated, they are still transmissible, able to colonise the nose (313), lead to bacteraemia (321), and even result in poorer clinical outcomes than *agr* functional strains in bacteraemic patients (320). This chapter aimed to determine the prevalence of *agr* dysfunction among invasive and non-invasive *S. aureus*

isolates in Tygerberg Hospital and to describe the correlation between the *agr* dysfunction and the genetic background.

5.2 Methods

The study design, patient population, included clinical isolates, DNA extraction and isolate genotyping methods are described in chapter 3.

5.2.1 Detection of delta-haemolysin

The *agr* functionality was determined by evaluating the level of delta-haemolysin production as it is encoded by RNAIII which is considered to be the *agr* locus activator (306). One bead of each stored isolate was inoculated into 10 ml brain heart infusion broth (BHI) and incubated at 37°C overnight. This was then subcultured onto horse blood agar (BA) plates (National Health Laboratory Service Media Laboratory, South Africa) and incubated overnight at 37°C; and a single colony of this subculture was then used for *agr* function assessment. Haemolysin activity was analysed by cross-streaking each isolate against RN4220 (kindly provided by the Network of antimicrobial resistance in *Staphylococcus aureus* (NARSA)) which is positive for only beta-haemolysin on tryptose blood agar base (Oxoid LTD) with 5% sheep blood (SBA). These agar plates were incubated at 37°C overnight and the level of delta-haemolysin production was measured by the enhanced haemolysis in the overlapping area of this haemolysin with the beta-haemolysin zone produced by RN4220 (Figure 20) (318).

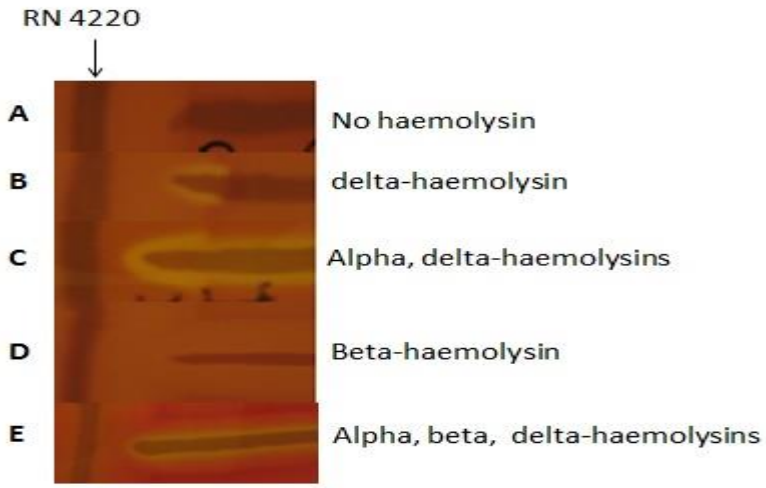

Figure 20 : Typical results for the assessment of agr functionality by measuring delta-haemolysin production on sheep blood agar.

RN4220 producing only beta-haemolysin is streaked vertically. The tested clinical isolates (A, B, C, D and E) are streaked horizontally.

5.2.2 *agr* typing

Multiplex PCR with specific *agr* primers (Table 33) were used to amplify the *agr* alleles of all blood and nasal isolates as previously described (366). Briefly, the PCR mixture consisted of 12.5 µl of Go Taq® Colorless Master Mix (Promega), 5 mM of the primers, 1 µl of the crude DNA, and 2.75 mM of additional $MgCl_2$. Cycling parameters included an initial denaturation step at 95˚C for 6 minutes, followed by 30 cycles of 95˚C for 0.45 s, 58˚C for 0.45 s and 72˚C for one minute. Following a final extension at 72˚C for 10 minutes, the PCR products were resolved on a 3.5% agarose gel. Nontypeable isolates were repeated as uniplex PCRs.

Table 33 : Primers used for *agr* locus typing

Primer description	Primer sequence	Band size/bp	Primer concentration
Agr-R	ATGCACATGGTGCACATGC	pan reverse primer	5 mM
Agr-1F	GTCACAAGTACTATAAGCTGCGAT	439	
Agr-2F	TATTACTAATTGAAAAGTGCCATAGC	572	
Agr-3F	GTAATGTAATAGCTTGTATAATAATACCCAG	321	
Agr-4F	CGATAATGCCGTAATACCCG	657	

5.2.3 Shifting of *agr* function

To determine *agr* function shifting between similar isolates obtained from the same patient, 96 patients who were colonised and infected with the same strain were included in this analysis.

5.2.4 Definitions

Isolates were classified as *agr* dysfunctional when they showed complete absence of delta-haemolysin production as indicated by the absence of enhanced haemolysis in proximity to the beta-haemolysin of RN4220 (Figure 20; sample A, D). Functional *agr* isolates showed enhanced haemolysis close to the beta-haemolysin zone due to delta-haemolysin production (Figure 20, samples B, C, E).

5.2.5 Statistical analysis

The association of *agr* dysfunction with methicillin resistance was assessed by the Chi-square test using Statistica version 9 (StatSoft, Tulsa, USA). A p value of less than or equal to 0.05 was considered significant.

5.3 Results

5.3.1 *agr* group

The *agr* gene was detected in 204 of 208 included isolates. *agr* could not be amplified in 4 strains and these were reported as nontypeable. The prevalence of *agr* groups was as follows: *agr* type I, 67.3% (n = 140); *agr* type II, 21.6% (n = 45); *agr* type III, 9.1% (n = 19). *agr* type IV was not identified during the study period.

5.3.2 Association between *agr* types and genetic background of included blood and nasal isolates

To investigate the association of identified *agr* types (I-III) with specific clones and genetic backgrounds, isolates represented by selected isolates for MLST analysis were included (n = 156). As shown in Table 34 all isolates belonged to CC8, CC22, CC45 and CC97 contain *agr* type I. Isolates belonging to CC12, CC15 and CC5 (except ST6) carried *agr* type II. Isolates belonging to CC30 carried *agr* III.

Table 34 : Association of identified *agr* types with isolates genetic background

***agr* types (N)**	**STs**	**MLST-CC**	**No.**	**Clone**
agr I (107)	1	1	2	ST1-MSSA
	6	5	7	ST6-MSSA
	8	8	10	ST8-MSSA (7) and ST8-MRSA-V (1)
	22	22	7	ST22-MSSA (5) and ST22-MRSA-IV (2)
	45	45	18	ST45-MSSA
	97	97	7	ST97-MSSA
	239	8	40	ST239-MRSA-III (9) and ST239-MRSA-*ccrC*/class A *mec* (31)
	612	8	16	ST612-MRSA-IV (14) and ST612-MRSA-NT (2)
agr II (38)	5	5	12	ST5-MRSA-*ccrC, ccrA1B1*/class B *mec* (10) and ST5-MSSA (2)
	12	12	2	ST12-MSSA
	461	5	5	ST461-MRSA- *ccrC, ccrA1B1*/class B *mec*
	2122	5	8	ST2122-MSSA
	2126	15	11	ST2126-MSSA
agr III (10)	1	1	2	ST1-MSSA
	36	30	4	ST36-MRSA-II
	1865	30	4	ST1865-MSSA
agr NT (1)	2126	15	1	ST2126-MSSA

Of the 208 isolates, 61 (29.3%) were *agr* dysfunctional. The prevalence of *agr* dysfunction was significantly higher in MRSA isolates (n = 50/90; 55.5%) compared to MSSA isolates (n = 11/118; 9.3%) (P = < 0.0001) as shown in Table 35.

Table 35 : Prevalence of *agr* dysfunctional isolates among MRSA and MSSA isolates

***agr* function**	**MRSA (%)**	**MSSA (%)**	***P* value**	**Total (%)**
Dysfunctional	50 (55.6)	11 (9.3)	< 0.0001	61 (29.3)
Functional	40 (44.4)	107 (90.7)	< 0.0001	147 (70.7)
Total	90 (100)	118 (100)	-	208 (100)

5.3.3 MRSA genotype and *agr* function

Of the 90 MRSA isolates, 68 belonged to *agr* group I, 15 to *agr* group II, 5 to *agr* group III, and 2 were untypeable. All *agr* group II isolates were *agr* functional. All the untypeable SCC*mec* types (*ccr*A1B1, *ccr*C/Class B *mec* (n = 15)) and SCC*mec* type IV (n = 21) were identified as *agr* functional isolates, while all isolates with SCC*mec* type III (n = 10) and the untypeable *ccr*C/Class A *mec* (n= 31) were identified as *agr* dysfunctional. Of the 5 isolates with SCC*mec* II, 4 were *agr* dysfunctional. MRSA STs 5, 8, 22, 461, and 612 were identified only as *agr* functional. In contrast, all STs 36 and 239 were found to be *agr* dysfunctional. All MRSA isolates belonging to *spa*-CC002 (n = 15) and 022 (n = 5) were *agr* functional. The majority (81%) of *spa*-CC64 was *agr* functional and *spa*-CC021 (93.87%) was *agr* dysfunctional (

Table 36). It is interesting to note that, among CC8 MRSA isolates, all isolates belonging to ST 239 were *agr* dysfunctional, while all the isolates belonging to ST8 and ST612 were *agr* functional.

5.3.4 MSSA genotype and *agr* function

The majority of MSSA isolates were identified as belonging to *agr* group I (n = 72) followed by *agr* group II (n = 30), with only 14 of the MSSAs belonging to *agr* group III. Eleven of the MSSA isolates showed *agr* dysfunction, with the distribution of *agr* groups, sequence types and *spa*-types shown in

Table 36.

The association of *agr* dysfunction with specific lineages was reported in ST36-MRSA-II, ST239-MRSA-III, and ST239-MRSA-*ccr*C/Class A *mec*. While other lineages: ST5-MRSA-*ccr*AB1/Class B *mec*, ST8-MRSA-V, ST22-MRSA-IV, ST461-MRSA-*ccr*AB1/Class B *mec* and ST612-MRSA-IV showed positive results for delta-haemolysin production.

To determine the shifting of *agr* functionality within the same strains from nose and blood isolates of a patient, all patients infected and colonised with the same strain were included in this analysis. Of the 164 included patients, 96 were infected and colonised with the same strains. Of these, 67 showed *agr* functionality in their blood and nasal isolates, while in 28, both isolates were *agr* dysfunctional. In one patient, the same strain was *agr* dysfunctional in the nose and functional in the blood.

Table 36 : Genotypic characteristics of *agr* functional/dysfunctional *S. aureus* isolates

Category		Genotype	No of isolates	
			***agr* dysfunctional**	***agr* functional**
Methicillin susceptibility		**MRSA (n = 90)**	**50**	**40**
***agr* genotype**		I	45	23
		II	0	15
		III	4	1
		NT	1	1
SCC*mec* type		*ccrA1B1*, *ccrC*/ Class B *mec*	0	15
		II	4	1
		III	10	0
		IV	0	21
		V	1	1
		ccrC/Class A *mec*	31	0
		NT	4	2
MLST		ST5	0	10
		ST461	0	5
	CC8	ST8	0	1
		ST239	41	0
		ST612	0	16
	CC22	ST22	0	2
	CC36	ST36	4	0
	Non applicable (NA)	NA	5	6
***spa*-CCs**		CC002	0	15
		CC021	46	3
		CC022	0	5
		CC064	4	17
Methicillin susceptibility		**MSSA (n = 118)**	**11**	**107**
***agr* genotype**		I	4	68
		II	2	28
		III	4	10
		NT	1	1
MLST	**CC1**	ST1	0	4
	CC5	ST5	0	2
		ST6	0	7
		ST2122	0	8
	CC8	ST8	0	9
	CC12	ST12	0	2
	CC15	ST2126	2	10

	CC22	ST22	0	5
	CC30	ST1860	1	3
	C45	ST45	1	17
	CC97	ST97	1	6
	NA	NA	6	34

Table 37 Cont. Genotypic characteristics of *agr* functional/dysfunctional *S. aureus*

***spa*-CCs**	CC002	0	12
	CC073/015	1	15
	CC021	2	6
***spa*-CCs**	CC022	0	8
	CC084	3	10
	CC064	0	20
	CC160	0	6
	CC174	1	5
	CC267	1	5
	No Founder	0	4
	Singletons	2	12
	NT	1	2
	Excluded	0	2

5.3.5 Association between *agr* group and virulence factors

Distribution of five virulence factor genes was found to be significantly different between the 3 identified *agr* groups (table 34).

Table 38 : Selected virulence factors that have significant differences between one of the *agr* groups compared to others

Virulence factor genes	***agr* I (n = 12)**	***agr* II (n = 6)**	***agr* III (n = 3)**	***P* value**
cna	7 (58.33%)	1 (16.67%)	3 (100%)	0.05 (*agr* II compared to *agr* III)
sak	12 (100%)	2 (33.33%)	3 (100%)	0.002 (*agr* II compared to *agr* I and III)
scn	12 (100%)	3 (50%)	3 (100%)	0.0125 (*agr* II compared to *agr* I and III)
sea	11 (91.67%)	2 (33.33%)	3 (100%)	0.0135 (*agr* II compared to *agr* III)
icaA	12 (100%)	6 (100%)	1 (33.33%)	0.0013 (*agr* III compared to *agr* I and *agr* II)

5.4 Discussion

5.4.1 Association with *agr* dysfunction, SCC*mec* type and genetic lineage

This study showed a significant association of *agr* dysfunction with MRSA compared to MSSA. A similar finding was reported in a South Korean hospital where 302 of 407 blood MRSA isolates were *agr* dysfunctional compared to 29 of 232 MSSA blood isolates (311). In the present study the dominant SCC*mec* type was SCC*mec* type II (90.7%). However, SCC*mec* element acquisition did not attenuate *agr* function in *S. aureus* clinical isolates obtained from hospitals in New York city (306), although this study did not categorise the SCC*mec* types of the MRSA isolates.

A previous study has reported an association between SCC*mec* types II and III, but not with SCC*mec* types I and IV, and *agr* dysfunction (311). Although our study also suggests an association between *agr* dysfunction and SCC*mec* types II, III as well as the untypeable element (*ccr*C/Class A *mec*), it is not clear whether this is related to the SCC*mec* element itself, or the genetic background of these isolates. SCC*mec* type II was found exclusively in isolates belonging to ST36, and both SCC*mec* type III and the untypeable SCC*mec* were found in ST239 (CC8). It is interesting to note that, among CC8 MRSA isolates, all isolates belonging to ST239 were *agr* dysfunctional, while all isolates belonging to ST8 and ST612 were *agr* functional.

All MRSA isolates carrying SCC*mec* type IV and the possible novel variant SCC*mec* type I (*ccr*A1B1, *ccr*C/Class B *mec*) were *agr* functional. Again, the majority of SCC*mec* IV elements (16/18, 88.8%) were found in isolates belonging to ST612, and the possible

SCC*mec* type I novel variant was found in isolates belonging to ST5 and ST461. Both of these are part of CC5.

Another study also reported a higher rate of *agr* dysfunction among MRSA harbouring SCC*mec* types I-III (43%) than SCC*mec* type IV and V isolates (3%) obtained from patients with *S. aureus* bacteraemia. However, only one isolate with SCC*mec* type I was included in this study, therefore the small number of samples included may not be representative of *agr* functionality in MRSA harbouring SCC*mec* type I (310). The difference in association of *agr* dysfunction with MRSA may be attributed to variable SCC*mec* element types amongst the included isolates, or due to the different lineages associated with methicillin resistance.

A previous study reported that isolates with impaired *agr* function are commonly identified in hospitals or previously hospitalised individuals (313). Paulander *et al*., suggested that the reduced *agr* functionality in *S. aureus* may allow isolates to adapt and survive in intensive antibiotic environments (317).

In accordance with a previous study that reported very infrequent shifting of *agr* functionality within-host (321), our study showed a similar finding. Of the 96 patients with identical nasal and blood culture isolates (28 *agr* dysfunctional and 68 *agr* functional), only one of these patients was found to carry an *agr* dysfunctional isolate in the nose and shifted into *agr* functional isolate in the bloodstream.

S. aureus virulence is regulated by the *agr* locus, but it has been suggested that *agr* function is not essential for colonising isolates to cause subsequent bacteraemia (321). This is supported by this study finding where, 27 of the 28 *agr* dysfunctional colonised isolates were identified from blood culture of the same patients.

5.4.2 Association of virulence factors with *agr* type

The presence of *sea* in *agr* type II isolates was significantly less than its prevalence in *agr* type I and III. This is similar to findings by Collery *et al*., that *agr* type II and type IV isolates were negative for *sea* genes (218, 408). There was no association between specific IEC types with any of the *agr* types (219). Our results showed that the IEC genes *scn* and *sak* were less prevalent in *agr* II isolates. In accordance with this study finding, Diep *et al*., found that *agr* type II isolates lacked the *cna* gene(218).

5.4.3 Association between *agr* type and genetic background

In accordance to previous study there was an association between CC8, CC22, CC45 and *agr* I, CC5, CC12, and CC15 and *agr* II and CC30 with *agr* III (389).

5.5 Conclusion

In conclusion, the prevalence of *agr* dysfunctional *S. aureus* isolates was significantly higher in MRSA than MSSA. In MRSA isolates, *agr* dysfunction was more commonly associated with isolates carrying SCC*mec* types II, III and *ccr*C/Class A *mec*. In this study, the association of *agr* dysfunction with ST36-MRSA-II, ST239-MRSA-III ST239-MRSA-*ccr*C/Class A *mec* was also reported. Furthermore, our study showed that *agr* function shifting in the same host was not common. Further studies are required to assess the vancomycin susceptibility of MRSA *agr* dysfunctional isolates, and to evaluate the clinical outcome of patients infected with these strains as previous studies reported an association between increased mortality and *agr* dysfunction.

CHAPTER 6 GENERAL DISCUSSION AND CONCLUSION

A hundred and sixty four patients with SAB were included in this study; and a nasal swab for *S. aureus* culture was obtained from all patients. Using PFGE, the second chapter aimed to describe the correlation between the blood and nasal isolates of 162 patients (two patients were excluded from this analysis). 79% (128/162) of the patients had nasal colonisation with *S. aureus,* and there was a significant correlation between the blood and nasal isolates in colonised patients. Interestingly, MRSA nasal colonisation was significantly associated with endogenous bacteraemia when compared to MSSA. Endogenous bacteraemia was more common than exogenous in adults and neonates.

In the third chapter our study focused on genotyping the invasive and colonising isolates. From 164 patients, 208 unique non-repeated isolates were obtained and characterised using PFGE, *spa* typing, MLST, *agr* typing and SCC*mec* typing. Based on the PFGE results, the isolates were categorised into 31 clones (4 major, 4 intermediate and 23 minor). Three *agr* groups were identified (I, II and III) while *agr* type IV was not detected. A range of *SCCmec* types (II, II, IV and V) were found in the MRSA isolates including two 2 untypeable SCC*mec* types. Further analysis of these untypeable SCC*mec* elements suggested two novel structures; *ccr*A1B1, *ccr*C/Class B *mec* (type I variant) and *ccr*C/Class A *mec* (novel SCC*mec* type in *S. aureus*). Interestingly, the potential novel SCC*mec* type was the predominant type (34.4%; n=31). Representative isolates from all major, intermediate and 11 minor PFGE clones were analysed further by MLST. The predominant clone was ST239-MRSA-*ccr*C/Class A *mec*. ST612-MRSA-IV, previously described in South Africa and Australia, was the second predominant clone and the first time one of these isolates was shown to be PVL positive. ST461-MRSA-*ccr*A1B1, *ccr*C/Class B *mec* was identified as MRSA for the first time.

Several findings support the local emergence and evolution of new MRSA clones: different SCC*mec* elements within one PFGE clone, MSSA and MRSA strains grouped in one PFGE clone, the same ST identified as MSSA and MRSA; and the finding of untypeable SCC*mec* elements which may represent a new SCC*mec* type (*ccr*C/ Class A *mec*) and a new type I variant (*ccr*A1B1, *ccr*C/ Class B *mec*). All but one of the 18 PVL positive isolates were MSSA.

Chapter four investigated the virulence factor gene distribution among different genetic lineages. Various groups of virulence factors were selected for screening: adhesins, superantigenic toxins, immune avoidance genes and hemolysins. In South Africa, there is limited data related to the prevalence of virulence factors among local *S. aureus* clones. Selected representative isolates (those selected for MLST analysis) were chosen for virulence factor profile screening. Our results showed the presence of the *nuc, coa, fnbA, fnbB, clfA* and *hld* genes in all the selected isolates. The IEC gene cluster was not associated with specific genetic lineages. However, there was an association between specific egc genes types and CC5, CC22, CC30 and CC45. As has been reported in previous studies, this study confirmed that the SAg genes were absent in CC15 and CC97. The *icaA* gene was amplified in all CCs except isolates belonging to CC30, while the *sei* gene was associated with CC8. Further analysis revealed that MSSA was associated with the presence of PVL and MRSA was associated to the *sei* genes.

The accessory gene regulator (*agr*) is the main quorum sensing system that controls virulence factor expression in *S. aureus*. Activation of *agr* function up regulates the expression of cell wall associated virulence factors and down regulates extracellular toxin production. Isolates with impaired *agr* function have been found to be less susceptible to vancomycin, associated with persistent SAB and in some studies have been associated with higher mortality rates.

The fifth chapter studied the prevalence of *agr* dysfunctional isolates among blood and nasal isolates as well as the association of *agr* dysfunction with genetic background. The prevalence of *agr* dysfunctional isolates was significantly higher among MRSA (50/90) isolates than compared to MSSA (11/118) (P=<0.0001). A similar result was reported in South Korean Hospitals where 302 of 407 MRSA blood isolates were *agr* dysfunctional. In MRSA, our study showed an association between *agr* dysfunction and SCC*mec* types II, III, and the *ccr*C/class A *mec*. On the other hand, all strains harbouring SCC*mec* type IV or *ccr*A1B1, *ccr*C/Class B *mec* were *agr* functional. Analysing the association between *agr* dysfunction and genetic background showed all ST239-MRSA-*ccr*C/Class A *mec* isolates to be *agr* dysfunctional. This was the predominant clone harbouring the new SCC*mec* type, and it is possible that acquisition of this new SCC*mec* type (*ccr*C/Class A *mec*) resulted in *agr* dysfunction. Since the isolates were obtained from the blood and via nasal carriage we aimed to determine the *agr* function shift and the importance or role of *agr* function in invasion. We found that *agr* function shift in the same host was a rare event and that *agr* function was not essential for invasion.

6.1 General conclusion

In South Africa, this is the first study describing the correlation between colonising nasal and invasive blood *S. aureus* isolates. The prevalence of virulence factor genes within *S. aureus* and their association with specific clones is also not well described in South Africa.

The significant association between blood and nasal isolates indicates that nasal carriage was a possible source of subsequent SAB. However, in all patients (colonised and non-colonised) nasal carriage was not the main source of SAB which highlights the impact of sources other than the colonised nostrils in developing SAB. MRSA bacteraemia was more commonly associated with MRSA nasal colonisation in patients admitted to adult surgical ICU and neonate units. From these study findings it is possible to conclude: (1) in colonised patients, nasal carriage is possible source of subsequent SAB; (2) *S. aureus* bacteraemia was acquired from sources other than colonised nostrils; and (3) endogenous bacteraemia was more commonly associated with MRSA nasal colonisation especially in adult ICU and neonates units.

Molecular characterisation of 208 unique non repeated isolates showed a predominance of ST239, ST612 and ST5. ST239 was identified with SCC*mec* type III and the untypeable *ccr*C/Class A *mec* elements. According to our knowledge ST612-MRSA-IV has been identified only in South Africa and Australia and this study reported one isolate as being PVL positive which may increase its virulence. It is known that ST5 has acquired different SCC*mec* elements many times in variable geographic regions. Our results showed ST5 carried an untypeable SCC*mec* *ccr*A1B1, *ccr*C/Class B *mec*. The emergence of ST461-MRSA-IV in South Africa was reported in this study as well as novel MSSA STs (ST2122 and ST 2126). PVL was more prevalent among MSSA isolates than MRSA isolates.

In summary, the genotypic characterisation showed: (1) predominance of ST239-MRSA-*ccr*C/Class A *mec*, (2) local emergence of new MRSA clones, (3) local acquisition of new SCC*mec* elements and; (4) the ability of the local clone ST612-MRSA-IV to acquire the PVL toxin.

Twenty one selected representative *S. aureus* (MRSA and MSSA) isolates were screened for the presence of 38 virulence factor genes. Limited studies in South Africa have investigated the presence of virulence factor genes other than PVL. The key findings in this section of the work included: (1) the *nuc, coa, clfA, clfB* and *hld* virulence factor genes were present in all included isolates, (2) the presence of the IEC was not restricted to any specific clones, while egc was associated with CC5, CC22, CC30 and CC45, (3) both CC15 and CC97 were negative for SAg genes, (4) the biofilm associated gene, *icaA* was highly prevalent (90.4%) among the included isolates and; (5) there genes of the *pvl* gene was associated with MSSA isolates, which may be important with respect to the evolution of PVL positive MRSA clones.

Impaired *agr* functionality in *S. aureus* has been associated with reduced susceptibility to vancomycin, persistent bacteraemia and sometimes with a higher mortality rate in the literature. Investigating the prevalence of *agr* dysfunctional isolates among both blood and nasal isolates led to the following conclusions : (1) *agr* dysfunction was significantly associated with MRSA, (2) the presence of SCC*mec* elements type II, III or *ccr*C/Class A *mec* was associated with *agr* dysfunction, while the presence of SCC*mec* type *ccr*A1B1, *ccr*C/Class B *mec* or SCC*mec* type IV was associated with *agr* function, (3) Although *agr* dysfunction theoretically reduces *S. aureus* virulence, a functional *agr* gene was not essential for colonising isolates to cause SAB and; (5) finally, shifting of *agr* function in the same host

was uncommon.

6.2 Limitations of the study

Von Eiff and colleagues in 2001 estimated the correlation between the nasal and blood *S. aureus* isolates by two approaches: (1) they swabbed the nostrils of any patient immediately after positive *S. aureus* blood culture was identified and; (2) in the another approach, nasal swabs were obtained from patients prospectively and followed up for 5 years (1994-1995). The correlation between nasal and blood isolates was 82.2% and 85.7% in the first and second approaches respectively. In our study the correlation of blood and nasal isolates was not determined by active surveillance which may raise the possibility of colonisation occurring after the development of SAB. From the limited clinical data available it was not possible to properly classify the isolates as CA-MRSA or HA-MRSA. The *S. aureus* isolates in this study only represented blood and nasal isolates as other clinical infection and colonisation sites were not included. The virulence factor profile screening was performed for selected isolates, and since limited clinical data was available it was not possible to link the presence of specific virulence factor genes to presentation or outcome.

6.3 Future work

Further studies and research questions have been raised by this study, including:: (1) will a "search and destroy" policy prove to be a feasible prevention control measure in colonised patients? (2) The two common untypeable SCC*mec* elements need further analysis using additional PCR typing methods as well as DNA sequence analysis; (3) the association between the virulence factors and the clinical presentation and outcome would need to be investigated using larger numbers of isolates in a prospectively enrolled cohort; (4) the reported association between *agr* dysfunctional isolates and reduced susceptibility to vancomycin as well as clinical outcome would likewise need to be investigated prospectively; and (5) further analysis of selected isolates using whole genome sequencing and proteomic analysis may shed further light on the consequences of *agr* dysfunction.

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Appendix A1: Final Ethics approval

UNIVERSITEIT·STELLENBOSCH·UNIVERSITY
jou kennisvennoot • your knowledge partner

09 December 2009 **MAILED**

Mr KJK Karayem
Department of Medical Microbiology
9th Floor, Tygerberg Hospital
Francie van zijl drive
Tygerberg
7505

Dear Mr Karayem

"A phenotypic and genotypic characterization of strain types, virulence factors and agr groups of colonizing S.aureus strains associated with associated with bloodstream infections."

ETHICS REFERENCE NO: N09/10/266

RE : FINAL APPROVAL

At a meeting of the Health Research Ethics Committee that was held on 4 November 2009, the above project was approved on condition that further information is submitted.

This information was supplied and the project was finally approved on 9 December 2009 for a period of one year from this date. This project is therefore now registered and you can proceed with the work.

Please quote the above-mentioned project number in ALL future correspondence.

Please note that a progress report (obtainable on the website of our Division: www.sun.ac.za/rds should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly and subjected to an external audit.
Translations of the consent document in the languages applicable to the study participants should be submitted.

Federal Wide Assurance Number: 00001372
Institutional Review Board (IRB) Number: IRB0005239
The Health Research Ethics Committee complies with the SA National Health Act No.61 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

Approval Date: 9 December 2009 Expiry Date: 9 December 2010

09 December 2009 14:28 Page 1 of 2

Fakulteit Gesondheidswetenskappe · Faculty of Health Sciences

Verbind tot Optimale Gesondheid · Committed to Optimal Health
Afdeling Navorsingsontwikkeling en -steun · Division of Research Development and Support
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Yours faithfully

MR FRANKLIN WEBER
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Appendix A2: Consent form used when the included patient was ≥ 18 years

Participant information leaflet and consent form

TITLE OF THE RESEARCH PROJECT:

A phenotypic and genotypic characterization of strain types, virulence factors and *agr* groups of colonizing *S. aureus* strains associated with bloodstream infections

PRINCIPAL INVESTIGATOR: Karayem. J. Karayem

ADDRESS: Division of Medical Microbiology, Department of Pathology, PO Box 19063, Tygerberg Hospital, 7505

CONTACT NUMBER: 021 9384032

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part.

This study has been approved by the **Committee for Human Research at Stellenbosch University** and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice

and the Medical Research Council (MRC) Ethical Guidelines for Research.

What is this research study all about?

- The study will be conducted in Tygerberg Hospital, on patients that suffer from serious infections caused by a bacterium called Staphylococcus aureus.
- All of us carry this organism on our skin and about 50% of healthy people in their nose, and usually it does not cause us any harm. Sometimes it may lead to skin infections, like boils, which are not very serious. We are interested why some patients suffer from more serious infections caused by this organism.
- No extra examinations or investigations (for example, no extra blood tests or any other test like X-rays) will be done on you other than those that your doctor request routinely. The only extra procedures of this study is the collection of nasal swabs of your nose to find out the association between nasal and bloodstream isolates, more information about your condition, and some extra tests will be done in the laboratory on the bacteria we isolate from your nose and/or blood that your doctor takes to diagnose your disease.
- We want to include all the patients in this hospital that suffer from this type of infection.
- Your involvement in this study will give the doctors more information about the possible reasons for your infection, but it will not change your treatment in any way.

Why have you been invited to participate?

- You have been invited to participate in this study because the bacterium Staphylococcus aureus had been isolated from blood that your doctor took to diagnose your infection.

What will your responsibilities be?

- You have no responsibilities, but the research staff may ask you questions about previous infections and perhaps whether you have recently had any antibiotic treatment for other infections. We would be glad if you can answer such questions as best you can.

Will you benefit from taking part in this research?

- By looking at the data we collect about your disease, we may be able to alert your doctor to certain risk factors you may have to get better less quickly or get this disease again. The information will also be used to help prevent complications in patients who get the same disease in future and it will provide clinically useful information towards infection control and prevention of invasive S. aureus diseases.

Are there in risks involved in your taking part in this research?

- As no extra investigations will be done on you, and as the study will not influence the treatment your doctor gives you in any way, this study has no risks for your health.

If you do not agree to take part, what alternatives do you have?

- Should you decide that you do not want to be included in this study, your treatment and care will continue exactly as before.

Who will have access to your medical records?

If you are part of this study, your medical records may be examined for relevant information by the staff participating in this study. These people are all registered health care professionals and they include a trained nursing sister, medical doctors and medical scientists all working for this hospital or the National Health Laboratory Services. The information about all the patients included in this study will be analysed in such a way that no personal

information about you will be published or made known in any way.

What will happen in the unlikely event of some form injury occurring as a direct result of your taking part in this research study?

There is no risk of injury as a direct result of this study, as no additional examinations, investigations or treatment forms part of this study.

Will you be paid to take part in this study and are there any costs involved?

No, you will not be paid to take part in the study and you do not need to come back to the hospital for any follow up appointments because of your involvement in this study. There will be no costs involved for you, if you do take p

Is there anything else that you should know or do?

- You can contact Prof E Wasserman Tel: 021 9384032 if you have any further queries or encounter any problems.
- You can contact the Committee for Human Research at 021-938 9207 if you have any concerns or complaints that have not been adequately addressed by your study doctor.
- You will receive a copy of this information and consent form for your own records.

Declaration by participant

By signing below, I ……..……. agree to take part in a genetic research study entitled **A phenotypic and genotypic characterization of strain types, virulence factors and agr groups of colonizing S. aureus strains associated with bloodstream infections.**

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.

- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.
- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (place) .....…… on (date) ……...…….. 2010.

Signature of participant **Signature of witness**

Declaration by investigator

I (name) ……..…… declare that:

- I explained the information in this document to ……
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did/did not use a translator. (If a translator is used then the translator must sign the declaration below.

Signed at (place) .....….....…… on (date) ……...…….. 2010.

..... ……..

Signature of investigator **Signature of witness**

Declaration by translator

I (name) ……..…… declare that:

- I assisted the investigator (name) …… to explain the information in this document to (name of participant) ……..… using the language medium of Afrikaans/Xhosa.
- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (place) ..... on (date) …….. 2010.

…… ……

Signature of translator **Signature of witness**

Appendix A3: Consent form used when the included patient was < 18 years

Participant information leaflet and assent form

TITLE OF THE RESEARCH PROJECT:

A phenotypic and genotypic characterization of strain types, virulence factors and *agr* groups of colonizing *S. aureus* strains associated with bloodstream infection

RESEARCHERS NAME(S): Karayem J. Karayem

ADDRESS: Division of Medical Microbiology, Department of Pathology,

PO Box 19063, Tygerberg, 7505

CONTACT NUMBER: 021 9384032

What is research?

Research is something we do to find new knowledge about the way things (and people) work. We use research projects or studies to help us find out more about disease or illness. Research also helps us to find better ways of helping, or treating children who are sick.

What is this research project all about?

The study will be about germs called *Staphylococcus aureus*. It lives on/in our skin and nose.

Sometimes it reaches the blood and causes illness. In this study we want to know how this germ can reach the blood, and why it sometimes only stays as normal living germs in others and doesn't cause a serious disease.

The Staphylococcus aureus germ was found in your blood, and caused you to become ill. We would like to know if this germ also lives in your nose. Therefore, we would like to take a simple swab of the inside of your nostrils to see if this germ is growing there. It is a simple procedure and it will not hurt at all. It will also not be uncomfortable.

No other examinations and investigations will be done on you other than those that your doctor requested routinely.

Why have I been invited to take part in this research project?

You have been invited to take part in this study because the germ Staphylococcus aureus had been found in your blood that was taken by your doctor.

Who is doing the research?

I am a PhD student at the Division of Medical Microbiology, Department of Pathology, Stellenbosch University. I am doing this study at Tygerberg Hospital to get PhD degree.

What will happen to me in this study?

The Staphylococcus aureus germ was found in your blood sample that had been taken by your doctor routinely. We need more information about your condition. We would glad if you answer some questions that will be asked to you. We will take swabs of your nose to know if you have this germ in your nose and to do more laboratory tests on the germs that we found in your blood and nose.

Can anything bad happen to me?

This study doesn't need any more investigations and does not change your treatment that your doctor gives you in any way. This study cannot harm you or cause you pain.

Can anything good happen to me?

From the information that we collect about your condition, and the extra laboratory tests that we will do on the Staphylococcus aureus germ that we found in your blood and/or nasal swabs, we will provide your doctor with advice which will help to cure you and to avoid complications or that you may get the same disease again.

We will also use this information to help other patients who have the same illness and to provide the nurses and doctors working in preventing infection with useful information.

Will anyone know I am in the study?

If you agree to take apart in this study, all your information will only be examined by the staff working in this study. All of them are registered health care professionals. Your name will not be connected to our findings and no personal information about you will be published or made known in any way.

Who can I talk to about the study?

- **You can contact Prof E Wasserman at** tel 0219384032**…… if you have any further queries or encounter any problems.**
- **You can contact the Committee for Human Research at** 021-938 9207 **if you have any concerns or complaints that have not been adequately addressed by your study doctor.**

You will receive a copy of this information and consent form for your own records.

What if I do not want to do this?

Only you have the right to decide whether you want to take part in this study or not, even if your parents agree to participate. As a participant you can leave the study at any time, while the study hasn't finished. You will not get any trouble and your treatment and care will continue exactly as before.

Do you understand this research study and are you willing to take part in it?

YES	NO

Has the researcher answered all your questions?

YES	NO

Do you understand that you can pull out of the study at any time?

YES	NO

_____ _____

Signature of Child Date

Appendix A4: Consent form used by parent or guardians when the included patient ≤17 years

Participant information leaflet and consent form for use by parents/legal guardians

TITLE OF THE RESEARCH PROJECT:

A phenotypic and genotypic characterization of strain types, virulence factors and agr groups of colonizing *S. aureus* strains associated with bloodstream infection

PRINCIPAL INVESTIGATOR: Karayem J. Karayem

ADDRESS: Division of Medical Microbiology, Department of Pathology

PO Box 19063, Tygerberg, 7505

CONTACT NUMBER: 021 9384032

Your child (or ward, if applicable) is being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how your child could be involved. Also, your child's participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you or your child negatively in any way whatsoever. You are also free to withdraw him/her from the study at any point, even if you do initially agree to let him/her take part.

This study has been approved by the **Health Research Ethics Committee at Stellenbosch University** and will be conducted according to the ethical guidelines and principles of the

international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

What is this research study all about?

The study will be conducted in Tygerberg Hospital, on patients that suffer from serious illnesses caused by a germ called *Staphylococcus aureus*.

All of us carry this germ on our skin and about 50% of healthy people in their nose, and usually it does not cause us any harm. Sometimes it may lead to skin diseases, like boils, which are not very serious. We are interested why some patients suffer from more serious illnesses caused by this organism.

No extra examinations or investigations (for example, no extra blood tests or any other test like X-rays) will be done on your child other than those that his doctor request routinely. The only extra procedures of this study is the collection of nasal swabs of your child nose to find out the association between nasal and bloodstream germs, more information about your child condition, and some extra tests will be done in the laboratory on the germs we isolate from your child nose and/or blood that his doctor takes to diagnose your child disease.

We want to include all the patients in this hospital that suffer from this type of infection.

Your child involvement in this study will give the doctors more information about the possible reasons for your child disease, but it will not change your child treatment in any way.

Why has your child been invited to participate?

Your child has been invited to participate in this study because the germ Staphylococcus aureus had been found in blood that your child doctor took to diagnose his illness.

What will your responsibilities be?

You and your child have no responsibilities, but the research staff may ask you questions about previous disease and perhaps whether your child has recently had any antibiotic treatment for other diseases. We would be glad if you can answer such questions as best you can.

Will your child benefit from taking part in this research?

By looking at the data we collect about your child disease, we may be able to alert his doctor to certain risk factors he may has to get better less quickly or get this disease again. The information will also be used to help prevent complications in patients who get the same disease in future and it will provide useful information to stop or reduce the disease that is caused by this germ.

Are there any risks involved in your child taking part in this research?

As no extra investigations will be done on your child, and as the study will not influence the treatment of your child doctor gives him in any way, this study has no risks for your child health.

If you do not agree to allow your child to take part, what alternatives does your child have?

Should you decide that you do not want your child to be included in this study, your child treatment and care will continue exactly as before.

Who will have access to your child's medical records?

If your child is part of this study, his medical records may be examined for relevant information by the staff participating in this study. These people are all registered health care professionals and they include a trained nursing sister, medical doctors and medical scientists

all working for this hospital or the National Health Laboratory Services. The information about all the patients included in this study will be analysed in such a way that no personal information about your child will be published or made known in any way.

What will happen in the unlikely event of your child getting injured in any way, as a direct result of taking part in this research study?

There is no risk of injury as a direct result of this study, as no additional examinations, investigations or treatment forms part of this study.

Will you or your child be paid to take part in this study and are there any costs involved?

You or your child will not be paid to take part in the study, but out-of-pocket expenses will be covered for each study visit. There will be no costs involved for you if your child does take part.

Is there anything else that you should know or do?

You should inform your family practitioner or usual doctor that your child is taking part in a research study. (Include if applicable)

You should also inform your medical insurance company that your child is participating in a research study (Include if applicable)

You can contact **Prof E. Wasserman** at tel **021 9384032. I**f you have any further queries or encounter any problems.

You can contact the **Health Research Ethics Committee** at **021-938 9207** if you have any concerns or complaints that have not been adequately addressed by your child's study doctor.

You will receive a copy of this information and consent form for your own records.

Declaration by parent/legal guardian

By signing below, I (name of parent/legal guardian) ……agree to allow my child (name of child) ……..… who is ……. years old, to take part in a research study entitled

A phenotypic and genotypic characterization of strain types, virulence factors and agr groups of colonizing S. aureus strains associated with bloodstream infection

I declare that:

- I have read or had read to me this information and consent form and that it is written in a language with which I am fluent and comfortable.
- If my child is older than 7 years, he/she must agree to take part in the study and his/her ASSENT must be recorded on this form.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to let my child take part.
- I may choose to withdraw my child from the study at any time and my child will not be penalised or prejudiced in any way.
- My child may be asked to leave the study before it has finished if the study doctor or researcher feels it is in my child's best interests, or if my child do not follow the study plan as agreed to.

Signed at (place) .....…..…. on (date) ……...……

Signature of parent/legal guardian **Signature of witness**

Declaration by investigator

I (name) ……..…… declare that:

- I explained the information in this document to ……
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understand all aspects of the research, as discussed above
- I did/did not use a interpreter (if a interpreter is used, then the interpreter must sign the declaration below).

Signed at (place) .....….....……on (date) ……...…….. 2005.

.....

Signature of investigator

Declaration by interpreter (Only complete if applicable)

I (name) ……..…… declare that:

- I assisted the investigator (name) ……..……. to explain the information in this document to (name of parent/legal guardian) ……..……... using the language medium of Afrikaans/Xhosa.
- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the parent/legal guardian fully understands the content of this informed consent document and has had all his/her questions satisfactorily answered.

Signed at (place) .....…….. on (date) ……...……

Signature of interpreter **Signature of witness**