Genotypic characterization of *Staphylococcus aureus* isolates causing bacteraemia in patients admitted to Tygerberg Hospital, Western Cape Province, South Africa

by

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DECLARATION

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the owner of the copyright thereof and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: March 2010

ABSTRAK

S. aureus veroorsaak ernstige infeksies in die hospitaalomgewing en in die gemeenskap. Wêreldwyd, neem metisillien-weerstandige *S. aureus* (MRSA) infeksies vinnig toe. Huidiglik by Tygerberg hospitaal is ongeveer 'n derde van *S. aureus* isolate MRSA. Hierdie is die eerste epidemiologiese studie by Tygerberg hospitaal wat prospektiewe kliniese data van pasiënte met *S. aureus* bakteremie saam met *spa* tipering en aantoning van die *mecA* en *pvl* gene in 'n multipleks PKR insluit. Klonale groepe (*spa*-CC) van MRSA en MSSA isolate is deur BURP analise verkry, en vergelyk met internasionaal belangrike klone. Die molekulêre epidemiologie van hospitaalverworwe (HA), gesondheidsorgverworwe (HCA) en gemeenskapsverworwe (CA) *S. aureus* bakteremie by hierdie hospitaal is ondersoek. Laastens, oorspronklike en daaropvolgende herhaal isolate is gekollekteer om moontlike organisme- faktore geassosieerd met persisterende en herhalende bakteremiese episodes te analiseer.

Ons het in totaal 113 *S. aureus* isolate van 104 pasiënte ondersoek (70% MSSA, 30% MRSA). Nege isolate (van 5 pasiënte) was herhaal isolate. Alle isolate was afkomstig vanaf bloedkulture wat gedurende die periode Maart 2008 tot Mei 2009 gekollekteer is. Fenotipiese en genotipiese aantoning van metisillien weerstandigheid het goed gekorreleer. Volgens die literatuur kan die meeste CA-MRSA isolate van HA-MRSA isolate onderskei word op grond van die teenwoordigheid van die PVL toksien. Geen CA-MRSA is egter in ons studie gevind nie, dus kon die assosiasie tussen HA-MRSA en CA-MRSA isolate nie ondersoek word nie. CA-MSSA was in 22% van alle MSSA geidentifiseer teenoor 0% CA-MRSA. PVL is in MSSA isolate gevind (22.7% van alle MSSA) maar glad nie in MRSA nie. Dit is opgemerk dat MRSA isolate hoofsaaklik in *spa* CC 701 en CC-012 kloongroepe voorkom, teenoor kloongroep CC-002 wat slegs MSSA isolate bevat het. Soortgelyk het HA-isolate wat die meerderheid van MRSA isolate verteenwoordig het ook in kloongroepe 1 & 2 gegroepeer.

Nege-en-veertig *spa* tipes is geïdentifiseer in 89.3% of alle isolate en 9.7% was nie-tipeerbaar. Vyf nuwe *spa* tipes is getoon. Ons het 'n diverse aantal *spa*-tipes geïdentifiseer wat met internasionale klone gekorreleer het. Die mees dominante *spa* tipe in ons omgewing was t037 (slegs in MRSA), gevolg deur t891. Volgens die literatuur word t037 met die Brasiliaanse/Hongaarse kloon geassosieer (SCC*mec* tipe III; ST 239). Ons bevindings, asook ander Suid Afrikaanse studies, dui aan dat t037 in

kliniese isolate vanaf talle provinsies in Suid-Afrika aangetoon is. Van belang is dat al die isolate van *spa* tipe t891 MSSA en PVL positief was.

Bakteremiese gevalle was hoofsaaklik geassosieer met kateter-sepsis, gevolg deur vel en sagteweefsel infeksies (SSTI). Slegs een persisterende bakteremiese geval was geïdentifiseer geassosieer met HA-SSTI. Herhalende bakteremiese episodes is in pasiënte op dialise vir kroniese nierversaking en in brandwonde pasiënte met intra-vaskulêre kateter infeksies aangetoon. Die lokale epidemiologie van *S. aureus* en die prevalensie koers van verskillende stamme is van belang. Hierdie inligting dra by tot kennis van die epidemiologie van stafilokokkale stamme wat in ons omgewing bakteremie veroorsaak. Hierdie insigte is nuttig vir optimale diagnostiese en terapeutiese riglyne. Die tegnieke wat ontwikkel is, kan gebruik word om uitbrake en herhalende infeksies te identifiseer.

ABSTRACT

S. aureus causes serious infections in the hospital and community settings. The rate of MRSA infections are rapidly increasing worldwide. Currently, at Tygerberg hospital, approximately a third of *S. aureus* isolates are MRSA. This was the first epidemiological study of *S. aureus* conducted at Tygerberg Hospital that included prospective clinical data on patients with *S. aureus* bacteraemia together with *spa* typing of strains and the detection of the *mecA* and *pvl* genes in a multiplex PCR. Clonal cluster groups of *S. aureus* isolates were obtained by BURP analysis and compared to international important clones. The molecular epidemiology of hospital acquired (HA), health-care associated (HCA) and community acquired (CA) *S. aureus* bacteraemic strains at this hospital was examined. Lastly, repeat isolates of patients were collected to analyse any possible organism-related factors associated with persistent and recurrent bacteraemia.

We investigated a total number of 113 *S. aureus* strains from 104 patients (70% MSSA, 30% MRSA). Repeat strains consisted of nine isolates (from 5 patients). All isolates were obtained from blood cultures collected during the period March 2008 to May 2009. Phenotypic and genotypic detection of methicillin resistance correlated well. According to the literature, most CA-MRSA strains are distinguishable from HA-MRSA strains based upon the presence of the PVL toxin. However, no CA-MRSA was detected in our study, therefore the association between HA-MRSA versus CA-MRSA strains could not be analysed. In this study, CA-MSSA was identified in 22% of all MSSA isolates versus 0% CA-MRSA. PVL positive strains were found in 22.7% of all MSSA isolates with no detection in MRSA isolates. It was noted that MRSA strains clustered in *spa* CC-701 and CC-012, whereas CC-002 only contained MSSA strains. Likewise HA-strains representing the majority of MRSA strains also clustered in *spa* CC-701 and CC-012.

Forty nine *spa* types were identified in 89.3% of all isolates, whereas 9.7% of these strains were non-typeable. Five novel *spa* types were revealed. We detected a diverse number of *spa*-types that correlated to international clones. The most predominant *spa* type found in our setting was t037 (only in MRSA), followed by t891. According to the literature, t037 is associated to the Brazilian/Hungarian clone (SCC*mec* type III;

ST 239). Our findings, as well as other South African studies, indicate that t037 has been identified in clinical strains from numerous provinces in South Africa. Interestingly, all isolates from *spa* type t891 were PVL positive MSSA.

Bacteraemia cases were predominantly related to catheter sepsis, followed by skin and soft tissue infections (SSTI). Only one persistent bacteraemia case was identified related to a HA-SSTI. Recurrent bacteraemia cases were found in patients on dialysis for chronic renal failure and in burns patients related to intravascular catheter infections. The local epidemiology of *S. aureus* and the prevalence rate of different strains are important to investigate. The information provided contributes to the epidemiology of staphylococcal strains causing bacteraemia in our setting. These insights are useful for optimal diagnostic and therapeutic measures. The techniques developed can be used to identify outbreaks and recurrent infections.

ACKNOWLEDGEMENTS

DEDICATION

To my parents and husband

for their

support, love and understanding

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LIST OF ABBREVIATIONS

µg	Microgram
µl	Microlitre
µM	Micromolar
agr	Accessory gene regulator
ATCC	American Type Culture Collection
BHI	Brain-heart infusion
Bp	Base pairs
BURP	Based upon repeat patterns
BURST	Based upon related sequence types
CA	Community-acquired
CA – MRSA	Community-acquired methicillin resistant *Staphylococcus aureus*
CC	Clonal complex
ccr	Cassette chromosome recombinase
CFU	Colony forming units
CLSI	Clinical Laboratory Standards Institute
CoNS	Coagulase-negative staphylococci
CSI	Catheter and prosthetic device-related sepsis
DLV	Double locus variant
DNA	Deoxyribonucleic acid
Dnase	Deoxyribonuclease
EARSS	European antimicrobial resistance surveillance system
EMRSA	International epidemic methicillin-resistant *Staphylococcus aureus*
ESBL	Extended-spectrum beta-lactamase
FnBPs	Fibronectin-binding proteins
GISA	Glycopeptide intermediate *Staphylococcus aureus*
HA	Hospital- acquired
HA–MRSA	Hospital- acquired methicillin resistant *Staphylococcus aureus*
HCA	Health-care associated
h-GISA	Heteroresistant glycopeptide intermediate *Staphylococcus*

	aureus
h-VISA	Heteroresistant vancomycin intermediate *Staphylococcus aureus*
ICU	Intensive care unit
IE	Infective endocarditis
IgG	Immunoglobulin G
IS	Insertion sequences
KZN	Kwazulu Natal
McF	McFarland
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing
MRSA	Methicillin resistant *Staphylococcus aureus*
MSA	Mannitol salt agar
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
MSSA	Methicillin susceptible *Staphylococcus aureus*
NHLS	National Health Laboratory Service
PAP-AUC	Population analysis profile- area under the curve
PB	Primary bacteraemia
PBP2	Penicillin binding protein 2
PCR	Polymerase chain reaction
PCR-RFLP	Polymerase chain reaction- restriction fragment length polymorphism
PEARLS	Pan-European Antimicrobial Resistance Using Local Surveillance
PFGE	Pulsed-field gel electrophoresis
pls	Plasmin-sensitive surface protein gene
PMN	Polymorphonuclear leukocytes
PN	Pneumonia
PRSA	Penicillin-resistant *Staphylococcus aureus*
PVL	Panton Valentine Leukocidin
REAP DNA	Restriction endonuclease analysis of plasmid DNA
RNA	Ribo Nucleic Acid
ROS	Reactive oxygen species

S. aureus	*Staphylococcus aureus*
SA	Septic arthritis and Osteomyelitis
SaPI	*Staphylococcus aureus* pathogenicity islands
SCC*mec*	Staphylococcal Cassette Chromosome *mec*
SCV	Small colony variants
SLST	Single-locus sequence typing
SLV	Single locus variant
SNP	Single nucleotide polymorphism
SOP	Standard operating procedure
spa	Staphylococcal Protein A
spa-**CC**	*spa*-clonal complex
SSI	Statens Serum Institute
SSR	Short sequence repeat
SSTI	Skin and soft tissue infection
ST	Sequence type
TBH	Tygerberg Hospital
TSS	Toxic shock syndrome
UPGMA	Unweighted pair-group matching analysis
USA	United States of America
VISA	Vancomycin intermediate *Staphylococcus aureus*
VRE	Vancomycin-resistant *E. faecium*
Vwf	von Willebrand factor

CHAPTER ONE

1 INTRODUCTION AND LITERATURE REVIEW

1.1 <u>Introduction</u>

Staphylococcus aureus is one of the most virulent microbial pathogens to cause nosocomial and community acquired infections. *S. aureus* frequently causes bloodstream infections, skin and soft tissue infections, pneumonia and post-operative wound infections (Orrett & Land, 2006; Randrianirina *et al.*, 2007). Other severe infections include septic arthritis, osteomyelitis and endocarditis with significant rates of morbidity and mortality. Furthermore, community-acquired methicillin resistant *S. aureus* (CA-MRSA) infections and hetero-resistance to the glycopeptides in the hospital setting have emerged worldwide, especially in the United States (Orrett & Land, 2006; Shittu & Lin, 2006).

Deep-seated staphylococcal infections are common in patients admitted to Tygerberg Hospital. These infections include bacteraemia associated with line-sepsis, osteomyelitis, septic arthritis, deep organ abscesses, and infective endocarditis. Previous experience has shown that bone and soft tissue *S. aureus* infections particularly tend to relapse or become chronic. Persistence may be related to a number of factors, namely endovascular sources, vancomycin treatment, metastatic infections, diabetes (Khatib *et al.,* 2006), HIV status or inappropriate therapy (Chang *et al.*, 2003). Another possible reason may be a population of *S. aureus* strains with enhanced virulence and antimicrobial drug resistance.

There is a paucity of local studies on the genotypic characterisation of invasive *S. aureus* strains as well as on the incidence of CA-MRSA infections. In 2007, the first report documenting a variety of MRSA epidemic clones throughout South Africa was presented. The typing methods used in the study included SCC*mec* typing using multiplex PCR, *spa* typing and PCR for the detection of PVL toxin (Oosthuysen *et al.*, 2007). In 2009, another South African study was published, reporting the first

multilocus sequence typing (MLST) of *S. aureus* strains collected from public hospitals in Kwazulu-Natal (Essa *et al.*, 2009).

Data on the genotypic characteristics of local *S. aureus* strains in relation to the clinical presentation of *S. aureus* infections, in particular with regards to persistent infection, and community-acquired versus hospital acquired infections, is lacking. This is the first study that was performed at Tygerberg Hospital (an academic hospital situated in the Western Cape province of South Africa), which included *spa* typing, together with the detection of the *mecA* and *pvl* genes. The information provided in this study contributes to the understanding of local epidemiology of *S. aureus* and the pathogenesis of different strains in our setting.

1.2 <u>Literature Review</u>

1.2.1 Characteristics of *Staphylococcus aureus*

The genus *Staphylococcus* is derived from the family *Staphylococcaceae,* which consists of more than thirty species of Gram positive spherical bacteria (Todar, 2008). In 1880, Sir Alexander Ogston revealed *S. aureus* as an important human pathogen, responsible for the formation of pus in wounds (AL-Haj *et al.*, 2009; Archer, 1998). Skinner and Keefer (1941) provided proof of its virulence as 82% of 122 patients with *S. aureus* bacteraemia died of this infection at Boston City Hospital, Harvard Medical School. From the 122 cases, only 22 patients recovered (Skinner & Keefer, 1941).

Bacteria of this genus are microscopically observed as single organisms, in pairs, but mainly forming grapelike clusters. The latter is derived from the Greek term *staphyle* meaning a bunch of grapes. The cell wall has a Gram positive structure, containing peptidoglycan and teichoic acid (Ryan & George Ray, 2004; Tolan *et al.*, 2009). In most *S. aureus* strains, the peptidoglycan layer is covered with surface proteins. One of these proteins, namely protein A is discussed in more detail in section 1.2.7, Staphylococcal Protein A (*spa)*.

Although *S. aureus* are constituents of the normal flora of the skin and nose in carriers, they are also found in the oral cavity and gastrointestinal tract (Todar, 2008). Studies report that most *S. aureus* infections are thought to be derived from colonization of the anterior nares with 30% of the population being colonized at any given time (Melles *et al.*, 2004; Sinha & Herrmann, 2005).

All species of staphylococci are catalase positive and can therefore be clearly distinguished from streptococci and enterococci which are catalase negative bacteria. A catalase test can be performed, which converts hydrogen peroxide (H_2O_2) to water and oxygen in order to differentiate between catalase positive and negative bacteria (Todar, 2008). *S. aureus* grow well aerobically but are facultative anaerobes that form relatively large yellow to golden colonies when grown on rich media. The organism is also non-spore forming, non-motile and non-flagellate. In addition, it has the ability to

resist high temperatures up to 50° C, drying, as well as high salt concentrations (AL-Haj *et al.*, 2009; Tolan *et al.*, 2009).

The genus is classified into two main groups based upon the presence of the enzyme coagulase. *S. aureus*, being the most virulent pathogen is a coagulase positive bacterium which possesses the ability to clot blood plasma. The second group are the coagulase-negative staphylococci (CoNS) which form relatively small white colonies when grown on rich media. There are a number of CoNS species, such as *S. epidermidis, S. haemolyticus* and *S. saprophyticus*. For many years, CoNS have been considered to be harmless bacteria that form part of normal skin flora. However, this perception changed over time as the pathogenicity of these organisms was recognized. Heubner & Goldmann (1999) reported CoNS as being the most common cause of bacteraemia related to indwelling devices in nosocomial infections (Heubner & Goldmann, 1999). Virulence factors of these organisms are not well understood, but their ability to grow as biofilms on catheters or medical implants are of utmost importance (Mims *et al.*, 2004).

1.2.2 Infections caused by *S. aureus*

Over the past several decades, *S. aureus* has been associated with a diverse range of mild to life threatening clinical infections. Strains of this human pathogen can arise from colonized sites and be transmitted in the community or hospital settings (Shittu & Lin, 2006). These infections may vary from superficial skin lesions namely impetigo, furuncles, carbuncles and urinary tract infections, to serious infections like pneumonia, meningitis, endocarditis and osteomyelitis (Figure 1.1).
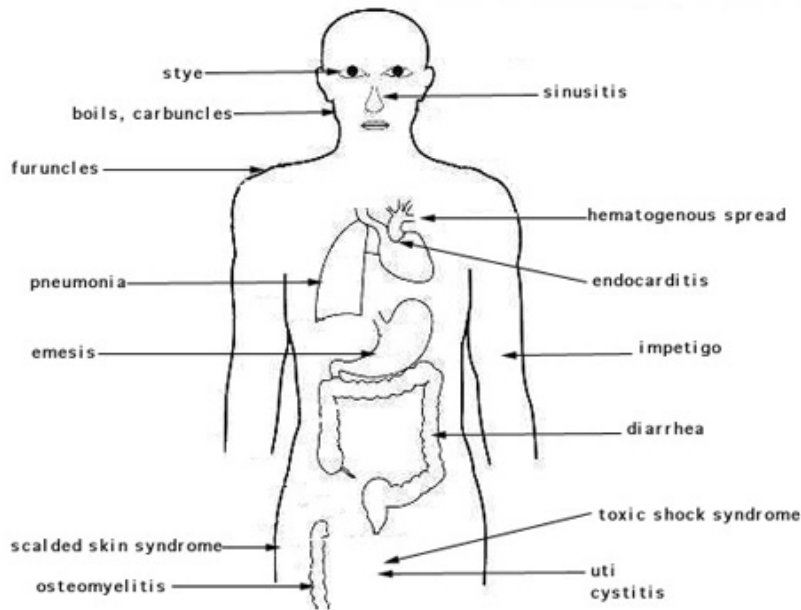
Figure 1.1: Diseases and infection sites of *S. aureus*

At least 30% of the population is colonized with *S. aureus,* mostly in the anterior nares but also in the axilla, perineum or vagina (Archer, 1998; Fischetti *et al.*, 2006). From a two week period up to a few months, the organism can be passed on asymptomatically onto the mucous membranes but only transiently carried on intact skin (Archer *et al.*, 1996). *S. aureus* is spread by means of hand carriage, as the organisms spread from the hands to other areas of the body. When colonization with *S. aureus* takes place, the organism may find entry through skin abrasions and cause skin infection, which could with further spread lead to more serious infections like endocarditis, osteomyelitis or toxemias (Fischetti *et al.*, 2006).

Community-acquired outbreaks are more often than not related to poor hygiene and fomite transmission from person to person. For instance, because *S. aureus* has the ability to survive lengthy periods of drying, clothing contaminated with pus from a previous infection could produce recurrent skin infections (Ryan & George Ray, 2004). The immune system also plays an important role as individuals who are at high risk of *S. aureus* colonization are usually patients on dialysis, intravenous (IV) drug users, diabetics, and patients with HIV-AIDS (Fischetti *et al.*, 2006).

Hospital-acquired outbreaks normally include patients who have undergone surgery or other invasive procedures. *S. aureus* can spread directly from infected open wounds to other patients on the hands of hospital staff (Ryan & George Ray, 2004). For this reason, health care workers and hospitalized patients have a much greater chance of becoming colonized with *S. aureus* for long periods of time (Fischetti *et al.*, 2006).

1.2.3 Methicillin resistant *S. aureus* (MRSA)

The introduction of penicillin in the 1940s resulted in a decrease in mortality rates due to *S. aureus* infections for a brief period. Soon after, penicillin-resistant *S. aureus* (PRSA) strains producing β-lactamase was established. Within the following 10 years, this resulted in a rise in penicillin resistance in up to 90% of hospital-acquired strains. In the late 1950s, methicillin was the treatment of choice for PRSA infections. Only six months after methicillin was marketed, methicillin-resistant *S. aureus* (MRSA) strains emerged in 1959, primarily in nosocomial settings (Boyle-Vavra & Daum, 2007; Grundmann *et al.*, 2006).

MRSA emerged due to the alteration of the penicillin binding protein 2 (PBP2) to PBP 2a, mediated by the *mecA* gene. This target change caused resistance to penicillinase-resistant penicillins (PRPs) and conferred resistance to all beta-lactam agents (Oosthuizen *et al.*, 2005). Since then, MRSA rates gradually increased, until it remarkably surged from the late 1990s (Amod *et al.*, 2005; Boyle-Vavra & Daum, 2007). Due to the dramatic increase in MRSA in nosocomial infections, the glycopeptides, vancomycin and teicoplanin, were until recently the last choice available for therapy (Boyle-Vavra & Daum, 2007; Nunes *et al.*, 2002; Robinson & Enright, 2003). These antibiotics and their resistance are further discussed in section 1.2.3.1 (Reduced susceptibility to vancomycin).

Currently, increasing rates of MRSA have been reported worldwide, especially in developing countries (Feng *et al.*, 2008; Randrianirina *et al.*, 2007). Afroz *et al* (2008), reported high MRSA rates of 32-63% in various cities of Bangladesh, which compares to countries in Europe and the United States (Afroz *et al.*, 2008). At present, hospital MRSA rates are more than 50% in Japan and the United States, whereas Sweden and Norway MRSA rates are less than 1% (Nübel *et al.*, 2008). The high

prevalence and growing rates of MRSA in hospitals is becoming a global problem, with intermediate occurrences in most of Australia, Europe, and several countries in South America and Africa (Nübel *et al.*, 2008).

Recent data on the prevalence rates of staphylococcal infections in Africa are limited. Early studies of MRSA in South Africa were reported during the 1980s and early 1990s (Shittu & Lin, 2006). A study conducted from 2001 to 2002 by the Pan-European Antimicrobial Resistance Using Local Surveillance (PEARLS), showed 18/54 (33%) of *S. aureus* isolates as MRSA in South Africa (Bouchillon *et al.*, 2004; Marais *et al.*, 2009). The PEARLS study provided baseline data of extended-spectrum β-lactamase (ESBL) producers in selected Enterobacteriaceae, vancomycin-resistant *E. faecium* (VRE) and MRSA strains from 17 participating countries. South Africa was one of these countries, together with 13 European and 3 Middle Eastern countries (Bouchillon *et al.*, 2004).

At Tygerberg hospital, (Cape Town, South Africa) during 1985, MRSA was isolated from 18% of 2681 pus swabs and 25% of 100 blood cultures (Peddie *et al.*, 1988). Currently, at this hospital, the laboratory statistics report showed that approximately a third of *S. aureus* isolates are methicillin resistant. This figure increases to approximately 60% for isolates from ICUs and approximately 45% for blood cultures isolates (Table 1.1).

Table 1.1: Percentage of *S. aureus* that are methicillin resistant isolated from (a) all specimens received from all hospital wards, (b) all specimens received from ICUs and (c) from blood cultures at Tygerberg Hospital. The time line for each year was from the 1st January to the 31st December.

Percentages	2006	2007	2008
(a) All specimens from all wards	34%	30%	29%
(b) All specimens from ICUs	64%	56%	64%
(c) Blood cultures	49%	42%	44%

Studies report that patients with MRSA bacteraemia have increased morbidity and mortality rates, higher medical costs and a longer duration of hospital stay, compared to patients with methicillin susceptible *S. aureus* (MSSA) bacteraemia (Nübel *et al.*, 2008; Shittu & Lin, 2006). The treatment of MRSA can be problematic if the location is at anatomical sites (for example in the treatment of bone infections or endocarditis) where there is reduced antimicrobial penetration (Duckworth, 2003; Shittu & Lin, 2006).

1.2.3.1 Reduced susceptibility to vancomycin

Vancomycin and teicoplanin are glycopeptides that are crucial for the treatment of life-threatening infections caused by multi-resistant Gram-positive bacteria (Tenover *et al.*, 2008). In the 1980s, empiric therapy for nosocomial staphylococcal infections changed to vancomycin, due to the universal occurrence of MRSA in many hospital settings (Tiwari & Sen, 2006). Vancomycin was believed to retain activity against all strains of *S. aureus*, but MRSA strains with reduced susceptibility to vancomycin have emerged during the last decade (Srinivasan *et al.*, 2002; Walsh & Howe, 2002).

GISA (glycopeptide intermediate *S. aureus*) and heteroresistant GISA (h-GISA) are the acronyms used when resistance occurs in both vancomycin and teicoplanin (Walsh

& Howe, 2002). Heterogeneous intermediate resistant strains test susceptible by standard susceptibility methods, but contain subpopulations composed of small numbers of bacterial cells with variable degrees of resistance to glycopeptides (Nunes *et al.*, 2006; Srinivasan *et al.*, 2002). Nonetheless, these isolates possess a minimum inhibitory concentration (MIC) below susceptible breakpoints, while containing bacterial subpopulations (ca. 10^{-6}) which grow in the presence of 4 µg/ml of vancomycin (Oosthuizen *et al.*, 2005). These strains, which are seen in both coagulase negative Staphylococci and *S. aureus*, are thought to occur more frequently than vancomycin intermediate *S. aureus* (VISA) or GISA (Oosthuizen *et al.*, 2005; Srinivasan *et al.*, 2002).

The automated VITEK 2 system and the vancomycin agar screening methods are not sensitive enough in detecting hetero-resistance. Therefore, it is recommended that the E-test macromethod, which correlates well with the gold standard, a modified population analysis profile- area under the curve ratio (PAP-AUC), should be used for selected isolates from patients not responding to vancomycin therapy (Walsh *et al.*, 2001). For the E-test macromethod a higher inoculum (2 McFarland) and a richer medium (brain heart infusion agar) is used to detect heteroresistance. This test performs well with a sensitivity of 96% and a specificity of 97%, thus making it a reliable and sensitive method (Oosthuizen *et al.*, 2005; Walsh *et al.*, 2001).

VISA and h-VISA strains have been reported from Asia, USA and Europe. In Africa, reports of VISA infections are limited. Two MRSA isolates from Johannesburg, South Africa, appeared to be intermediately resistant to vancomycin (Standard E-test of 6 and 8 µg/ml). However, these strains were not confirmed to be VISA or h-VISA by the broth dilution MIC or the population analysis results (Amod *et al.*, 2005). Nevertheless, Amod *et al* (2005) reported the first confirmed clinical h-VISA infection from a South African patient. The patient presented with a ventriculitis caused by a MRSA strain with reduced susceptibility to vancomycin. The MRSA strain was confirmed to be an h-VISA by both the macro-dilution E-test and the PAP-AUC method. However, no genotyping methods were included in this study.

In 2007, at Tygerberg hospital, a study was performed using the E-test macromethod to determine the presence of staphylococcal isolates with resistance to the

glycopeptides (Salaam, 2007). No GISA and h-GISA was detected in this study. However, soon after the completion of the above mentioned study, the first strain of hetero-glycopeptide intermediate resistant *S. aureus* (h-GISA) was isolated from an infant on vancomycin therapy at Tygerberg hospital. This infant developed a bacteraemia following umbilical catheter site infection. Despite removal of the catheter and vancomycin therapy, osteomyelitis of the tibia developed. The child subsequently improved after numerous surgical debridements and linezolid therapy. Due to the lack of strain typing methods available at that stage, it could not be established if the hetero-resistant strain evolved from the initial vancomycin-susceptible strain isolated from the same patient.

The detection of resistance is important in order to optimize treatment and guide preventative measures to contain further spread of these multi-resistance organisms in the hospital setting. The current procedures to detect h-GISA strains at Tygerberg Hospital are described in Chapter 3 (section 3.2).

1.2.3.2 Hospital, Health-Care and Community acquired infections

MRSA isolates from the community amongst previously healthy individuals with few or no record of healthcare associated risk factors for MRSA have recently been described (Boyle-Vavra & Daum, 2007). The first community-acquired MRSA (CA-MRSA) strain was reported in Western Australia during 1993 in patients with no known risk-factors for MRSA colonization (Deurenberg *et al.*, 2007). This signified remarkable changes in the epidemiology of MRSA, as previously all Staphylococcal community-acquired infections were due to MSSA (Boyle-Vavra & Daum, 2007; Deurenberg *et al.*, 2007).

Hospital acquired bloodstream infections are defined by a positive blood culture collected more than 48 hours after admission without evidence of a *S. aureus* infection at the time of admission. If the patient was transferred from another hospital, the duration is considered from the date of the first hospital admission. Health-care associated bloodstream infections are defined by positive blood cultures at the time or within 48 hours of hospital admission from patients with the following history:

- Patients who have been to a hospital or hemodialysis clinic or obtained intravenous chemotherapy within 30 days before the infection
- Patients who have been hospitalized for 2 or more days within 90 days (3 months) before the infection
- A patient who is a resident in a nursing home or long-term care facility (Friedman *et al.*, 2002)

Community acquired bloodstream infections are defined by a positive blood culture collected in an outpatient setting or within 48 hours of hospital admission from patients without a history of health-care exposure. Therefore, these patients should not have a history of MRSA infection or colonisation, surgery, dialysis, admission to a nursing home and also no history of hospitalization in the past year. Lastly, to be considered in this category is that the patient must have no permanent indwelling catheters or medical devices passing through the skin into the body (Deurenberg *et al.*, 2007; Friedman *et al.*, 2002)

It was primarily thought that CA-MRSA strains were nosocomial strains that spread from the hospital to community settings. Nonetheless, it is indicated that CA-MRSA strains are indeed different from those prevalent in hospital settings due to the susceptibility of CA-MRSA to non beta-lactam antimicrobials and the link with clinical syndromes that are more characteristic of MSSA strains. It has also been established that CA-MRSA developed from MSSA strains commonly spread in the community. This was concluded from studies that have shown clear genotypic differences between CA-MRSA and HA-MRSA (Boyle-Vavra & Daum, 2007; Deurenberg *et al.*, 2007). These genotypic markers include the genetic lineages; the architecture of genetic elements in methicillin resistance and the presence of the Panton Valentine Leukocidin (PVL) gene (refer to section 1.2.4.1).

CA-MRSA appears to be more virulent than HA-MRSA (Boyle-Vavra & Daum, 2007). The clinical infections associated with CA-MRSA include severe skin and soft tissue infections and necrotizing pneumonia. Boyle-Vavra & Daum (2007) mentions a high mortality rate of patients who are hospitalised within 24-48 hours with severe sepsis associated with necrotizing pneumonia. Furthermore, these cases have occasionally been correlated to purpura fulminans, disseminated intravascular

coagulation (DIC) and bilateral adrenal haemorrhage. The latter is usually related to Waterhouse-Friderichsen syndrome (Boyle-Vavra & Daum, 2007). CA-MRSA outbreaks have occurred in participants in team sports, military personnel and correctional facility inmates. Risk factors for these infections include poor hygiene and over populated areas (File, 2008).

1.2.4 Virulence factors

S. aureus may cause a wide range of infections due to an extensive number of virulence factors (Archer, 1998). Generally, *S. aureus* has been considered as an extracellular pathogen, until recent data revealed the organism's ability to infect a variety of host cells, both professional and non-professional phagocytes. These non-professional phagocytes include fibroblasts, osteoblasts, epithelial and endothelial cells (Fischetti *et al.*, 2006; Krut *et al.*, 2003; Que *et al.*, 2005).

In general, pathogenicity is associated with the bacteria's ability to adhere to surfaces, invade the tissues or cells, and cause harmful toxic effects to the host. Virulence factors of *S. aureus* are comprised of cell surface components (surface proteins) and extracellular proteins (secreted proteins or exoproteins). Cell surface components include capsular polysaccharide, protein A, fibronectin-binding protein, collagen-binding protein, elastin-binding protein and the clumping factor. Extracellular proteins include coagulase, hemolysins, enterotoxins, exfoliatins, toxic shock syndrome toxin, and Panton-Valentine leucocidin (PVL) (Figure 1.2) (Holmes *et al.*, 2005; Jarraud *et al.*, 2002; Lina *et al.*, 1999).
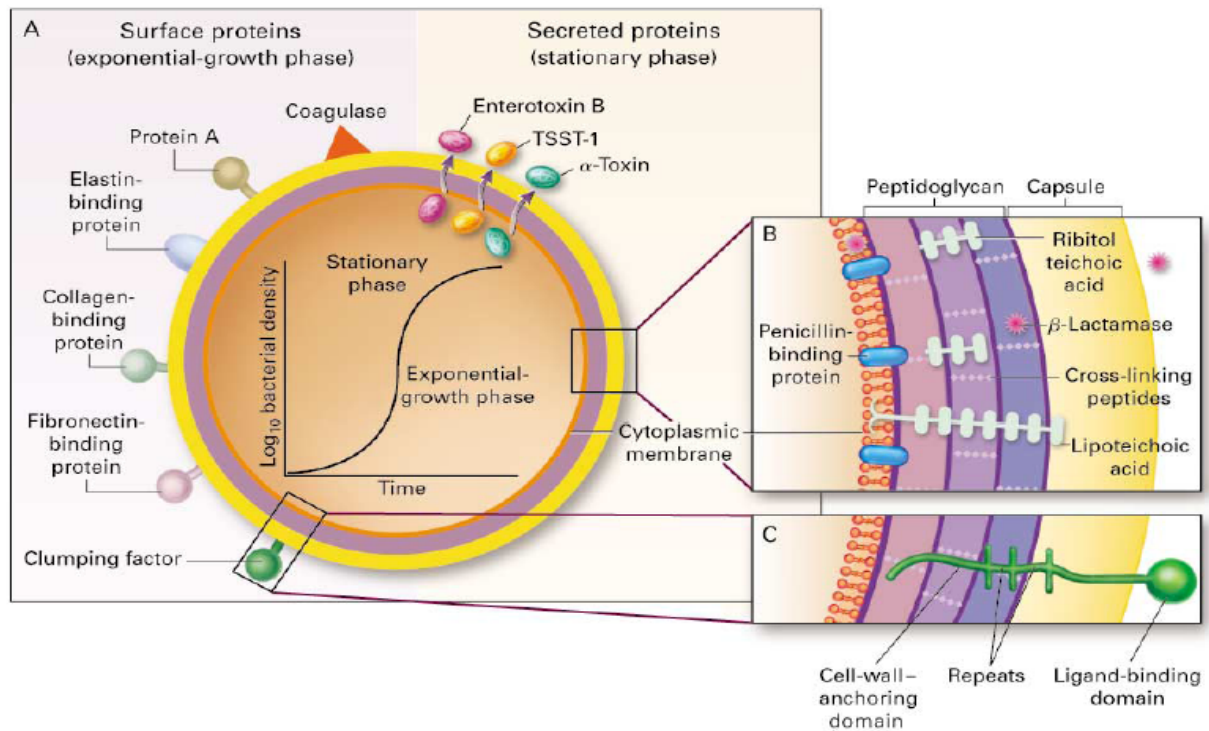
Reproduced from Lowy 1998

Figure 1.2: A diagram illustrating the surface and secreted proteins of *S. aureus* together with its growth phases. These growth phases are controlled by regulatory genes such as *agr*

In humans, *S. aureus* presents itself as a highly versatile pathogen known to cause three basic syndromes. Firstly, it can cause superficial skin lesions such as wound infections and skin abscess. Secondly, it can cause deep-seated and systemic infections namely osteomyelitis, endocarditis, pneumonia and bacteraemia. Thirdly, it may cause toxaemic syndromes related to the production of extracellular proteins. These include toxic shock syndrome (TSS), staphylococcal food poisoning (due to various enterotoxins), scalded-skin syndrome (due to exfoliatins) and necrotizing pneumonia (due to the PVL toxin) (Dinges *et al.*, 2000; Fischetti *et al.*, 2006; Jarraud *et al.*, 2002; Lina *et al.*, 1999). The pathogenicity of *S. aureus* is multifactorial, particularly on a molecular basis where the precise role of any given factor is difficult to determine. This depends largely on the expression of accessory gene products that constitute of surface proteins and extracellular proteins (Figure 1.2) (Jarraud *et al.*, 2002).

The accessory gene regulator (*agr*) induces the expression of extracellular proteins while suppressing the expression of surface proteins. Moreover, the surface proteins are produced during the early exponential growth phase, when the bacteria are at low density (Figure 1.2). As the adhesion molecules are expressed during the initial stages of infection, the bacteria adhere to and colonise host cells and implanted medical devices. On the contrary, the synthesis of secreted proteins is produced during the stationary phase which favours the spread to adjacent tissues (Figure 1.2). The bacteria therefore produce these toxic secretory proteins at higher densities, which allow the survival and spread of bacteria thus leading to infection (Korem *et al.*, 2003; Lowy, 1998).

Sinha & Hermann (2005) describes the mechanism of adherence and invasion of *S. aureus* causing serious infections, such as infective endocarditis in molecular detail. They report a vast diversity in the invasion of *S. aureus* and its effect to host cells, which may be due to multiple virulence factors. Therefore, further studies are needed in order to better understand the pathogenesis of *S. aureus*.

Furthermore, Sinha & Hermann (2005) discuss covalently cell wall-anchored adhesins which are referred to as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). It was believed that MSCRAMMs were mono-specific for a given host protein. This reasoning changed as more than one ligand has been recognized for many of these adhesins. The members belonging to the MSCRAMMs class are Spa (staphylococcal protein A), FnBPs (fibronectin-binding proteins), Cna (collagen adhesin), ClfA and ClfB (clumping factors, and other fibrinogen-binding proteins). In addition, pls (plasmin-sensitive protein) exists in this class (Nashev *et al.*, 2004; Sinha & Herrmann, 2005). The MSCRAMMs allow bacteria to initially adhere to host tissue components in order to withstand phagocytosis and other host defences. For instance, fibronectin-binding protein facilitates incorporation by epithelial and endothelial cells.

Although *S. aureus* is usually classified as an extracellular pathogen, the ability of these organisms to survive intracellularly has been established in epithelial cells as well as neutrophils (Kielian *et al.*, 2001). The intracellular environment protects staphylococci from both host defence mechanisms and bactericidal effects of

antimicrobial agents (Kielian *et al.*, 2001; Lowy, 2000). It has been reported that variants of *S. aureus,* specifically small colony variants (SCV) due to their colony morphology, has adapted an ability to persist intracellularly (Fischetti *et al.*, 2006; Krut *et al.*, 2003). These variants, protected by the intracellular environment may cause persistent or recurrent infections. Hence, further studies are needed to investigate whether invasion and cytotoxicity are characteristic of clinical *S. aureus* isolates and if these factors are associated to their pathogenicity.

1.2.4.1 Panton Valentine Leukocidin (PVL)

The cytolytic toxin PVL was discovered in 1894 by Van de Velde. This toxin has the ability to lyse leucocytes (Ellington *et al.*, 2007; Pathirage, 2008). In 1932, the toxin was named after Panton and Valentine when they associated it with severe soft tissue infections (Pathirage, 2008). PVL is also closely related to CA-MRSA clones that have recently emerged worldwide (Dumitrescu *et al.*, 2007). Genetic analysis shows that these clones emerged in various continents, and not from a single clone that spread worldwide (Pathirage, 2008). The CA-MRSA clones, USA300 and USA400 have been identified in Canada and the United States. The USA300 clone has been detected in approximately 50% of community-acquired skin infections in the United States (McDonald *et al.*, 2005; Tinelli *et al.*, 2009). Thus, CA-MRSA has become an increasing threat worldwide, as newly emerging strains has been reported in various studies with rates of 77% to 100% (McClure *et al.*, 2006; Naas *et al.*, 2005; Naimi *et al.*, 2003; Shukla *et al.*, 2004). Evidence that PVL is a major virulence factor in CA-MRSA is discussed in more detail in section 1.2.4.1 a).

It has been documented that the toxic effects produced by PVL is encoded by two contiguous genes, namely, *lukF-PV* and *lukS-PV*. These genes occur in several temperate bacteriophages. The two genes act as subunits that assemble in the membrane of host cells, mainly in neutrophils, macrophages and monocytes (Feng *et al.*, 2008; Pathirage, 2008). The subunits form a ring with a central pore when in close contact, through which the cell contents escape, acting as superantigens (Ellington *et al.*, 2007; Holmes *et al.*, 2005; McDonald *et al.*, 2005; Pathirage, 2008). According to published reports, PVL toxin is produced by 2-10% of *S. aureus* isolates (Ellington *et al.*, 2007; Holmes *et al.*, 2005; McDonald *et al.*, 2005).

This bicomponent cytotoxin causes damage in the leucocytes and tissue necrosis, resulting in severe skin and soft tissue infections as well as necrotizing pneumonia (McClure *et al.*, 2006; Pathirage, 2008; Tinelli *et al.*, 2009). The mortality rates of these PVL-producing *S. aureus* infections are high (approximately 75%) (Dumitrescu *et al.*, 2007). Furthermore, Melles *et al* (2004) reported the presence of PVL in a high number of *S. aureus* strains causing abscesses and arthritis in contrast to colonizing strains. Another study detected PVL genes in isolates that were responsible for burn infections, bacteraemia, scalded skin syndrome as well as community acquired-pneumonia (Holmes *et al.*, 2005). Many reports found that PVL causes severe necrotizing pneumonia, specifically among children, young adults and immunocompetent patients (Labandeira-Rey *et al.*, 2007; Pathirage, 2008; Yamasaki *et al.*, 2005).

Lina *et al* (1999), noted the first connection between the genes for PVL and community acquired (CA) pneumonia in *S. aureus* strains. Lina and colleagues (1999) developed a PCR assay for the detection of PVL genes. They discovered an association between the presence of the locus with severe necrotizing CA-pneumonia in 8% of their cases in comparison to none of the hospital-acquired pneumonia cases. They also confirmed the findings of previous reports proving that PVL genes are related to primary cutaneous infections, particularly furunculosis. Furthermore, studies of CA-pneumonia due to PVL-positive *S. aureus* strains have been reported in the United Kingdom, the Netherlands, France and Sweden (Holmes *et al.*, 2005).

Outbreaks of PVL-associated skin infections have been reported in schoolchildren in Switzerland, among homosexual men in the Netherlands, and among hospital staff in Scotland (Holmes *et al.*, 2005). Likewise, outbreaks of severe skin infections have also occurred in the United States among homosexual men, prison inmates and schoolchildren (Holmes *et al.*, 2005).

PVL, as well as γ-hemolysin, are derived from the synergohymenotropic toxin family. These toxins act on the cell membranes by the synergy of two classes of secretory proteins namely, S (slow-eluted) and F (fast-eluted). These proteins are further separated by column chromatography into the following:

- HIgA, HIgC, LukS-PV (belonging to class S)
- HIgB and LukF-PV (belonging to class F)

It is important to note that all PVL positive isolates produce both the S and F class proteins. Pathirage (2008) mentioned that PVL (LukS-PV and LukF-PV) is detected in less than 5% of *S. aureus* isolates, while the three proteins forming γ-hemolysin (HIgA, HIgC and HIgB) is detected in more than 99% of *S. aureus* isolates (Johnsson *et al.*, 2004; Lina *et al.*, 1999). Therefore, these strains have the ability to produce three class S and two class F proteins, which leads to six biologically active pairs in the S and F classes, respectively (Table 1.2) (Johnsson *et al.*, 2004; Lina *et al.*, 1999).

Table 1.2: A table to illustrate the secretory protein subclasses: S (slow-eluted) and F (fast-eluted)

Class (S)	Class (F)
HIgA	HIgB
HIgC	LukF - PV
LukS – PV	
6 (S + F) Biologically Active Pairs	
1. HIgA + HIgB	
2. HIgC + HIgB	
3. LukS - PV + HIgB	
4. HIgA + LukF – PV	
5. HIgC + LukF – PV	
6. LukS - PV + LukF – PV	

A study was done on the purification of PVL components (LukS-PV and LukF-PV); from a V8 strain (ATCC 49775) (Fink-Barbancon *et al.*, 1991; Johnsson *et al.*, 2004). The size was determined to be 32 and 38 kDa, respectively. The genes are 939 and 978 nucleotides in size while separated by a single thymine nucleotide and transcribed as a single mRNA molecule. This gene was found on the genomes of different *S. aureus* strains in its various prophages (Fink-Barbancon *et al.*, 1991; Johnsson *et al.*, 2004).

Furthermore, PVL does not produce haemolytic activity on human erythrocytes unlike γ-hemolysin, but is leucotoxic for human and rabbit polymorphonuclear cells and macrophages. PVL causes severe inflammatory lesions after intradermal injection in a rabbit's skin, which may lead to capillary dilation, skin necrosis, chemotaxis and polymorphonuclear karyorrhexis (rupture of cell nucleus) (Holmes *et al.*, 2005). On the other hand, γ-hemolysin in the rabbit's skin model also causes inflammatory but no skin necrosis (Johnsson *et al.*, 2004; Lina *et al.*, 1999).

1.2.4.1 a) Verification that PVL is a virulence factor

Up to now, there have been a limited number of studies on the pathogenesis of PVL. Research has shown the role of PVL in dermonecrosis in rabbits; however the role it might play in necrotizing pneumonia, severe sepsis and necrotizing fasciitis is unidentified (Boyle-Vavra & Daum, 2007). A study conducted by Labandeira-Rey *et al.,* (2007), showed PVL to be a virulence factor in an acute pneumonia mouse model. Their study used sets of isogenic strains for PVL, demonstating that PVL is a virulence factor (Dumitrescu *et al.*, 2007).

LukS-PV binds to an unidentified receptor on polymorphonuclear leukocytes (PMN) membranes, while connecting to its dimer LukF-PV (Figure 1.3). Consequently, both components, LukF-PV and LukS-PV eventually form a pore-forming heptamer by means of consistent alternate serial binding (Figure 1.3). It is important to note that PVL is not haemolytic (do not cause destruction to blood cells), unlike other *S. aureus* pore-forming leukocidins (Boyle-Vavra & Daum, 2007; Kaneko & Kamio, 2004). Furthermore, a host protein kinase (A or C) phosphorylates LukS-PV when binding to PMNs. This follows the induction of Ca^{++} ion channels (Kaneko & Kamio, 2004) suggesting that the events leading to signal transduction may trigger the production of interleukins and inflammatory mediators (Boyle-Vavra & Daum, 2007). Subsequently, high concentrations of PVL can cause lysis of PMN, yet low concentrations of PVL results in apoptosis of PMN via a novel pathway. This pathway involves the attachment of PVL-mediated pore formation onto the mitochondrial membrane, releasing cytochrome *c* and induction of caspases 9 and 3 (Figure 1.4) (Boyle-Vavra & Daum, 2007).
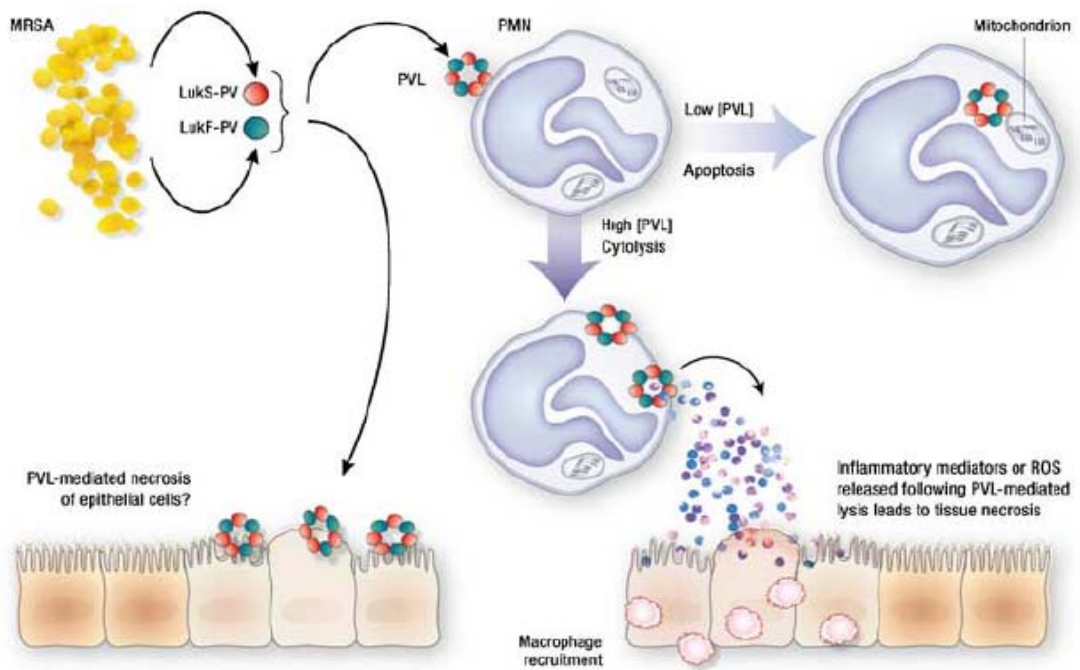
(Boyle-Vavra & Daum, 2007)

Figure 1.3: A model demonstrating how PVL might mediate tissue necrosis
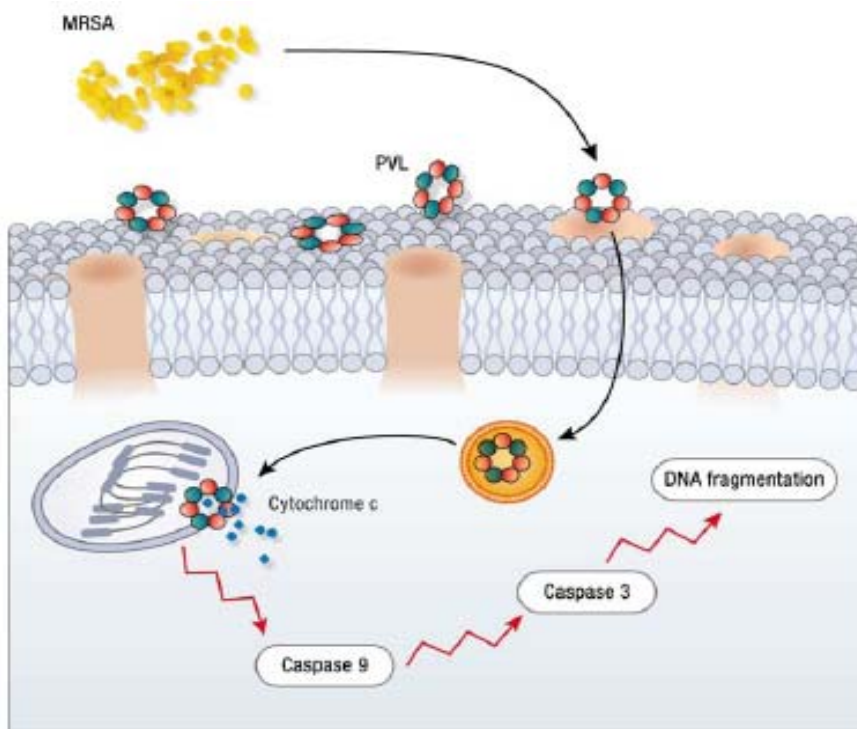
(Boyle-Vavra & Daum, 2007)

Figure 1.4: Apoptosis of polymorphonuclear leukocytes (PMN) membranes via a novel pathway

Studies have shown that neutropenia associated with PVL-positive *S. aureus* necrotizing pneumonia, is related to the PMN cytolytic activity of PVL (Adem *et al.*, 2005; Boyle-Vavra & Daum, 2007; Gillet *et al.*, 2002). According to Boyle-Vavra & Daum (2007), the first step in pathogenesis, mediated by the evasion of the first line of host defence, may be defined by PVL-mediated PMN lysis and apoptosis. However, the pathway leading to tissue necrosis and severe sepsis is not very clear. A few possibilities are outlined in figure 1.3. It was observed that purified PVL does not have a direct necrotic effect on epithelial cells (Boyle-Vavra & Daum, 2007; de Bentzmann *et al.*, 2004). Tissue necrosis and sepsis could occur from the release of granule contents from lysed PMNs (Boyle-Vavra & Daum, 2007). PVL-mediated lysis results in reactive oxygen species (ROS) being released and a variety of inflammatory mediators from granulocytes (Boyle-Vavra & Daum, 2007; Kaneko & Kamio, 2004).

1.2.4.1 b) The emergence of PVL producing CA-MRSA

Boyle-Vavra & Daum (2007) explains the origin of CA-MRSA (Figure 1.5). The model illustrates how a PVL phage, namely phiSLT, infects a MSSA strain. The *mecA* gene encoded by a methicillin resistant cassette (SCC*mec* IV, V or V_T) is horizontally transferred into the MSSA strain containing the *pvl* gene. The gene cassette incorporates itself into the genome in a location that is separate from that of the phiSLT integration site. This results in the integration of a methicillin resistant cassette into genomes of various MSSA ancestoral clones circulating in different geographic regions. HA-MRSA emerged from MSSA in the 1960s. Hence, it is possible that CA-MRSA also emerged from MSSA strains with the addition of *pvl* (Boyle-Vavra & Daum, 2007).

MSSA   PVL phage infects MSSA   Prophage integrated into *S. aureus* chromosome carrying pvl genes   MRSA produces PVL toxin

Chromosome

Methicillin resistance cassette (SCCmec IV or V) enters cell and integrates into chromosome

PVL toxin
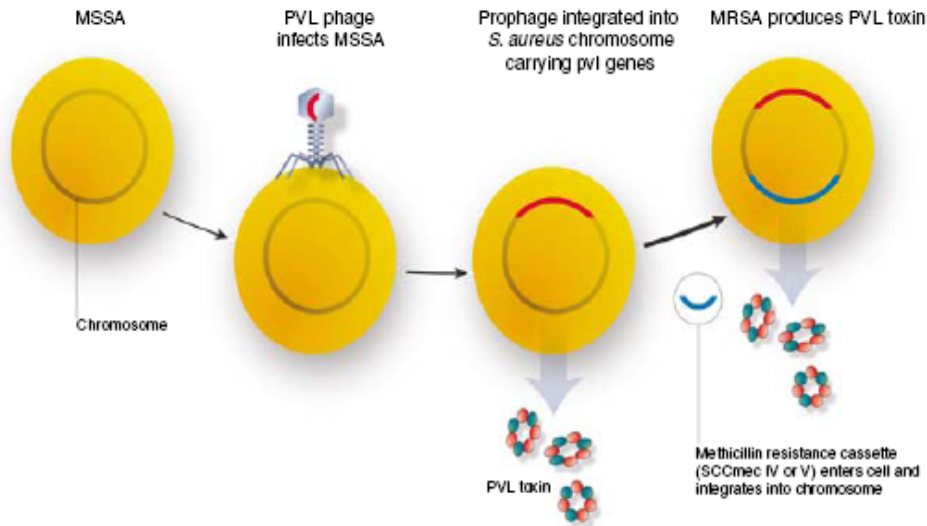
Reproduced from (Boyle-Vavra & Daum, 2007)

<u>Figure 1.5</u>: A model illustrating the origins of community-acquired MRSA

Global selective pressure is emerging worldwide as diverse genetic backgrounds carry one small methicillin resistance cassette as well as a phiSLT phage consisting of *pvl* (Boyle-Vavra & Daum, 2007). More frequently now, studies are reported on MRSA strains carrying *pvl* together with community-acquired genotypes (SCC*mec* IV, ST8) in hospital acquired infections (File, 2008; Maree *et al.*, 2006). Consequently, these *pvl* positive CA-MRSA colonizing isolates could have found a portal of entry during invasive procedures conducted in the hospital setting. In many hospitals, CA-MRSA strains may now be endemic (Boyle-Vavra & Daum, 2007).

1.2.5 Staphylococcal Cassette Chromosome *mec* (SCC*mec*)

Numerous pathogenicity islands have been identified in the genome of *S. aureus,* namely SaPI-1, SaPI-2, SaPI-3 (*S. aureus* Pathogenicity Island) and so forth. These islands are chromosomal regions which are acquired by horizontal transfer from other species. Furthermore, staphylococcal cassette chromosome with methicillin-resistance (SCC*mec*) is also classified as a pathogenicity island. The *mecA* gene is carried on these mobile genetic elements, called the SCC*mec* cassettes. These consist of five cassettes (SCC*mec* type I-V) that vary in size and genetic structure (Boyle-Vavra & Daum, 2007; Enright *et al.*, 2002; Foster, 2004; Ito *et al.*, 1999).

(Ito *et al.*, 1999) described the structures and origins of SCC*mec* type I to IV as follows:

- SCC*mec* type I: (34kb) Identified in 1961 in the United Kingdom in an MRSA strain (strain NCTC10442).
- SCC*mec* type II: (52kb) Identified in 1982 in Japan in an MRSA strain (strain N315).
- SCC*mec* type III: (66kb) Identified in 1985 in New Zealand in an MRSA strain (strain 82/2082)
- SCC*mec* type IV: (20 – 24kb) Independently identified among representatives of the Pediatric clone in two community-acquired MRSA strains

SCC*mec* type V is 28kb in size (Deurenberg *et al.*, 2007). The SCC*mec* are composed of two crucial genetic components (the *mec* and *ccr* gene complexes), and the junkyard (J) region DNA segments. The *mec* gene complex consists of IS431*mec*, *mecA* and regulatory genes, *mecR1* and *mecI*. Furthermore, this complex contains various classes, discussed in more detail in section 1.2.6 (The *mecA* gene). The *ccr* gene complex is mainly responsible for the mobility of these SCC*mec* elements. This complex consists of four allotypes namely types 1, 2, 3 and 5 (Zhang *et al.*, 2005). The remaining part of these elements is comprised of the J regions (regions J1, J2 and J3) which are allocated between and surrounding the *mec* and *ccr* complexes as follows:

- J1 region is situated between the chromosomal left junction and the *ccr* complex.
- J2 region is situated between the *ccr* complex and the *mec* complex.
- J3 region is situated between the *mec* complex and the chromosomal right junction.

The structural organisation of SCC*mec* can therefore be demonstrated as J1-*ccr*-J2-*mec*-J3. SCC*mec* types are distinguished by the various combinations of classes in the *mec* gene complex and allotypes in the *ccr* complex (Zhang *et al.*, 2005). Furthermore, the SCC*mec* elements are classified into subtypes according to the differences in their J region DNA within the same *mec-ccr* combination. These regions contain non-essential components of the cassette, but in some instances they

are capable of carrying further antibiotic resistance determinants (Milheirico *et al.*, 2007; Oliveira *et al.*, 2002; Zhang *et al.*, 2005).

CA-MRSA is generally associated with smaller SCC*mec* variants, either type IV (24 kb), or type V (or variant, V_T). In contrast, HA-MRSA are usually associated with large SCC*mec* types I, II or III (34-67 kb) (Boyle-Vavra & Daum, 2007; Deurenberg *et al.*, 2007). However, studies have shown SCC*mec* types I, II and III linked to CA-MRSA strains. Another study also reported a SCC*mec* type IV associated with HA-MRSA strains. This clearly shows that SCC*mec* types do not completely distinguish between community and hospital acquired MRSAs (Deurenberg *et al.*, 2007).

Notably, an integrated bacteriophage (phiSLT), carrying the *pvl* genes in CA-MRSA, is important in distinguishing CA-MRSA from HA-MRSA. The *pvl* gene widely associates itself with SCC*mec* IV and occasionally with SCC*mec* V or V_T. These associations are still unclear. Some authors have reported an association between CA-MRSA, SCC*mec* type IV and PVL production, but others disagreed. However, less than 5% of MRSA strains with SCC*mec* types I-III carry *pvl*, but 40-90% of MRSA strains with SCC*mec* type IV also carry *pvl* (Deurenberg *et al.*, 2007). Further investigation is thus needed in order to determine the association between *pvl* and SCC*mec* types in CA-MRSA.

Studies have shown differences between the genetic backgrounds of CA-MRSA and HA-MRSA clones circulating within distinct geographic regions by using typing techniques, mainly pulsed-field gel electrophoresis and multi-locus sequence typing. For instance, the most predominant CA-MRSA strain in the United States now is ST8/pulsotype USA 300. Before, most CA-MRSA isolates carried SCC*mec* IV in the genetic backgrounds ST1/pulsotype USA 400. The most widespread multi-resistant HA-MRSA clone in the United States is ST5 which is a carrier of SCC*mec* II (Boyle-Vavra & Daum, 2007).

1.2.6 The *mecA* gene

In Staphylococcus species, the *mec* gene complex consists of four genetic classes. In all of these classes, IS*431* is present downstream of *mecA*. IS*431* is one of the

insertion sequences (IS) for staphylococci (Archer *et al.*, 1996). Insertion sequences are short DNA sequences, carrying no genetic information hence acting as transposable elements. Only class A and class B *mec* gene complexes have been found in *S. aureus*. The structure of class A *mec* gene complex is as follows: *mecI-mecR1-mecA-* IS*431mec*. In class B, another insertion sequence, IS*1272* is found. The structure in this class is shown as IS*1272-ΔmecR1-mecA*-IS*431*. A study done by Archer *et al* (1996) showed that IS*1272* is more prevalent in *S. haemolyticus,* suggesting that SCC*mec* type I was transferred from *S. haemolyticus* to *S. aureus* in the past. Class C complexes are mainly found in *S. haemolyticus,* whereas *S. hominis* were only identified in the class D complex. Both class C1 and C2 has a deletion in the left side of class A *mec* complex followed by an insertion of IS*431*. It should be noted that IS*431* is inserted in the same direction as *mecA* in class C1, whereas IS*431* is inserted in the opposite direction to *mecA* in class C2. Class D demonstrates no insertion sequence adjacent to its deletion point. The direction of transcription is indicated by means of arrows in figure 1.6 (Hiramatsu *et al.*, 2001; Katayama *et al.*, 2001).
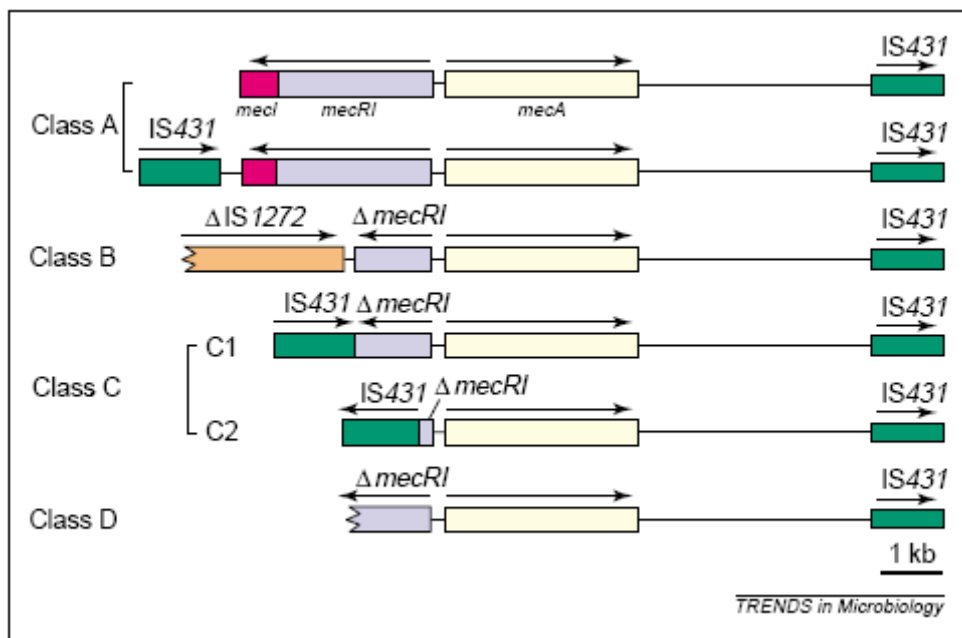
Reproduced from (Hiramatsu *et al.*, 2001)

Figure 1.6: Illustration of the *mec* gene complex with its four classes in Staphylococci

MRSA emerged due to the alteration of the penicillin binding protein 2 (PBP2) to PBP 2a, mediated by the *mecA* gene. This target change caused resistance to penicillinase-resistant penicillins (PRPs) and conferred resistance to all beta-lactam antibiotics (Oosthuizen *et al.*, 2005). PBP2a is also a transpeptidase that catalyzes crossbridge formation in the bacterial peptidoglycan cell wall (Hiramatsu *et al.*, 2001).

The *mecA* gene complex contains a two-gene operon *mecR1-mecI* that is transcribed in different directions containing overlapping promoter-operator regions with *mecA*. When *mecA* transcription is repressed by *mecI*, induction by means of MecR1 can occur as follows: MecR1 detects B-lactam antibiotics with its extracellular penicillin-binding domain, thereby activating its cytoplasmic domain as a protease by autocatalytic cleavage. It then cleaves the MecI repressor protein by direct or indirect action. The binding of MecI repressor protein to the operator region of the *mecA* gene causes the release in repression of *mecA* gene transcription (Hiramatsu *et al.*, 2001).

β-lactamases are enzymes produced by certain bacteria, in this case staphylococci that become resistant to penicillin. The gene *blaZ* is encoded by β-lactamase. The production of β-lactamase is also controlled by a two-gene operon, *blaR1-blaI* that is transcribed in different directions from each other. Hence, these gene products also have amino acid homology to *mecR1* and *mecI*. Experimental studies have proven that purified Mec1 and BlaI binds to *bla* promoter-operator sequences in DNase assays. Due to the resemblances between *blaZ* and *mecA* regulons, both BlaI and MecI repressor proteins are responsible for the regulation of *mecA* transcription. Furthermore, BlaR1 and MecR1 are the two signal transduction proteins responsible for regulating repressor activity (McKinney *et al.*, 2001).

It has been discovered that *mecA* gene transcription is fully repressed by *mecI* and *mecR1* if clinical strains remain susceptible to methicillin (AL-Haj *et al.*, 2009; Katayama *et al.*, 2001). These strains are known as pre- methicillin resistant *S. aureus* (pre-MRSA) and pre-methicillin resistant coagulase negative staphylococci (pre-MRCNS) (AL-Haj *et al.*, 2009). These pre-MRSA strains were first isolated in Japan during the early 1980s, having a marginal level of resistance to methicillin (MIC < 8 mg/liter) (Hiramatsu *et al.*, 2001; Katayama *et al.*, 2001). Therefore in MRSA strains,

mutations occur in the *mecI* genes or the *mecA* operator regions (to where the MecI repressor protein should bind). As a result, the MecI repressor protein inhibits the activity of the *mecA* operator gene. This finding corresponds to a study done by Hiramatsu (1995) who found mutations in either the *mecI* genes or *mecA* operator regions in majority of their clinical MRSA strains (Hiramatsu, 1995). These strains were carrying SCC*mec* type II or III, in the class A *mec* complex. To conclude, in MRSA strains a loss or inactivation of the MecI repressor protein leads to the activation of the *mecA* operating gene, known as derepression. This leads to the occurrence of *mecA* transcription in MRSA strains.

1.2.7 *Staphylococcal* Protein A (*Spa*)

Protein A, historically known as Jensen's antigen A, is one of the MSCRAMM surface proteins covalently anchored to the cell wall of *S. aureus* (Figure 1.2) (Dossett *et al.*, 1969). This protein consists of approximately 2,150 base pairs with a molecular weight of 42,000 kilo Dalton (42kDa) (Frenay *et al.*, 1994). Furthermore, staphylococcal protein A (Spa) is produced by more than 95% of *S. aureus* strains (Adesida *et al.*, 2006; Wann *et al.*, 1999).

There are four different two-component systems in *S. aureus* that are responsible for virulence control. They are Agr, Sae, Srr and Arl. A study conducted by Fournier & Klier (2004) showed how the *spa* gene is regulated by DNA supercoiling in response to environmental conditions like high osmolarity, and the ArlS-ArlR two-component system.

Staphylococcal protein A is well known for its ability to interact directly with the Fc region of immunoglobulins of most mammalian species (Adesida *et al.*, 2006; Callegan *et al.*, 1994; Dossett *et al.*, 1969; El-Sayed *et al.*, 2006; Forsgren, 1970). Spa interacts with several host components, not only with immunoglobulins (mainly G, A and E), but also with platelets and von Willebrand factor (vWF) (Fournier & Klier, 2004). The latter is a large multifunctional glycoprotein that mediates platelet adhesion at sites of endothelial damage. Hartlieb *et al* (2000) found an interaction between protein A in *S. aureus* and vWF which suggests a potential role of endovascular *S. aureus* pathogenesis. Consequently, all these factors support the role

of protein A as an important virulence factor in *S. aureus* infections (El-Sayed *et al.*, 2006; Fournier & Klier, 2004; Genovese *et al.*, 2000; Graille *et al.*, 2000; Hartlieb *et al.*, 2000; Marone *et al.*, 1987; Palmqvist *et al.*, 2002) .

Immunoglobulin G (IgG) contain two binding sites to which protein A attaches itself to. They are the Fcγ constant region which is involved in effector functions and the Fab region responsible for antigen recognition in a non-antigen specific manner (El-Sayed *et al.*, 2006; Graille *et al.*, 2000).

The *spa* gene is composed of a N-terminal region and a C-terminal region (Frenay *et al.*, 1994; Guss *et al.*, 1985; Löfdahl *et al.*, 1982). The N- terminal region encodes five homologous immunoglobulin binding units. These repeat regions are designated E, D, A, B and C, each unit having the ability to bind one human IgG molecule when isolation occurs separately (Figure 1.7) (El-Sayed *et al.*, 2006; Guss *et al.*, 1985; Palmqvist *et al.*, 2002). The C- terminal part, also called region X, encodes the COOH-terminal that binds protein A to peptidoglycan of the cell wall with no IgG-binding capacity (Figure 1.7) (Guss *et al.*, 1985; Löfdahl *et al.*, 1982). Region X or short sequence repeat (SSR) region contains a polymorphic repetitious Xr part. Xr is composed of a variable number of 21-24 bp repeats in length (Hallin *et al.*, 2007; Kuzma *et al.*, 2005; Moodley *et al.*, 2006). The Xc region is located upstream to the X region and encodes a non-repetitive sequence for 81 amino acids (El-Sayed *et al.*, 2006; Shopsin *et al.*, 1999). The sequence variation in the X region can arise from both duplications and deletions of repetitive units or by point mutations within the repeat sequence (Figure 1.7) (Mitani *et al.*, 2002; Shopsin *et al.*, 1999).

It has also been noted that with increased amounts of protein A, *S. aureus* strains tend to resist phagocytosis (by polymorphonuclear leukocytes), causing a decrease in free receptor sites needed for complement C3b. On the other hand, *S. aureus* strains containing decreased or no protein A are phagocytosed. Therefore, protein A encoded by the *spa* gene, prevents the occurrence of opsonization and phagocytosis (Dossett *et al.*, 1969; Kuzma *et al.*, 2005).
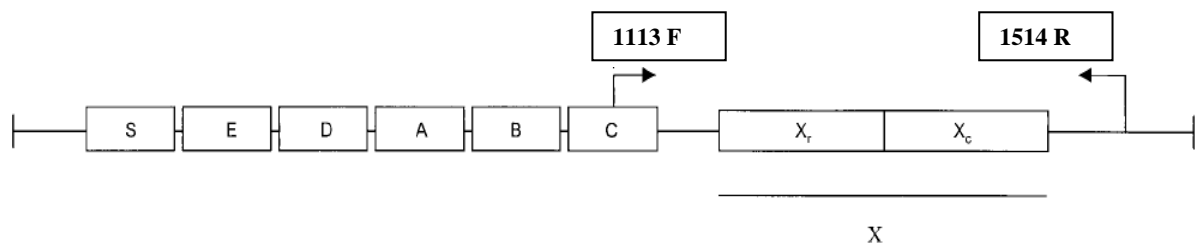
The abbreviations signify the following: [S] signal sequence; [A-D] IgG binding regions; [E] a region homologous to A-D; [X] COOH terminus; [Xr] SSR region; [Xc] cell wall attachment sequence. (Shopsin *et al.*, 1999)

Figure 1.7: A diagram of the *spa* gene with each box illustrating segments of the gene together with forward and reverse primers.

1.3 Genotyping of *S. aureus*

Typing of *S. aureus* clinical isolates is important to investigate strain origin, clonal relatedness, as well as the epidemiology of outbreaks (Cookson *et al.*, 2007; Shopsin *et al.*, 1999). The relatedness of isolates may be determined by a range of typing methods (Shopsin, 2001).

Typing methods should have high discriminatory power and typeability, have good reproducibility and portability, be easy to perform and be affordable (Cookson *et al.*, 2007). Typing methods are divided into two categories namely phenotypic methods and genotypic methods. Phenotypic methods are observable characteristics of gene expression products to differentiate between strains. Examples of phenotypic properties observed in the laboratory are biochemical profiles, antigens present on the surface of cells, bacteriophage types as well as antimicrobial susceptibility profiles. On the other hand, genotypic methods include the analysis of genetic structure of an organism which includes various molecular techniques (Tenover *et al.*, 1997).

For *S. aureus*, genotyping techniques have largely substituted traditional phenotypic typing methods. These genotypic techniques include pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), SCC*mec* typing and *spa* typing.

1.3.1 Pulsed-Field Gel Electrophoresis (PFGE)

PFGE is currently the gold standard for typing Gram positive and Gram negative bacteria. This method has a high discriminatory power and is commonly used for studying nosocomial outbreaks (Cookson *et al.*, 2007). This method was used successfully to identify the relatedness of isolates from MRSA outbreaks during the 1980s and 1990s (Enright *et al.*, 2002; Kim, 2009).

PFGE typing of *S. aureus* isolates involves the digestion of purified chromosomal DNA with the restriction enzyme *SmaI*, followed by the electrophoresis of large DNA fragments in agarose gel. Instead of electric fields being applied in one direction on a standard gel electrophoresis, the orientation of electric field is alternated in opposite directions for defined time periods. The DNA fragments migrate through the gel by means of an electric field current that provides pulses that alternate from three sets of electrodes (Tenover *et al.*, 1997). This allows the separation of DNA fragments according to size (Deurenberg *et al.*, 2007; Palvecino, 2007). For *S. aureus* the *SmaI* restriction enzyme is used for macro-restriction of DNA to allow the fragments to move across the gel (Kim, 2009). Tenover *et al* (1995) proposed a scheme for the analysis of PFGE patterns using the Dice coefficient and unweighted pair-group matching analysis (UPGMA) settings (Deurenberg *et al.*, 2007).

The foremost disadvantage of PFGE and other methods that comparatively analyses DNA fragments on gels, is the difficulty in comparing the results obtained in different laboratories due to differences in PFGE protocols and nomenclature. At a national level, databases have been established to adhere to standardized protocols. However, international efforts have been unsuccessful in producing common nomenclature and databases (Deurenberg *et al.*, 2007; Strommenger *et al.*, 2008a). Another disadvantage of PFGE is that the equipment required is relatively expensive and the method is labour intensive. Results are obtained within two to four days depending on which organisms need to be tested (Moodley *et al.*, 2006; Tenover *et al.*, 1997; van Belkumm *et al.*, 2007). Nonetheless, once this method has been established in a laboratory, it can be used to differentiate between strains of a variety of bacterial species with minimal changes necessary.

Chang *et al* (2003) used PFGE to differentiate between *S. aureus* isolates in recurrent episodes of bacteraemia in a multicentre prospective study. In this study, recurrence could then further be subdivided into re-infection (different PFGE patterns) or relapse (the same PFGE patterns). The results showed that relapse occurred earlier than re-infection (median, 36 and 99 days respectively, p<0.06). Another study published in 1995 reported that at that time PFGE was the most widely used molecular typing method for studying the local and global epidemiology of MRSA (Bannerman *et al.*, 1995). More recently, Cooksen *et al* (2007) showed that PFGE (using common protocols) and *spa* typing data from different laboratories can be compared to reference patterns or sequences which correlated well with the more expensive MLST/SCC *mec* typing method.

1.3.2 Multi-Locus Sequence Typing (MLST)

MLST, which was developed by Maiden *et al* (1998), characterizes isolates for each species by relying on sequences of internal fragments of housekeeping genes (Maiden *et al.*, 1998). Housekeeping genes are present with variations in each species, resulting in various alleles of each locus. For *S. aureus*, seven housekeeping genes of about 450 base pair (bp) each are generated by PCR and sequenced in both directions (Fischetti *et al.*, 2006; Trindade *et al.*, 2003). These genes include the following: carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE)*, glycerol kinase (*glpF*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), triosephosphate isomerase (*tpi*), and acetyl coenzyme A acetyltransferase (*yqiL*) (Deurenberg *et al.*, 2007; Fischetti *et al.*, 2006; Ward *et al.*, 1981).

Gene sequences that are identical are allocated the same allele number. The combination of alleles at each locus, for each strain, defines its sequence type (ST) (Enright *et al.*, 2002). Furthermore, this typing technique has been developed and validated for *S. aureus* strains, providing good discriminatory power for investigating the clonal evolution of MRSA. The MLST online database, http://www.mlst.net, is easily accessible via the internet to compare DNA sequences to previously identified alleles at each locus (Kim, 2009). Also, clonal complexes (CCs) can be used to analyse evolutionary events through this database (Enright *et al.*, 2002). The CCs are based upon related sequence types (BURST). If five of the seven housekeeping genes

of *S. aureus* strains have exactly the same sequences, they are grouped together within a single CC by BURST analysis (Deurenberg *et al.*, 2007). Enright *et al* (2000) have successfully used BURST in studying the evolutionary history of MRSA (Enright *et al.*, 2000). The MLST database is easily accessible for local and international surveillance purposes as clinical information and geographic locations can be stored for each isolate (Kim, 2009).

Enright *et al* (2000) validated the procedure of MLST using PFGE as a reference method. Two major clones that are circulating in British hospitals were found in this study namely, clone EMRSA-16 (ST36) and clone EMRSA-15 (ST22). When tested with MLST and confirmed by PFGE, a close similarity was found in three STs (12, 15, 22) containing both MRSA and MSSA strains. Enright and colleagues (2002) considered a close relationship between the genotypes of MRSA and MSSA strains with the same allelic profiles. Furthermore, this suggested that MRSA strains were descendents of MSSA strains that acquired methicillin resistance through horizontal transfer of the *mecA* gene. Even though MLST is a highly discriminatory method of characterizing bacterial isolates, it can also be a quite laborious and time-consuming method (Deurenberg *et al.*, 2007; Enright *et al.*, 2002; Hallin *et al.*, 2007). MLST requires the performance of seven PCRs and 14 DNA sequencing reactions per isolate, resulting in very high costs (Cookson *et al.*, 2007; Kim, 2009). This makes MLST difficult to conduct in a routine laboratory, unless the necessary equipment and facilities are readily available for PCR and sequencing (Harmsen *et al.*, 2003; Palvecino, 2007). Moreover, MLST is more useful in studying long-term genetic changes in contrast to PFGE which is used to study genetic changes and hospital outbreaks during short periods of time (Arakere *et al.*, 2005).

1.3.3 SCC*mec* Typing

The importance of SCC*mec* typing is being recognized as an increasing amount of community acquired clonal outbreaks is occurring around the world (Zhang *et al.*, 2005). In the past, traditional PCR methods were used to type SCC*mec* genes. These methods were problematic due to the need of many primer sets and multiple individual PCR testing (Zhang *et al.*, 2005). As a consequence, multiplex PCR methods were developed that are more rapid and useful in both the clinical and

research laboratory settings. This method simultaneously amplifies two or more loci in the same reaction. A disadvantage of this method is that it requires an increasing amount of primers as new alleles are being discovered rapidly (Cookson *et al.*, 2007).

Oliveira *et al* (2002) developed a multiplex PCR method for SCC*mec* typing. This method includes the detection of *mecA* and six different loci on SCC*mec* (Deurenberg *et al.*, 2007). Milheirico *et al* (2007) reported an updated version to improve characterization of SCC*mec* type IV. In addition, this method detects the recently discovered SCC*mec* type V. Amplification by PCR of the *mec* and *ccr* complexes has been developed by Ito *et al* (2001) and Okuma *et al* (2002). Nonetheless, characterization of SCC*mec* type of the same MRSA strain gave diverse results with this method (Deurenberg *et al.*, 2007; Okuma *et al.*, 2002). Furthermore, a real-time PCR developed by Francois *et al* (2004) also characterized SCC*mec* types I-IV on the basis of the *mec* and *ccr* gene complexes (Francois *et al.*, 2004). Zhang *et al* (2005) developed a multiplex PCR assay that competently detects and classifies all the currently described SCC*mec* types (I-V), as well as their major subtypes.

A novel classification scheme has been proposed by Chongtrakool *et al* (2006) for the nomenclature of SCC*mec*. A number is assigned to the *ccr* genes, whereas an upper-case letter is assigned to the *mec* complex for example, SCC*mec* type 1A (type I) (Chongtrakool *et al.*, 2006; Deurenberg *et al.*, 2007). Initially, in 2002, it has been proposed that a standard nomenclature for MRSA classification be used based on the MLST, resistance phenotype and SCC*mec* type. This proposal has been accepted by the International Union of Microbiological Societies subcommittee on *S. aureus* typing. This nomenclature may however change, as other genotypic methods such as *spa* typing are becoming more popular (Kim, 2009).

1.3.4 *spa* Typing

For *S. aureus*, a single-locus sequence typing (SLST) method was developed by Frenay *et al* (1996) by sequencing the polymorphic X region of the *S. aureus* protein A (*spa*) gene. As mentioned in section 1.2.7 (Chapter 1), this region contains a variable number of 21-24 bp repeats mainly due to deletions, duplications or point mutations within the repeat sequence (Deurenberg *et al.*, 2007).

Spa typing and MLST are the most frequently used sequence-based typing methods for *S. aureus* (Faria *et al.*, 2008). However, *spa* typing involves the sequencing of only a single locus, making it much simpler and more affordable than MLST (Karynski *et al.*, 2008). *Spa* typing has discriminatory power that lies between that of PFGE and MLST. A recent study conducted by Faria *et al* (2008) showed that *spa* typing had a great predicative power of the clonal relationships defined by eBURST (software analysis of MLST method). PFGE was less precise for that purpose. *Spa* typing provides improved typeability and discriminatory power, compared to MLST. According to their results, PFGE together with *spa*-typing provided much better results especially for MRSA strains, whereas *spa* typing alone would be sufficient for MSSA strains. However, irrespective of methicillin resistance, the most affordable combination of methods would be PFGE and *spa* typing without needing to perform MLST. Faria and colleagues (2008) concluded that the use of these two methods for local and long-term epidemiological surveillance studies provides the necessary discriminatory power and typeability. Similarly, a study conducted by Robinson & Enright (2003) showed the diversity of *spa* typing to be greater than MLST.

Other studies found *spa* typing to be more rapid and easier to perform and interpret than other genotyping methods (Palvecino, 2007). Hence, *spa* typing can be used to investigate the epidemiology of hospital or community outbreaks and the molecular evolution of MRSA and MSSA strains (Deurenberg *et al.*, 2007). *Spa* typing may also be useful for long-term epidemiological and population-based studies (Kahl *et al.*, 2005).

Ridom StaphType software (Ridom GmbH, Würzburg, Germany) has generally been used for the analysis of *spa* sequences in Europe. This software package determines various *spa*-types and *spa*-repeats, as well as implementing Based upon repeat pattern (BURP) clonal cluster analysis of *spa* types. The software also allows data management and retrieval, as well as Internet-based assignment of new *spa* types with automatic quality control of DNA sequence chromatograms (Harmsen *et al.*, 2003). Strains that are typed are easily synchronized via the Internet with a central *spa* server (http://www.spaserver.ridom.de) so that nomenclature and access to the *spa* typing data can be viewed universally. New *spa* types and repeats can be submitted online to

be included into the reference database. The server is curated by the European SeqNet.org initiative which currently includes 57 laboratories and 29 European countries (http://www.seqnet.org). At present the *spa* server database consists of 5501 *spa*-types, consisting of 311 *spa*-repeats from a total number of 92 900 isolates that were typed in 66 countries. A rapid increase in numbers can be immediately noticed as only 1200 *spa*-types, 100 *spa*-repeats from a total number of 13 000 isolates typed in 36 countries was reported in the year 2007 (Deurenberg *et al.*, 2007). Hence, for *S. aureus* this software package is one of the most leading sequence-based typing databases accessible all over the world (Deurenberg *et al.*, 2007). The database can also be used to continuously collect *spa* typing data for infection control purposes. If preferred, an automatic warning can be assigned into the database in order to detect MRSA outbreaks in hospitals or regions with heterogeneous *spa* types.

Several *spa* types can be detected within one sequence type (ST), as determined by MLST due to the high discriminatory power of *spa* typing. In addition, these *spa* types are still assigned to a clonal cluster. The accomplishment of BURP analysis makes clonal cluster groups of *spa* types easier to determine. It is suggested that future studies should interpret BURP analysis together with clonal complexes (CCs) that are established by MLST (Deurenberg *et al.*, 2007). Strommenger and colleagues (2008) as well as other studies demonstrated that *spa* type groups obtained by BURP analysis were in agreement with the classifications obtained by other methods such as MLST-BURST analysis or *SmaI* macrorestriction and cluster analysis (Hallin *et al.*, 2007; Ruppitsch *et al.*, 2006; Strommenger *et al.*, 2006). Furthermore, a study done by Mellmann and colleagues (2008) reported BURP clustering to be 96.5% in concordance with MLST and a 94.9% in concordance with PFGE (Mellmann *et al.*, 2008).

In general, single locus *spa* typing seems to be gaining more popularity as a useful and reliable method, in contrast to typing seven housekeeping genes in MLST. *Spa* typing might also replace MLST as the typing method of choice, since the sequencing of one gene is much more affordable and quicker to perform than sequencing seven genes for each strain. The RidomStaph software package, including BURP analysis for MRSA and MSSA types is user friendly, easy to interpret and performs well.

1.3.5 Multiplex PCR of the *mecA, pvl* and *spa* genes

Multiplex PCR is a technique that amplifies more than two loci in a single PCR reaction. A recent study conducted by Larsen *et al* (2008), validated a method to detect the presence of the *mecA, pvl* and *spa* genes in a single multiplex PCR. In addition, this multiplex PCR method allows the sequencing of one PCR gene (*spa* gene) for *spa* typing directly from the mixture of PCR amplicons (*mecA; pvl* and *spa* genes). After successful validation, this method was introduced routinely at the Statens Serum Institute (SSI), Copenhagen, Denmark during January 2007. Implementation of this method at SSI minimises the use of reagents and the amount of PCRs required. The method also provides reduced risk of sample mishandling and offers rapid typing results. Thus, the multiplex PCR method described in this study proves to be less expensive and less laborious than the single reaction methods for the detection of these three important genes used for MRSA surveillance (Larsen *et al.*, 2008).

1.4 <u>Genotyping of *S. aureus* in South Africa</u>

In 2007, the first report documenting a variety of MRSA epidemic clones throughout South Africa was presented (Oosthuysen *et al.*, 2007). MRSA isolates were collected from private and state laboratories from 9 provinces in South Africa. Phenotypic methods were used to identify and determine resistance profiles. Genotypic methods included SCC*mec* typing using multiplex PCR, *spa* typing and PCR for the detection of PVL toxin. Of the 302 MRSA isolates tested for *SCCmec* typing, the most predominant types included type IV (36.7%), type II (25.2%) and type III (23.2%), including its variant type IIIA. SCC*mec* type I and IA amounted to 13.2%, whereas 1.7% of these isolates were non-typeable. For *spa* typing, 27 different *spa* types were found and grouped into five *spa* clonal complexes (*spa*-CC) via BURP analysis. Interestingly, only 2/314 MRSA strains (0.6%) were detected as PVL-positive in this study. This is in contrast to studies from Tristan *et al* (2009) and Aires-de-Sousa *et al* (2006), reporting an association between CA-MRSA and PVL. However, the study did not include clinical information with regards to hospital and community acquired

infections. It was concluded that the population structure of MRSA consists of different epidemic clones.

In 2008, ninety-one MRSA strains from hospitals within Cape Town (Western Cape Province, South Africa) were collected to determine the SCC*mec* contents (Jansen van Rensburg *et al.*, 2009). It was found that SCC*mec* types IV (46.1% of strains) and I (40.7% of strains) were the most prevalent. MLST was used to analyse four strains representing each of SCC*mec* types I-IV. Sequence types (STs) of SCC*mec* types I, II and III strains were STs 5, 36 and 239, which are frequently identified worldwide. However, the ST of SCC*mec* type IV strain, ST 612 is not found frequently elsewhere. This suggests the emergence of a local clone.

Zinn *et al* (2004) obtained *S. aureus* strains from twenty one hospital laboratories globally. They found high levels of MRSA (28-63%) in South Africa, the United States and Asia, and Southern European countries (Zinn *et al.*, 2004). A dominating MRSA clone as well as several other clones was distinguished as international epidemic MRSA (E-MRSA) by typing with PFGE.

In 2006, a study was conducted in the KwaZulu-Natal (KZN) province, South Africa in order to investigate the antimicrobial resistance of *S. aureus* (Shittu & Lin, 2006). Detection of the *mecA* and *nuc* genes by PCR confirmed the identification as MRSA of strains resistant to oxacillin, methicillin and cefoxitin by the disk diffusion method. In addition, resistance to mupirocin was confirmed by the MIC value with E-test, as well as the detection of the *mupA* gene. This study was able to successfully differentiate between MSSA and MRSA strains with PCR-RFLP (polymerase chain reaction- restriction fragment length polymorphism) of the coagulase gene.

Shittu *et al* (2007) published the first report on the molecular identification and characterization of 5/227 (2.2%) mannitol-negative MRSA isolates from clinical samples in KZN, South Africa. Phenotypic tests such as MSA (mannitol fementation), coagulase (tube method) and DNase tests, as well as PCR detection of the *nuc, mecA* and coagulase gene signified mannitol-negative isolates as *S. aureus*. However, 2 mannitol-negative MRSAs were misidentified as *S. lugdunensis* by the API STAPH (Biomerieux, France) test. These MRSA isolates were confirmed as being positive with detection of the *mecA* gene. All of these isolates carried SCC*mec* type IV.

In 2009, a study was done on the phenotypic and molecular characterization of *S. aureus* isolates with low-level (in KZN, South Africa) and high-level mupirocin resistance (in Nigeria and South Africa) (Shittu *et al.*, 2009). Mupirocin is a topical antimicrobial agent used to treat skin and postoperative wound infections, including the prevention of nasal carriage of MRSA. Antibiogram, PCR-RFLP of the coagulase gene and PFGE was performed on 17 mupirocin-resistant (high-level) *S. aureus* isolates obtained from clinical samples from Nigeria and South Africa. PCR detection of the *mupA* gene confirmed high-level mupirocin resistant in these isolates. The transfer of the mupirocin plasmid by curing and conjugation experiments encoding high-level mupirocin resistance was determined in this study. Authors found high-level mupirocin resistance by the conjugative transfer of the 41.1 kb plasmid alone or with co-transfer with other resistance determinants.

Essa *et al* (2009) published a report on the first multilocus sequence typing (MLST) of 241 *S. aureus* strains collected from sixteen public hospitals in Kwazulu-Natal during January 2001 to December 2002. Randomly selected MRSA strains were subjected to agar-based methods, the E-test, screen latex agglutination methods and confirmed with detection of the *mecA* gene by PCR. The mobility of SCC*mec* type IV amounted to 79% with STs 5, 8 and 45, respectively. Also, ST 239 was found in SCC*mec* type III (13%), whereas ST8 was found in SCC*mec* type II (8%), respectively (Essa *et al.*, 2009).

Another study conducted in Kwazulu-Natal during March 2001 to August 2003, characterized MRSA isolates (Shittu *et al.*, 2009). Genotyping included PFGE, MLST, SCC*mec* and *spa* typing. PFGE of *Sma*I- digested genomic DNA indicated seven types assigned A-G, with types A, F and G most commonly present. These three major pulsotypes together with the other typing techniques provided useful information on the geographical distribution of MRSA clones in KZN province.

In 2008, a study was published on the molecular characterization of *S. aureus* isolates, acquired from uncomplicated skin infections during five phase III global clinical trials of retapamulin, a recent topical antibiotic agent (Goering *et al.*, 2008). Genotyping techniques included MLST, PFGE, SCC*mec* (MRSA only) and PVL analysis. The most common PVL-positive methicillin-resistant clone was isolated in the United States, with a SCC*mec* type IV, multilocus sequence type 8 and a pulsed-field type

USA300. Interestingly, the most common PVL-positive methicillin-susceptible clone was found in South Africa and the Russian Federation with a multilocus sequence type 121 and pulsed-field type USA1200. All other clones were limited in their geographic distribution and frequencies. This study demonstrated extensive genetic diversity within pulsed-field types and MLST clonal complexes throughout the world.

Similarly, a study by Campbell *et al* (2008) genotyped a collection of *S. aureus* strains collected for an international clinical trial (FAST II) evaluating telavancin in order to treat complicated skin and skin structure infections (cSSSI). This trial included 99 *S. aureus* isolates from 11 sites in the United States (56 isolates) and 7 sites in South Africa (34 isolates). Genotyping consisted of SCC*mec, agr* and PFGE to determine the presence of 31 virulence genes. Briefly, results from South Africa showed that it's more common for MSSA isolates to carry certain virulence genes which include *sdrD, sea* and *pvl.* In contrast, MRSA isolates from the United States were more common to carry these virulence genes than in MSSA isolates. The outcome was that virulence genes are distributed differently within various geographic regions (Campbell *et al.*, 2008).

It was believed that the proliferation of MRSA strains resulted from the global distribution of a few highly epidemic clones. However, Nübel *et al* (2008) provided substantial data in their study stating that the population of MRSA in one of the clones (ST5) is geographically structured. This proves that MRSA emerged very frequently in various regions of the world through independent imports of methicillin resistance into their genomes (Nübel *et al.*, 2008). South African studies are therefore needed in order to understand the clonal distribution of *S. aureus.*

Data on the genotypic characteristics of local *S. aureus* strains in relation to the clinical presentation of *S. aureus* infections, in particular with regards to persistent infection, and community-acquired versus hospital acquired infections, is lacking. In this present study conducted at Tygerberg Hospital, we have decided to implement the multiplex PCR method discussed in section 2.1.5. This is the first epidemiological study of *S. aureus* that has been conducted at Tygerberg Hospital, and the first to our knowledge in the region that included *spa* typing, together with the detection of the *mecA* and *pvl* genes.

The information provided in this study contributes to our understanding of the local epidemiology of *S. aureus* and the pathogenesis of different strains at Tygerberg hospital.

CHAPTER TWO

2 AIM AND OBJECTIVES OF STUDY

2.1 <u>Aim of this study</u>

The aim of this study is to describe specific genotypic characteristics of *S. aureus* strains causing bacteraemia in our setting. This study forms part of a larger study "Characterization of *Staphylococcus aureus* causing bacteraemia in patients admitted to Tygerberg Hospital, Western Cape Province, South Africa" (approved by the ethics committee on 2 June 2008; N08/02/046).

2.2 <u>Objectives</u>

- To characterize staphylococcal isolates, from patients with staphylococcal bacteraemia, according to the presence of *mecA* and *pvl* genes, as well as to strain-type these isolates (stored blood culture isolates from study: N08/02/046) using molecular typing methods.

- To compare genotypic profiles of staphylococcal strains (obtained from *spa* typing) with profiles of international important clones.

- To determine clonal cluster groups by BURP (Based Upon Repeat Patterns) analysis via RidomStaph software.

- To analyse the *spa* typing data, as well as the presence of the *pvl* gene, together with prospectively collected clinical data for possible organism-related factors associated with persistent and recurrent bacteraemia (clinical data will be obtained from the database of study: N08/02/046)

- To analyze MSSA and MRSA strains; community-acquired, health-care associated and hospital-acquired *S. aureus* strains in order to better understand the epidemiology of staphylococcal bacteraemia in patients admitted to Tygerberg Hospital.

CHAPTER THREE

3 MATERIALS AND METHODS

3.1 <u>Study Design</u>

This research study takes the form of a prospective descriptive study. The study was conducted in the molecular laboratory of the Division of Medical Microbiology, Tygerberg hospital. This study forms part of a larger Staphylococcal project that aims to describe the clinical course of deep-seated Staphylococcal infections in Tygerberg hospital, and to phenotypically and genotypically characterize the strains responsible for these infections. The larger project also aims to describe the incidence of persistent *S. aureus* bacteraemia in patients admitted to this hospital. Furthermore, clinical and organism-related risk factors associated with persistent infections in the population group will be identified in this larger project. In this specific study, specific genotypic characteristics of *S. aureus* strains causing bacteraemia in patients admitted to Tygerberg hospital were described.

3.1.1 Ethical Considerations

This research project is incorporated as part of a leading Staphylococcal group study, namely the 'Characterization of *Staphylococcus aureus* causing bacteraemia in patients admitted to Tygerberg hospital, Western Cape Province, South Africa', and the genotypic analyses that was undertaken in this study, comprised part of the objectives set for this larger study. Ethical approval for the larger integrated study has been granted on the 2nd June 2008, by the Committee of Human Research at Stellenbosch University (Project number: N08/02/046).

For this specific study, ethical approval has also been granted by the same committee on the 8th October 2008 (Project number: N08/09/252). Patient consent for the storage of clinical isolates and data was obtained as part of the larger study, whereby the necessary clinical information regarding patient demographics and clinical data was collected by a research assistant (nursing practitioner). This study required no

additional samples or clinical information, as the study material consisted of subcultures from the stored strains. As in the larger study, patient identity remained anonymous, as all samples were coded with specific study numbers.

3.2 Phenotypic testing of bacterial strains

Staphylococcal isolates were obtained from routine blood cultures processed at the NHLS diagnostic Medical Microbiology laboratory at Tygerberg Hospital. The collection studied included all methicillin-susceptible *S. aureus* (MSSA) strains as well as methicillin-resistant *S. aureus* (MRSA) strains. Mixed cultures and duplicate isolates from subsequent blood culture specimens taken less than 3 days after appropriate therapy initiated, were excluded from this study. All isolates were identified routinely in the laboratory by conventional tests, according to the laboratory's standard operating procedures (SOPs). These tests included Gram stains, deoxyribonuclease or DNase test, mannitol salt agar (MSA) test and a latex agglutination test (Pastorex® Staph-Plus test, Bio-Rad, Marnes-la-Coquette, France). Resistance to methicillin was detected with the cefoxitin disk (30μg) diffusion test (Kirby Bauer method) and interpreted according to CLSI criteria (CLSI document M100-S19. Wayne, Pennsylvania). Furthermore, the vancomycin agar screen method was used to screen the strains for reduced resistance to vancomycin. Also, a standard vancomycin minimum inhibitory concentration (MIC) test was performed on all MRSA strains using the E-test method (AB Biodisk, Solna, Sweden). In cases where the MIC result was 1.5-2 μg/ml, an E-test macromethod (AB Biodisk, Solna, Sweden) was performed to detect possible heteroresistant glycopeptide intermediate *S. aureus* (h- GISA). All agar plates that were used in the laboratory and in this study were prepared by Greenpoint media laboratory, Cape Town.

Briefly, the Gram stain differentiates between Gram positive and Gram negative bacteria. A suspension of the organism to be stained is mixed with a drop of saline solution on a glass slide. The smears are then fixed by flaming and then stained consecutively with crystal violet, iodine, a decolourizer (alcohol) and counterstained with carbol fuchsin. Consequently, Gram positive bacteria will stain purple, whereas Gram negative bacteria will stain pink.

For the DNase test, each plate was inoculated with a single colony of up to 8 selected strains. These included positive (*S. aureus* ATCC 25923) and negative (*S. epidermidis* ATCC 49134) control strains. The plates were incubated at 35ºC for 24 hours, followed by flooding of the plate with 1M HCl. After about a minute, the excess HCl was discarded, with a positive result indicated by a clear zone around the inoculum, whereas with a cloudy precipitate of non-hydrolysed DNA around the inoculums were interpreted as a negative result.

MSA plates were also inoculated with a single colony of up to 8 selected strains, including control strains. The plates were incubated at 35ºC for 24 hours. Yellow colonies indicated mannitol fermentation (*S. aureus*), whereas pink colonies with no colour change to the agar indicated no fermentation of mannitol, and documented as MSA negative.

A positive DNAse and MSA confirmed the identification of *S. aureus*. A negative DNAse and MSA was further analysed as coagulase negative Staphylococci.

A latex agglutination test (Pastorex Staph Plus test®, Bio-Rad, Marnes-la-Coquette, France) was performed when the DNase and MSA tests were inconclusive or not in accordance. This test detects the fibrinogen affinity (clumping factor), protein A, and capsular polysaccharides of *S. aureus*. A drop of the latex test reagents were deposited onto one of the circles on the agglutination card provided in the kit. Negative and positive controls were deposited in one of the other circles. A few colonies were emulsified onto the drop of latex by means of a plastic stir rod. The card was gently rotated to homogenize the latex reagents with the colonies. After 30 seconds, a positive reaction produced aggregates of latex particles with a cloudy, pink background. A negative reaction did not produce aggregates and appeared to be milky.

The coagulase test was performed to differentiate between *S. aureus* and coagulase negative *Staphylococcus* (CoNS) species. This test was only carried out where there were discrepancies between genotypic and phenotypic results with regard to *S. aureus*. Briefly, an isolated staphylococcal colony was inoculated into a glass tube

containing 0.5 ml of rabbit plasma (Bio-Rad, Marnes-la-Coquette, France). The tubes were incubated at 35 to 37°C for 4 to 24 hours. After 4 hours of incubation, the tubes were observed for the presence of a clot. However, if no clot was observed after 4 hours, the tubes were re-incubated and observed again after 24 hours. A clot formation at 4 or 24 hours indicated a positive reaction, confirming the identification as *S. aureus*. If no clot formed, and the plasma continued to flow freely once the tube is tipped, the organism was considered to be negative and identified as a CoNS. *S. aureus* ATCC 25923 and *S. epidermidis* ATCC 14990 were used as quality controls for this test.

The Kirby Bauer disk diffusion antibiotic susceptibility testing method was performed according to the CLSI guidelines (CLSI document M100-S19, Wayne, Pennsylvania, January 2009). Single colonies were suspended in 1ml sterile saline solution until a turbidity of 0.5 McFarland (McF) (1.5×10^8 cfu/ml) was reached. The suspension was evenly spread onto Mueller-Hinton agar plates with a sterile cotton swab. Thereafter, a 30μg cefoxitin antibiotic disk was placed onto the inoculated plate and incubated at 35ºC for 24 hours. Zone diameters were measured with the use of sliding callipers and interpreted according to the CLSI criteria for *S. aureus*. The criteria states that zone diameters greater or equal to 22mm are susceptible and can be classified as methicillin-susceptible *S. aureus* (MSSA), whereas zone diameters less than or equal to 21mm indicate methicillin-resistant *S. aureus* (MRSA).

For the vancomycin agar screen method, brain-heart infusion (BHI) agar plates were used with a vancomycin concentration of 6μg/ml. A few colonies grown on blood agar plates were suspended in sterile saline solution to produce a suspension matching a turbidity of 0.5 McF. Bacterial suspensions were spotted onto BHI agar with an area of 10-15 mm diameter. This was done by placing a swab in the suspension and expressing the excess liquid. Plates were then incubated at 35°C for 24 hours before being viewed. Reduced susceptibility to vancomycin is indicated by more than one colony or a light film of growth. Up to eight isolates and two quality control organisms were tested per plate. Quality control was performed using *Enterococcus faecalis* ATCC 29212 as the susceptible control and *Enterococcus faecalis* ATCC 51299 as the resistant control.

In the laboratory, vancomycin MICs was determined by using the E-test method (AB Biodisk, Solna, Sweden). E-tests consist of plastic coated strips calibrated with minimum inhibitory concentration (MIC) values covering 15 two – fold dilutions. A predefined antibiotic gradient is impregnated on the opposite side of the MIC scale. The MIC is read at the point where the growth intersects the E strip. A few colonies grown overnight were inoculated into sterile saline solution until a turbidity of 0.5 McF was reached. Suspensions were swabbed in three directions over the entire Mueller Hinton agar plate. Vancomycin E-test strips was applied after plates were air dried for 10 minutes. The plates were then incubated at 35 °C for 24 hours. *S. aureus* ATCC 29213 was used to quality control the method. MIC results were interpreted according to CLSI criteria (Table 3.1).

An E-test macromethod (AB Biodisk, Solna, Sweden) was performed on those strains with an MIC of 1.5-2 μg/ml. The E-test macromethod was performed in the same manner as the standard E-test method. However, a heavier inoculum adjusted to 2 McFarland standard was used to inoculate brain-heart-infusion agar medium (BHI). Plates were read after 24 and 48 hours incubation at 35 °C in ambient air. *S. aureus* ATCC 29213 was used as quality control. Readings were recorded and interpreted according to the AB Biodisk guidelines: GISA/hGISA: vancomycin ≥ 8 and teicoplanin ≥ 8, or teicoplanin ≥ 12 (EAS 003, AB Biodisk).

Table 3.1: 2009 CLSI Interpretive criteria (μg/ml) for MIC testing of *S. aureus*

Glycopeptides	MIC (μg/ml) Interpretive standard for *S. aureus*		
	Susceptible	Intermediate	Resistant
Vancomycin	≤ 2	4 - 8	≥ 16
Teicoplanin	≤ 8	16	≥ 32

In this study, all stored *S. aureus* isolates from the integrated study (N08/02/046) that were collected since March 2008 up until May 2009 were included. In addition, repeat isolates from persistent bacteraemia (positive blood culture ≥3 days after appropriate

therapy initiated) and from recurrent bacteraemia (defined as the return of *S. aureus* bacteraemia after documenting a negative blood culture and/or clinical improvement after the completion of a course of appropriate antibiotics) were included (Chang *et al.*, 2003). After strain typing, recurrent bacteraemia was subdivided into re-infection (different strain types) and relapse (the same strain type). Clinical and demographic data were prospectively collected and entered on the computer database for *S. aureus* as part of the larger study (N08/02/046).

3.2.1 Analyses of clinical data

The origin of bacteraemia was considered hospital acquired (HA) if a positive blood culture specimen was collected more than 48 hours after admission without evidence of a *S. aureus* infection at the time of admission. If the patient was transferred from another hospital, the duration was considered from the date of the first hospital admission. Health-care associated (HCA) bloodstream infections were defined by positive blood cultures at the time or within 48 hours of hospital admission from patients with the following history:

- Patients who have been to a hospital or hemodialysis clinic or obtained intravenous chemotherapy within 30 days before the infection
- Patients who have been hospitalized for 2 or more days within 90 days (3 months) before the infection
- A patient who is a resident in a nursing home or long-term care facility (Friedman *et al.*, 2002)

Community acquired bloodstream infections were defined by a positive blood culture specimen collected in an outpatient setting or within 48 hours of hospital admission from patients without a history of health-care exposure (Deurenberg *et al.*, 2007; Friedman *et al.*, 2002)

The catheter and prosthetic-device related sepsis clinical category (CSI) consisted of intravascular catheter as well as other catheter related infections and prosthetic-device related infections. Catheter sepsis was defined as the isolation of the same *S. aureus* strain (identical antibiograms) from a quantitative ($>10^2$ CFU/catheter segment) culture of the catheter tip as from the blood culture and/or clinical evidence of catheter site infection (Liao *et al.*, 2008). Other prosthetic device related sepsis were

defined as the isolation of the same organism from the culture of the prosthetic device and/or clinical evidence of prosthetic device infection. Septic arthritis (SA) was defined as inflammation of a joint due to *S. aureus* infection. This category included osteomyelitis (Liao *et al.,* 2008). Infective endocarditis (IE) was defined according to Duke criteria, as microorganisms demonstrated by culture or histological examination of a vegetation, a vegetation that has embolized, or an intracardiac abscess specimen. The definition also includes pathological lesions; vegetation or intracardiac abscess confirmed by histological examination showing active endocarditis (Durack *et al.*, 1994). Skin and soft tissue infections (SSTI) was defined as clinical skin and soft tissue infection with bacteraemia (Liao *et al.,* 2008). Pneumonia (PN) was defined as a positive culture for *S. aureus* in purulent sputum samples and the presence of newly developed lung infiltrates; or the patient had a positive blood culture, pulmonary infiltrates and no other obvious source of a staphylococcus infection (Liao *et al.,* 2008). If no primary focus could be identified, the bacteraemia was defined as primary (PB) (Harbarth *et al.*, 1998). Isolates were classified in the unknown clinical category when the clinical significance was not clear and blood culture contamination was considered e.g. no clinical evidence of infection, no antimicrobial therapy given, and normal septic parameters such as white cell count and C-reactive protein.

3.2.2 Collection and storage of bacterial strains

All isolates were collected daily from the routine blood culture laboratory at Tygerberg Hospital. The relevant microbiological and clinical data was recorded onto specific forms (appendix A) and then captured onto an excel sheet for the staphylococcal database. Colonies of pure staphylococcal isolates were selected from Mueller-Hinton plates by using a sterile cotton swab. The colonies were then inoculated into MicroBank[TM] (Pro-Lab Diagnostic, Cheshire, UK) beads (Figure 3.1). Each vial contains numerous beads for subculture of the original organism isolated.

Figure 3.1: Illustration of MicroBank storage beads

MicroBank[TM] is a sterile cryovial, containing approximately 25 porous beads that are preserved in cryobroth solution (Figure 3.1). The beads allow the microorganism inoculated to readily adhere onto its surface. The excess cryobroth was aspirated by using a glass pipette, thereby leaving the inoculated beads free of liquid. The cryovial was labelled according to the allocated study numbers. After inoculation, each cryovial was stored at −80 ºC until required for further testing. This method was conducted aseptically and according to the instructions specified by the manufacturer.

3.2.3 Preparation of bacterial strains for genotypic analysis

All work was performed under aseptic conditions in the Medical Microbiology laboratory. When stored isolates were required, a single bead was removed from its cryovial and used directly to inoculate a suitable bacteriological culture medium as follows:

A single bead was placed into a glass tube containing 2ml Mueller Hinton broth with the use of a sterile inoculating loop. The tube was then vortexed in order to release the micro-organisms adhering to the bead into the broth. Thereafter, tubes were incubated aerobically at 37 °C, where the turbidity of the broth was monitored. If broths were clear after a few hours of incubation, tubes were incubated overnight and evaluated the following day for turbidity. The turbidity was assessed against a laboratory standard, to fulfil the CLSI requirement of 0.5 McFarland, which indicate colony forming units (CFU) of 1.5 x 10^8 organisms / ml. The staphylococcal suspensions in the turbid tubes were cultured onto Tryptose blood agar plates and incubated for a further 24 hours at 37 °C. Effective streaking techniques were used in order to produce single colonies from the plates for further experimental testing of these isolates.

3.3 DNA preparation for Multiplex PCR

InstaGene[TM] matrix solution (Bio-Rad, Marnes-la-Coquette, France) rapidly isolates small amounts of genomic DNA for polymerase chain reactions (PCR's). The solution eliminates various steps that are required in traditional genomic DNA purification methods. These include deproteinization, organic extraction, dialysis and alcohol

precipitation. An alternative method can also be used instead of the InstaGene matrix method, by using the enzyme lysozyme or lysostaphin for cell wall lysis. However, this alternative method requires a suitable DNA preparation kit and proves to be more expensive and time consuming. For this reason, the InstaGene Matrix method was preferred in this study as it reduces the use of reagents and proved to be less expensive and easier to perform.

InstaGene includes a cell lysis boiling step in the presence of the matrix. The cell lysis products interfering with the process of PCR amplification are rapidly absorbed by the matrix. The InstaGene matrix method includes DNA extraction protocols suitable for PCR amplification from whole blood, cultured mammalian cells and bacteria. For this study, the procedure for bacteria was performed according to the manufacturer's instructions (InstaGene[TM] Matrix; Catalog # 732-6030: Bio-Rad, Marnes-la-Coquette, France).

The InstaGene DNA preparation method was executed by selecting a few colonies that grew overnight on blood agar plates (refer to section 3.2.2). These colonies were suspended into 2ml reaction tubes containing 500µl of distilled water. The tubes were then vortexed, followed by immediate centrifugation at 12.000 x g for 60 seconds. The excess liquid, referred to as the supernatant, was removed from the tubes with the use of a pipette tip, whereby 100µl of the 6% InstaGene matrix solution (Bio-Rad, Marnes-la-Coquette, France) was added to the remaining pellet in the tube. Thereafter, all tubes were incubated in a water bath at 56°C for 20 minutes. Following the incubation period, all tubes were thoroughly vortexed, and placed immediately in a heating block for 8 minutes at 100°C. After the boiling step, all tubes were again thoroughly vortexed and centrifuged at 12.000 x g for 3 minutes. Finally, 80µl of the supernatant containing the DNA was transferred into new 1.5ml reaction tubes. All the tubes containing the final DNA products were stored at -20°C until further testing with multiplex PCR.

3.3.1 Measurement of DNA concentrations

The DNA concentrations of all these products were measured with a Nanodrop ND-1000 Spectrophotometer V3.1.0 instrument (Nanodrop Technologies, Inc., Wilmington, DE, USA) at the Division of Virology, Stellenbosch University. Briefly,

a spectrophotometer is an instrument measuring the amount of molecules that absorb a specific wavelength of energy. The nanodrop has the ability to analyse 1 to 2 µl samples which can either be DNA, RNA, proteins, dyes or microbial cell culture.
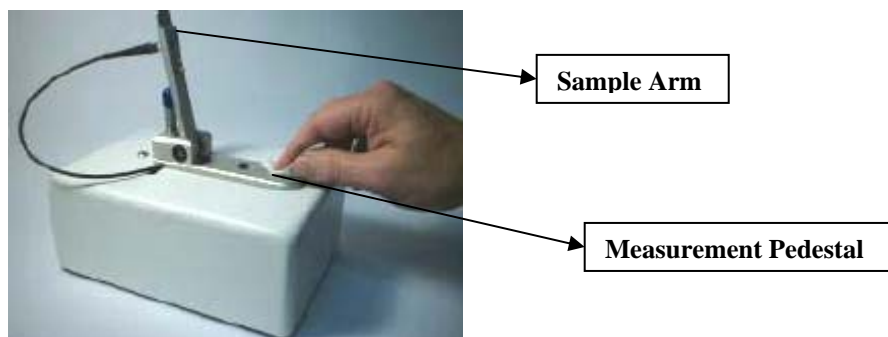
Reproduced from Nanodrop ND-1000 users manual.

Figure 3.2: The Nanodrop ND-1000 spectrophotometer V3.1.0 instrument

The DNA concentrations of all samples were performed on the Nanodrop instrument according to the instruction manual (Nanodrop Technologies, Inc., Wilmington, DE, USA). The ND-1000 V 3.1.0 icon on the computer desktop was opened, following the selection of the Nucleic Acid icon. The sample arm and measurement pedestal was carefully cleaned with ethanol (Figure 3.2). With the use of a pipette, 2 µl of nuclease-free water (Promega, Madison, USA) was added onto the measurement pedestal in order to make a blank measurement. This was done by lowering the sample arm in its horizontal position so that the nanodrop could initialize by clicking on the 'OK' button. A new blank measurement was made for a second time following the same above mentioned steps. The blanking cycle ensures that the instrument is working well and that any samples that might have been carried over from previous measurements do not occur. Thereafter, 2 µl of the DNA sample was loaded onto the measurement pedestal with a pipette. The sample arm was lowered whereby a DNA measurement was taken. An example of the resulting readout can be seen in figure 3.3. A spectrum of DNA should be observed over a wavelength range of 220 to 350 nm with a peak that is close to 260 nm (figure 3.3).
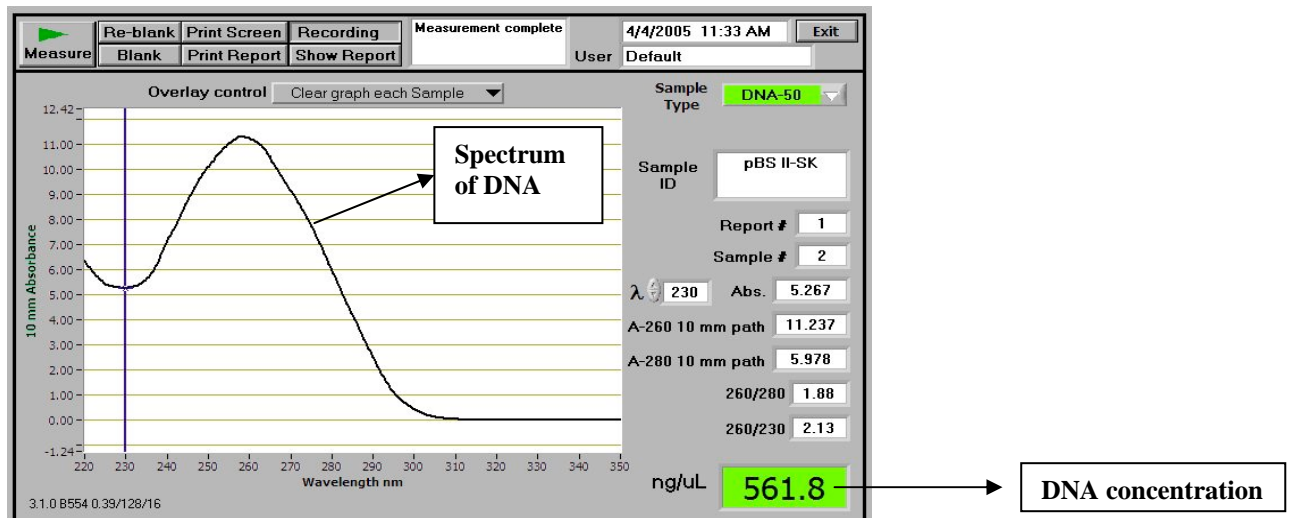
(reproduced from Nanodrop ND-1000 users manual).

Figure 3.3: Illustration of a Nanodrop spectrophotometer readout

3.4 DNA amplification of Multiplex PCR

Multiplex PCR was used to amplify the *mecA, pvl* and *spa* gene. Only the *spa* gene was sequenced for *spa* typing. The QIAGEN Multiplex PCR kit was developed for multiplex PCR reactions, including a master mix which is easy to use, with little or no optimization needed. The kit provides pre-optimized concentrations of specific reagents. These reagents include HotStarTaq DNA polymerase, Magnesium Chloride ($MgCl_2$), dNTPs and a PCR buffer. It is only necessary to add the specific primers and DNA templates for the preparation of the final amplification mix. Furthermore, the kit provides highly sensitive and specific results that are suitable for many types of multiplex PCR applications. These applications include the following:

- Typing and analysis of transgenic organisms
- Amplification and analysis of microsatellites
- Typing and detection of bacteria and viruses
- Amplification of multiple DNA regions for single nucleotide polymorphism (SNP) analysis.

For this study, the multiplex PCR kit was applied for typing bacterial strains. All strains were tested by using the validated method provided by (Larsen *et al.*, 2008). PCR amplification was performed in the PCR laboratory at the Division of Medical

Microbiology, Tygerberg Hospital. This method worked well in our setting as no optimisation was necessary. Each PCR reaction contained a total volume of 50µl, which were placed in 0.2 ml thin-walled PCR tubes (Table 3.2). The multiplex PCR reaction included the *spa, mecA* and *pvl* (forward and reverse) primers as specified in Table 3.3.

Table 3.2: The preparation of Multiplex PCR for simultaneous detection of *spa, mecA* and *pvl* genes

PCR reagents	N =1
PCR Multiplex Mastermix 2x	25 µl
PCR water	9 µl
Primermix (*spa* & *mecA*)	8 µl
Forward Primer: *pvl*	3 µl
Reverse Primer: *pvl*	3 µl
DNA	2 µl
Final Volume	**50 µl**

(Courtesy of Larsen A.R, Denmark) The column N =1 refers to the amount of reagents used to amplify one strain only. To test more than one strain, the amount of reagents in the N=1 column should be multiplied by any preferred number of strain amplifications.

Initially, a *spa* and *mecA* primermix was prepared, containing 100 pmol/µl of the reverse and forward primers. The *pvl* forward and reverse primers (10pmol/µl) were additionally included to the multiplex PCR reaction. The protocol for the standard multiplex PCR was conducted according to the manufacturer's instructions (Qiagen Inc., Valencia, USA).

Generally, the PCR reaction mix contains all the components required for amplification apart from the DNA template. For this study, these components contained all the relevant primers (Table 3.3), the RNAse-free PCR water together with the 2x Multiplex PCR Master mix (final concentration of 3mM $MgCl_2$ (Qiagen Inc., Valencia, USA).

Table 3.3: Description of the *spa, mecA* and *pvl* forward and reverse primers, as well as the 5' to 3' primer sequences.

Genes	Primer Sequence (5' – 3')
spa– 1113f (forward primer)	5'-TAAAGACGATCCTTCGGTGAGC -3'
spa – 1514r (reverse primer)	5'-CAGCAGTAGTGCCGTTTGCTT – 3'
mecA – P4 (forward primer)	5'-TCCAGATTACAACTTCACCAGG – 3'
mecA – P7 (reverse primer)	5'-CCACTTCATATCTTGTAACG – 3'
pvl – FP (forward primer)	5'-GCTGGACAAAACTTCTTGGAATAT – 3'
pvl – RP (reverse primer)	5'-GATAGGACACCAATAAATTCTGGATTG – 3'

Thereafter, 2 µl of each DNA sample was added to 48 µl of each of the PCR reaction tubes. A negative control included a tube of mastermix with no DNA template added. Positive controls included a *pvl* positive, MSSA strain (ATCC 49775) and a *pvl* negative, MRSA strain (ATCC 43300). All PCR reaction tubes as well as the negative and positive controls were amplified in the Thermocycler (Applied Biosystems GeneAmp® PCR System 9700) (Figure 3.4). Thermal cycling conditions for each reaction consisted of an initial activation step at 95ºC for 15 minutes, followed by 30 cycles of DNA denaturation at 94ºC for 30 seconds, primer annealing at 57ºC for 90 seconds, primer extension at 72ºC for 90 seconds with a final extension period at 72ºC for 10 minutes (Qiagen, Standard multiplex PCR protocol).

Figure 3.4: Thermocycler (GeneAmp® PCR system 9700, Applied Biosystems)

3.5 <u>Gel electrophoresis</u>

Approximately 2 grams of Seakem® LE Agarose gel powder (Whitehead Scientific, Cape Town, South Africa) was measured in a weighing boat. A 2% agarose gel was prepared with 1xTAE buffer (Qiagen Inc., Valencia, USA). The gel mixture was heated in a microwave oven on full power for a few seconds until agarose granules have completely dissolved and the solution remained clear. The mixture was given a period to cool down the temperature before adding 15µl of ethidium bromide (concentration of 1 µg/µl) (Promega, Madison, USA) to the melted agarose gel. The necessary safety precautions were taken into account when working with ethidium bromide as it is carcinogenic. The beaker was swirled in order to mix the solution before pouring the gel into its appropriate mould. The gel was carefully poured into its mould in order to prevent any air bubbles before placing an appropriate size comb into the gel. The gel was left at room temperature for approximately 30 minutes until it completely solidified.

Following solidification of the gel, the comb was carefully removed and placed into a gel tank ensuring effective coverage of the gel and electrodes with 1xTAE buffer. The buffer provides continuous flow of the electric current during electrophoresis and prevents melting of the gel. For each sample, 8µl of specific PCR products were then mixed with 3µl of a blue/orange 6x loading buffer (Promega, Madison USA) and loaded into individual wells of the gel with the use of a pipette. Also, a 100 base pair (bp) DNA molecular marker (Promega, Madison USA) was loaded into the well of the gel to determine the individual product sizes of the double-stranded DNA ranging from 100 – 1,500 bp. Negative and positive control PCR products were also loaded into the wells of the gel.

After loading the PCR products, a lid was placed onto the tank whereby the negative and positive electrodes were connected to the PowerPac^{TM} Basic Power supply box (Bio-Rad, Marnes-la-Coquette, France). The power was switched on and the gel was run at 100 volts in a 25x20 cm gel cast for approximately one and a half hours at room temperature.

Subsequent to gel electrophoresis, individual DNA bands were visualized on agarose gels. The current was switched off in order to remove the gel from its tank. The gel

was carefully placed in a UV machine in the Division of Chemical Pathology, Tygerberg Hospital, in order to visualize the DNA bands with the GelDoc XR computer system (Bio-Rad,Marnes-la-Coquette, France). All DNA bands were then viewed, to ensure that the *spa, mecA* and *pvl* bands correspond to its relevant molecular sizes by comparing them to the DNA marker, as well as positive and negative controls.

3.6 <u>Sequencing of the *spa* gene</u>

Sequencing of all PCR products to determine gene sequences of the *spa* bands were done at the Central analytical sequencing facility at Stellenbosch University. The purification of PCR products was performed at the facility in order to remove any un-incorporated dNTPs and primers. The *spa* reverse primers were sent to the facility at 5 µl per reaction with a concentration of 1.1 pmol / µl. Together with the primers, 5 µl of amplified DNA samples were also provided. Sequences were reported in ABI and SEQ file formats and further edited and analysed in the Ridom StaphType[TM], version 1.4 (Ridom Gmb, Würzburg, Germany) software package.

3.7 <u>Sequence analysis with Ridom StaphType[TM]</u>

Ridom StaphType was used to determine various *spa*-types and repeats (Chapter 2: section 2.1.4). This software package is a modernised, Internet database used to manage genotyping data. The software consists of three modules which include the following: a) a sequence editor, b) a database, c) a report generator module (Figure 3.5). The ABI sequencing file format was chosen as an input sequence in the software. Other file formats such as FASTA format or SCF-chromatograms could also be used.
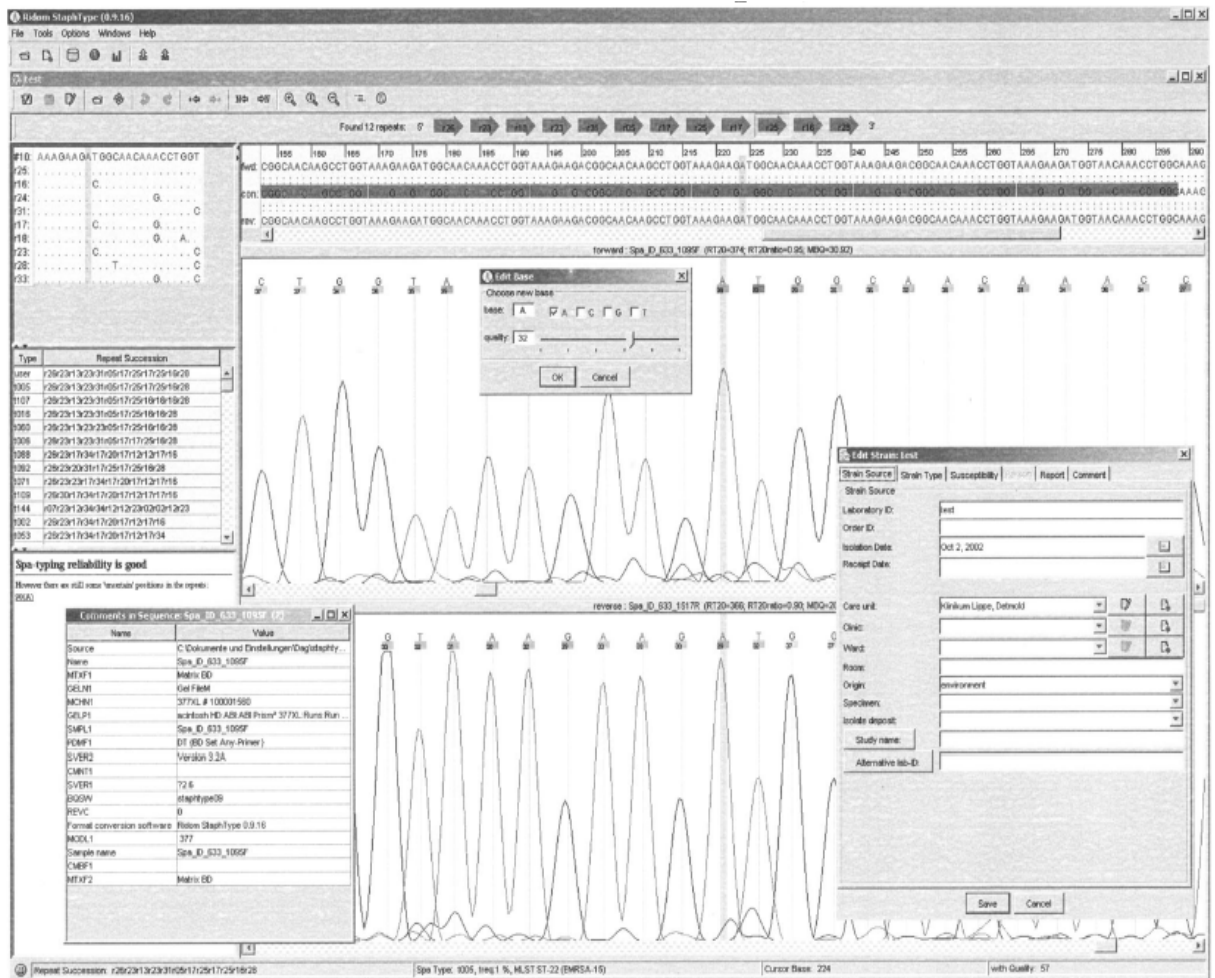
Reproduced by (Harmsen *et al.*, 2003). The figure includes a quality-based sequence editor, a database and a report generator (not shown) module

Figure 3.5: A screen shot of Ridom StaphType software.

The software was utilised according to the following description:

After the chromatogram file is loaded, a quality value is assigned to each base which determines any possible sequence errors depending on the quality value. The software then constructs a consensus sequence whereby *spa* repeats and *spa* types are automatically detected. No further manual editing is required in almost 90% of all input sequences. However if editing is required, sequences can be manually edited by a versatile graphical user interface.

For a *spa* type code, none of the sequence information up or downstream of the repeat region is taken into account. The *spa* types are produced from chromatograms and 5'

and 3' signatures which are unambiguously detected. The software detects 5' – 3' signature sequences at a correct distance to ensure that no beginning or end repeat is missed. All sequencing editing are stored in a sequence history log-file that can be viewed and printed by the user at any time.

For a standardized nomenclature, the *spa* types and repeats consists of two groups, namely local and global strains. The *spa* type is identified with the letter "t" and a unique number, whereas the repeat is identified with a letter "r" and a unique number. Should it occur that a *spa* type or repeat is unknown, it is assigned a new local *spa* type or repeat in the database beginning with the letters "tx" or "rx". These local strains are then synchronized with the spa server to be transferred into new global codes (Table 3.4).

Table 3.4: The assignment of *spa*-types and *spa*-repeats (Ridom StaphType userguide)

	spa-types	*spa-repeats*
global	t001, t002, ...	r01, r02, ...
local	txAA, txAB, ...	rxAA, rxAB, ...
in process	"unknown"	r?, r??, ...

After all sequencing editing was completed, the *spa* typing results and relevant epidemiological data was saved locally in a database system incorporated in the software program. Database information is easily retrieved by Boolean searches and exported in a tab-delimited spreadsheet format. In addition, the integrity of the database is regularly ensured and its information can be backed up to protect against data leakage.

For confidentiality purposes, the database contents are cryptographically secured. In addition, it is possible to create various configurable reports which are stored internally as PDF files. These files cannot be tampered with as it is encrypted to read-only. Thus, Adobe Acrobat Reader software (version 5.0 or higher) has to be installed in order to view or print these files.

To view or submit all new *spa* type and repeats codes, the software synchronizes with its website, http://www.ridom.de/spaserver/. This is achieved by either direct access via the http protocol or file based (e.g., via email). Preferably, all new *spa* types and repeats that meet the quality criteria can be synchronized to the *spa* server to acquire a final *spa* code. Furthermore, all *spa* types and repeats synchronized by users worldwide are transferred to the Ridom StaphType client software. Local or novel *spa* type frequencies are also transmitted to the server. They are assigned to a global *spa* type or if a newly discovered local strain, a new *spa* type code is allocated. The website is accessible to all users whereby *spa* types and repeats can be downloaded (Harmsen *et al.*, 2003).

BURP (Based upon repeat pattern) analysis was performed within the software in order to align and create various *spa* cluster groups or *spa*-clonal complexes (*spa*-CC). The *spa*-type alignments were first downloaded before accessing the BURP functions. Once accessed, the strain data were selected to begin the clustering process. The algorithm then searches for clusters of *spa* types to show relatedness between each *spa*-type via a graphical representation. Each individual *spa* type can only be a member of one cluster. *Spa* types shorter than five repeats were excluded from the analysis as it is impossible to deduct an evolutionary history from these *spa* types. The dimension of clusters were defined by implementing a value less than or equal to six as the calculated cost between members of a group. Lastly, a UPGMA dendogram was constructed by using MEGA software to show the relationship between all *spa* clonal complexes.

3.8 Statistical analysis

Phenotypic and genotypic and clinical data were analysed by using Statistica (Version 8, Statsoft, USA). A chi-square test was used to analyse data where a P value less than 0.05 was considered to be statistically significant.

CHAPTER FOUR

4 RESULTS

4.1 <u>Descriptive data</u>

A total number of 113 *S. aureus* strains from 104 patients were collected during the study period. In five patients isolates from more than one bacteraemic episode were included in the analysis (refer to Table 4.1). These repeat isolates were assigned a number (patient study number) and an alphabetical letter (isolate order number), for instance TBH 26.b (second isolate), indicating the first repeat isolate per patient; TBH 26.c (third isolate), indicating the second repeat isolate per patient. According to the laboratory results, 3 of these repeat strains were MRSA and 6 were MSSA. The results of these 5 patients with repeat strains are further analysed in section 4.3 (Persistent / Recurrent bacteraemia). These repeat isolates were categorised as different episodes of bacteraemia based on the available clinical data and time intervals between isolates.

Table 4.1: *S. aureus* repeat strains

Study number	Time apart from previous strain	MSSA/MRSA
TBH 7.a (1st isolate)	-	MSSA
TBH 7.b (2nd isolate)	1 month	**MRSA**
TBH 26.a (1st isolate)	-	MSSA
TBH 26.b (2nd isolate)	3 months & 13 days	MSSA
TBH 26.c (3rd isolate)	1 month & 28 days	MSSA
TBH 30.a (1st isolate)	-	MSSA
TBH 30.b (2nd isolate)	2 months & 12 days	MSSA
TBH 30.c (3rd isolate)	1 month & 23 days	MSSA
TBH 30.d (4th isolate)	4 months & 27 days	MSSA
TBH 30.e (5th isolate)	3 months & 7 days	MSSA
TBH 40.a (1st isolate)	-	**MRSA**
TBH 40.b (2nd isolate)	9 days	**MRSA**
TBH 110.a (1st isolate)	-	MSSA
TBH 110.b (2nd isolate)	12 days	**MRSA**

4.1.1 Phenotypic characteristics of strains

34/113 (30.1 %) of the strains collected were identified as MRSA, whereas 79/113 (69.9%) of the strains collected were identified as MSSA at the NHLS routine laboratory. Only one h-GISA (TBH 107) was detected in this study (Table 4.2).

Table 4.2: Summary of phenotypic characteristics of *S. aureus* strains

Phenotypic Characteristics	Total number of strains (%)
MSSA	79/113 (69.9%)
MRSA	34/113 (30.1%)
h-GISA (MRSA)	1/34 (2.9%)

4.1.2 Description of Patient Demographics

All descriptive and clinical data are included in appendix B. Of the 104 patients included in the study, 53/104 (51%) were female and 51/104 (49%) were male. Twenty-seven patients (26%) were less than a year old and 12 patients (12%) were between the ages 1 to 13 (Paediatric patients). The majority of staphylococcal isolates were from adult patients, defined as patients 13 years and above (65; 63%) [Figure4.1]. This group consisted of ages 13 to 40, 32 patients (31%); ages 40 to 65, 22 patients (21%) and >65 years old, 11 patients (11%).
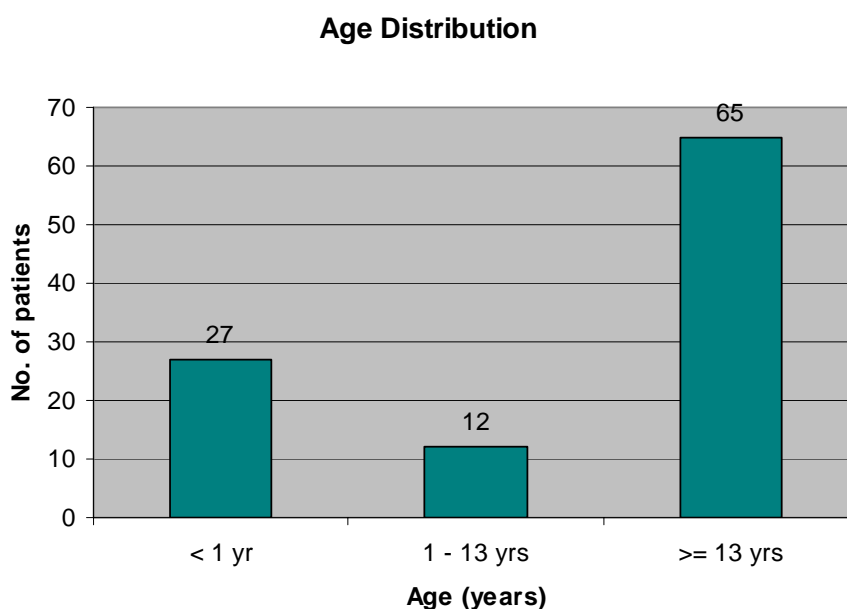
Age Distribution

Figure 4.1: Age distribution of all patients admitted to Tygerberg Hospital.

The distribution of patient wards is presented in appendix B (table B1). Figure 4.2 compares the number of MRSA to MSSA strains in wards with more than 7 isolates. Isolate TBH 43 was excluded from this analysis because of a discrepancy between the phenotypic and genotypic results of this isolate (Table 4.3). The wards with the highest number of isolates included the burns unit (A1B) with 12 isolates, the renal unit (A7) with 11 isolates and the surgical ICU (A1W) with 9 isolates. The surgical ICU had the highest number of MRSA isolates (44% (4/9) of the isolates from A1W were MRSA).

All wards were further categorised into ward groups, apart from intensive care units, renal and burns unit. The number of MRSA to MSSA isolates per ward group and units are presented in figure 4.3. It was noted that a high number of MRSA isolates (8) were seen in the paediatric ward group. For MSSA strains, ward groups with more than 7 isolates included the medical and paediatric ward groups, the renal and burns unit.
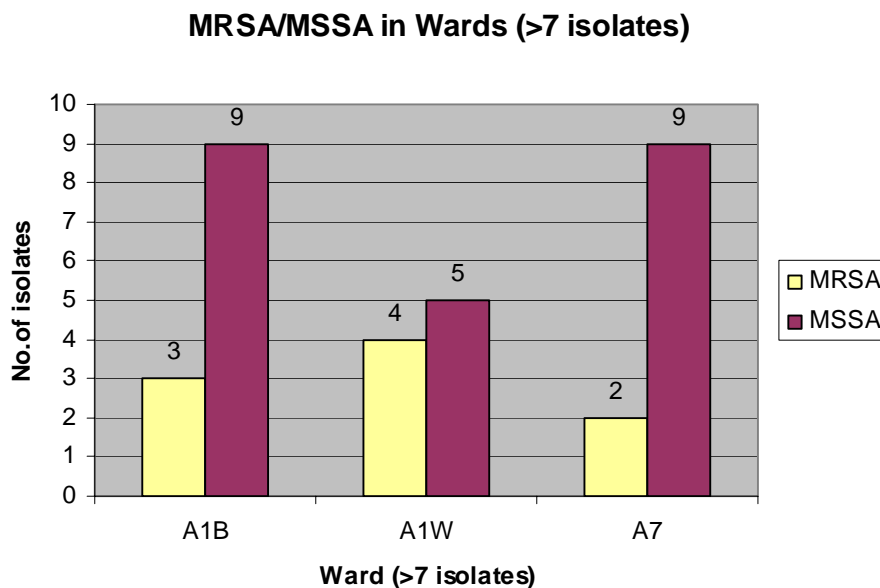
A1B = burns unit; A1W = adult surgical ICU; A7 = renal unit

Figure 4.2: Comparison of the number of MRSA to MSSA strains in wards with 7 isolates or more.

MRSA/MSSA in Ward groups and Units
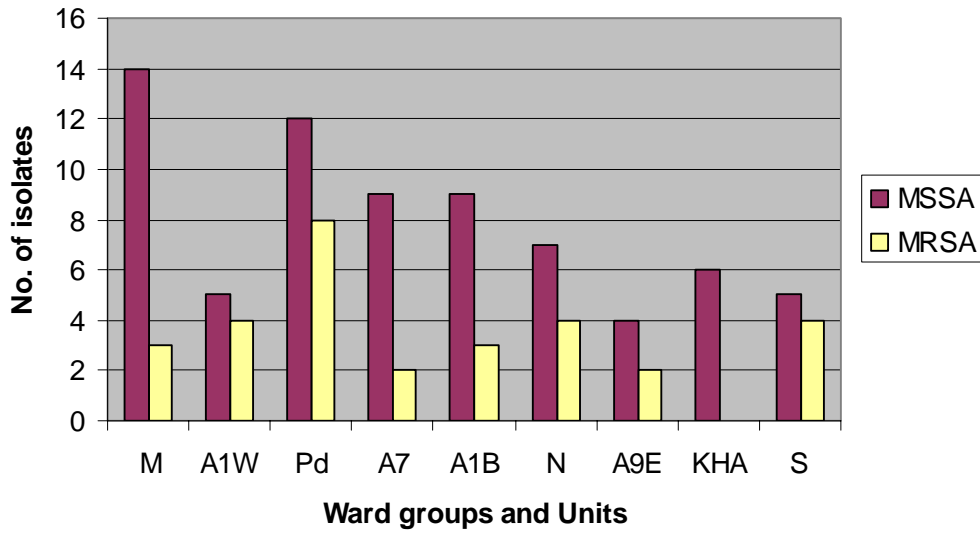
Adult medical ward group (M), Surgical ICU (A1W), Paediatric ward group (Pd), Renal unit (A7), Burns unit (A1B), Neonatal ward group (N), paediatric ICU (A9E), Khayelitsha hospital (KHA), Surgical ward group(S).

Figure 4.3: Comparison of the number of MRSA to MSSA strains in ward groups and units.

4.1.3 Genotypic characteristics of strains

4.1.3.1 The *mecA* gene

All genotypic results are presented in appendix B (table B2). All gel images obtained after multiplex PCR with detection of the *mecA, pvl* and *spa* genes are presented in appendix C (Figures C1 to C10). The *mecA* gene was detected in 33 of 34 (97%) strains, phenotypically characterised as MRSA. The *mecA* gene was absent in all 79 (100%) phenotypically characterised MSSA strains, thus confirming methicillin susceptibility.

The one isolate (TBH 43) with discrepant results was identified by the laboratory as MRSA (Table 4.3). However, no *mecA* gene was detected with multiplex PCR, even after repeat testing. On retesting of this strain for methicillin resistance using a cefoxitin disk and the Kirby Bauer diffusion method, the zone diameter was equivalent to 24 mm. According to the CLSI criteria for *S. aureus*, this signifies methicillin susceptibility (Refer to Chapter 3, section 3.2). However, MRSA was also isolated from other specimens in this patient and the possibility of an initial mixed culture could not be ruled out. Therefore this isolate was excluded from the analyses which compared MSSA and MRSA strains.

Table 4.3: Discrepancies in *mecA* gene results

Study number	**Phenotypic result:** Kirby Bauer method	**Genotypic result:** Multiplex PCR	**Retest:** Kirby Bauer method
TBH 43	MRSA	No *mecA* gene	MSSA

4.1.3.2 The *pvl* gene

18/113 (15.9%) of strains were positive for the *pvl* gene. All of these PVL positive strains were MSSA. The number of PVL negative strains amounted to 95/113 (84 %). All isolates were included in this analysis, including repeat isolates, because these represented different bacteraemic episodes.

4.1.3.3 The *spa* gene

The *spa* gene was detected in 101/113 (89.3%) of strains. In the remaining 11/113 strains (9.7 %) no *spa* gene was detected on initial and subsequent testing (re-testing) with the multiplex PCR. These strains were therefore grouped as non-typeable strains. Two of the non-typeable strains were resistant to methicillin (MRSA), whereas the remaining nine were all sensitive to methicillin (MSSA). All isolates were *spa* typed, including repeat isolates, because these represented different bacteraemic episodes.

4.1.3.4 *Spa* typing

The 101 protein A positive genes were sequenced and strain typed into the Ridom StaphType software program. Of these strains, 31/101 (30.7%) were MRSA and 70/101 (69.3%) were MSSA.

After all DNA nucleotides were analysed in the Ridom StaphType program, a total number of 49 different *spa* types were identified. Of these *spa* types, five were identified as novel (local *spa* types) after synchronisation with the Ridom *spa* server (Table 4.4). Two of the novel *spa* types, t5396 (14 repeats) and t5474 (12 repeats) together with *spa* type t253 (12 repeats) were the longest. Moreover, *spa* types t026 and t287 were found to be the shortest representing 3 repeats each.

Table 4.4: Novel (local) *spa*-types identified after synchronisation with the Ridom *spa*-server.

Study number	Novel *Spa*-type (before synchronisation)	Novel *Spa*-type (after synchronisation)
TBH 47	txAC	t5474
TBH 85	txAD	t5396
TBH 89	txAE	t5472
TBH 91	txAF	t5473
TBH 109	txAG	t5471

The *spa* types were further divided into the most prevalent (major) and minor *spa* types. Major *spa* types included five or more occurrences of an individual type, whereas minor *spa* types included occurrences of three or four of an individual type (Table 4.5).

The most prevalent *spa* types that were found in this study were *spa* type t037 and t891. t037 presented with repeat pattern 15-12-16-02-25-17-24 with nine occurrences in this study. t891 presented with repeat pattern of 26-23-13-23-31-05-17-25-17-25-28, with seven occurrences in this study. The following *spa* types, t002, t015 and t1257 each occurred six times, whereas t021 occurred five times in this study.

All *spa* types, including major/minor *spa* types that were detected in this study were further compared to the global frequency and international clones according to Ridom SpaServer (www.spaserver.ridom.de) (Table 4.5). Ridom sequences and the geographical spread amongst all *spa* types in this study is included in appendix D.

Table 4.5: Frequencies of all *spa*-types compared to global frequencies in association with international clones:

Spa-type (Major/Minor)	No. of isolates (%)	Global Frequency (%)*	Clone (Comments)*
t037 (Major)	9 (9%)	3%	CC8/239, Vienna MRSA, Brazilian/Hungarian, ST239 ORSA III, ST240 ORSAIII, EMRSA-1,4,7,9,11
t891 (Major)	7 (7%)	0%	
t1257 (Major)	6 (6%)	0%	
t002 (Major)	6 (6%)	6%	CC5, Rhine Hesse MRSA(prototype), EMRSA-3, New York clone, Japan clone, Pediatric, USA 100 ORSA II, USA800 ORSA IV, ST5 ORSA I
t015 (Major)	6 (6%)	1%	
t021 (Major)	5 (5%)	1%	CC30, prototype of ST-30, cMRSA (lukS-lukF+) widely disseminated in Australia
t1597 (Minor)	4 (4%)	0%	
t174 (Minor)	3 (3%)	0%	
t1443 (Minor)	3 (3%)	0%	
t701 (Minor)	3 (3%)	0%	
t012 (Minor)	3 (3%)	1%	
t064 (Minor)	3 (3%)	0%	CC8, Archaic/Iberian, USA500 ORSA IV, USA500 ORSA II, ST8 ORSA I, ST8 ORSA IV, ST8 ORSA III
t451 (Minor)	3 (3%)	0%	
Other *spa* types	40 (40%)	0%	*spa* types detected <u>twice</u>: t451, t030, t253, t1476, t148 *spa* types detected only <u>once</u>: t085, t214, t287, t292, t317, t375, t409, t433,t465, t521, t888, t1848, t2360, t2393, t2623, t2763, t4576, t018, t045, t306, t318, t267, t311 t026, t127, t275, t346, t5474, t5396, t5472, t5473, t5471
Total no. *spa* type strains	**101 (100%)**		

* According to Ridom SpaServer; 0%: *spa* types that are infrequently found globally

Table 4.6: *Spa* typing BURP cluster analysis of *S. aureus* strains at Tygerberg Hospital.

Cluster group	*spa*-CC (Clonal Complex)	*spa*-types	# strains	Total # strains (%)	Total # *spa*-types (%)
1	*spa*-CC 701	t064	3	25 (25% of all strains)	11 (22% of all *spa*-types)
		t292	1		
		t451	3		
		t701	3		
		t1257	6		
		t1443	3		
		t1476	2		
		t2360	1		
		t5472	1		
		t5473	1		
		t5474	1		
2	*spa*-CC 012	t012	3	26 (26% of all strains)	10 (20% of all *spa*-types)
		t018	1		
		t021	5		
		t030	2		
		t037	9		
		t253	2		
		t275	1		
		t318	1		
		t433	1		
		t1848	1		
3	*spa*-CC 002	t002	6	10 (10% of all strains)	5 (10% of all *spa*-types)
		t045	1		
		t214	1		
		t306	1		
		t311	1		
4	*spa*-CC 015	t015	6	9 (9% of all strains)	4 (8% of all *spa*-types)
		t465	1		
		t2623	1		
		t4576	1		
5	*spa*-CC 174	t127	1	5 (5% of all strains)	3 (6% of all *spa*-types)
		t174	3		
		t5471	1		
6	*spa*-CC 346/085	t085	1	3 (3% of all strains)	3 (6% of all *spa*-types)
		t346	1		
		t5396	1		
7	No founder	t267	1	2 (2% of all strains)	2 (4% of all *spa*-types)
		t521	1		
Singletons	Singletons	t148	2	19 (19% of all strains)	9 (18% of all *spa*-types)
		t317	1		
		t375	1		
		t409	1		
		t888	1		
		t891	7		
		t1597	4		
		t2393	1		
		t2763	1		
Excluded	Excluded	t026	1	2 (2% of all strains)	2 (4% of all *spa*-types)
		t287	1		

4.1.3.5 BURP cluster analysis

Spa types were clustered into seven cluster groups, which were assigned as either, *spa* clonal complexes, no founders, singletons or excluded *spa* types (Table 4.6). This was performed by means of the BURP algorithm that is installed in Ridom StaphType software. Clustering parameters excluded *spa* types shorter than five repeats from analysis as the length of repeat pattern is insufficient to deduct evolutionary history from these *spa* types. For this reason, two *spa* types (Table 4.6) were excluded from BURP analysis. The dimension of clusters were defined by implementing a value less than or equal to six as the calculated cost between members of a group (Faria *et al.*, 2008; Hallin *et al.*, 2007).

There is usually a repeat or several repeats commonly found in *spa* types that belong to a specific *spa*-clonal complex (*spa*-CC). *Spa* types can evolve due to; insertions, deletions or duplications of an individual repeat unit or group of repeats; or point mutations within a specific repeat unit, leading to the formation of a different repeat unit.

Graphical representation of the relationship of *spa*-types in cluster groups one to seven is shown in figures 4.4 to 4.10. Founders of each of these cluster groups are coloured blue, whereas sub-founders are coloured yellow. The size of each *spa*-type node refers to the number of clustered strains belonging to each specific *spa*-type. In addition, a UPGMA (unweighted - pair group method using average linkages) dendogram using MEGA software, was included to show the relationship between all *spa* clonal complexes (Figure 4.11).

4.1.3.5.1 *Spa*-Clonal Complex 701 (Cluster 1)

The graphical representation of the relation in *spa*-CC 701 showed t701 as the founder *spa* type (Figure 4.4).
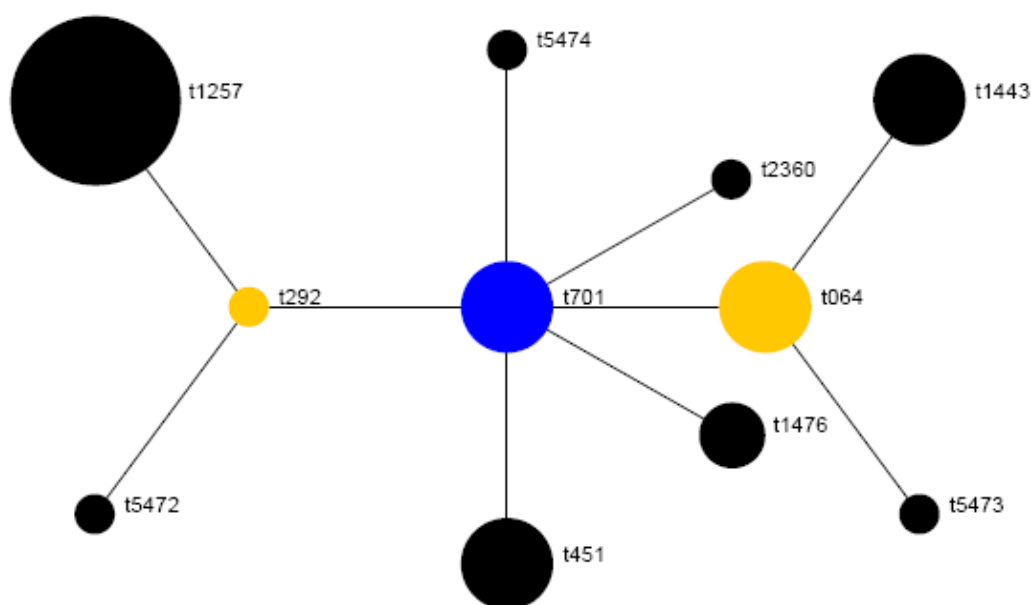
Figure 4.4: The relatedness of *spa*-types grouped into *spa* clonal complex 701 (Cluster 1)

Six *spa* types, namely t064, t292, t451, t2360, t1476 and t5474 evolved from *spa* type t701. In addition, two *spa* types, t064 and t292 were subfounders of *spa* type t701. It appears as if *spa* type t1257 and t5472 are more closely related to *spa* type t292, than it to *spa* type t701. A similar trend is seen for *spa* type t1443 and t5473 as they are more closely related to *spa* type t064, than to type t701.

All repeat patterns of these *spa* types were aligned (Appendix E, Table E1), indicating the presence of motifs 11-19, 11-12 or 11-10 at the beginning. This was followed by motif 17-34-24 (excluding t5473) and motif 34-22-25 (excluding t5472). The alignment of these *spa* types shows various deletions, duplications and insertions of repeat units, as well as point mutations within the nucleotide sequences of any repeat unit.

Alignment of repeat units r19 and r12 (Appendix F, Table F1) showed two point mutations at the twelfth (T – C) and the last nucleotide position (C – T). Furthermore, the same nucleotide sequence was revealed in the alignment of repeats r19 and r10 (Appendix F, Table F2), except for a point mutation that occurred at the last nucleotide (C – T). If one compares *spa* type t1257 and type t701, an insertion of r34 at the beginning motif, and a deletion of r25 at the end is noted.

A point mutation occurred in unit r21, which resulted in the formation of a new *spa* type (Appendix F, Table F3). The same pattern occurred in the alignment of repeat units r21 and r12 (Appendix F, Table F4) with a point mutation at the last nucleotide position (C – T).

As repeat motif 17-34-24 was identified in all the *spa* types, except *spa* type t5473, the alignment of repeat units, r17 and r12 was performed. Two point mutations occurred at the tenth and eleventh nucleotide positions (both G – A) (Appendix F, Table F5). *Spa* type t701 acquired an additional repeat unit r25, while *spa* type t2360 possessed two duplicated repeat units r25 at the end. Moreover, *spa* type t5474 developed three duplications of repeat unit r25.

4.1.3.5.2 *Spa*-Clonal Complex 012 (Cluster 2)

All repeat patterns of the *spa* types in *spa*-CC 012 were aligned (Appendix E, Table E2). It was revealed that motif 15-12-16-02, motif 02-16-02-25 and motif 17-24 were each detected in 70% (7/10) of the *spa* types. Various deletions and duplications of repeat units were detected amongst the *spa* types in *spa*-CC 012, whereas no point mutations were detected. However, there was an insertion of repeat unit r17 in motif 15-12-16-12, which occurred in *spa* type t1848.
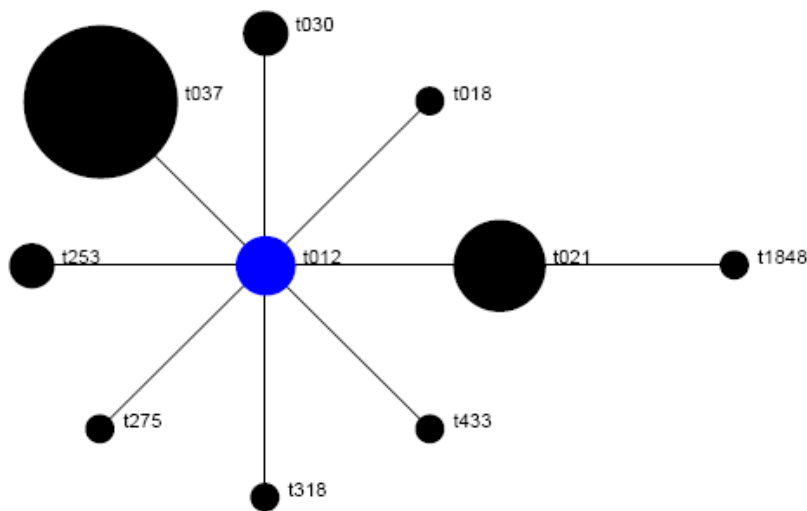
Figure 4.5: The relatedness of *spa*-types grouped into *spa* clonal complex 012 (Cluster 2)

4.1.3.5.3 *Spa*-Clonal Complex 002 (Cluster 3)

The graphical representation of *spa*-CC 002 showed that *spa* type t045, t214, t306 and t311 evolved from *spa* type t002. Alignment of the repeat patterns of the *spa* types identified motif 26-23-17-34 (excluding t045), followed by motif 20-17-12-17 in all *spa* types with repeat unit r16 (end) (Appendix E, Table E3). Deletions of motif 23-17-34 occurred in *spa* type t045, whereas a deletion of repeat unit r17 occurred in *spa* type t311. In *spa* type t306, a duplication of repeat unit r17 occurred at the end of the repeat motif. Another duplication arose of repeat unit r16, in *spa* type 214 towards the end of the repeat motif.
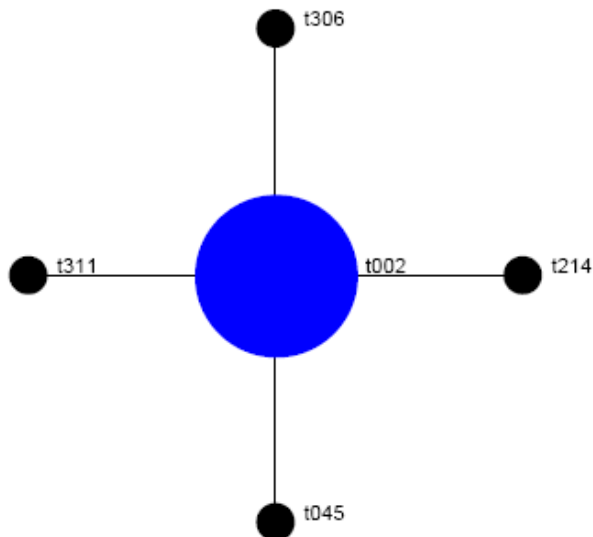
Figure 4.6: The relatedness of *spa*-types grouped into *spa* clonal complex 002 (Cluster 3)

4.1.3.5.4 *Spa*-Clonal Complex 015 (Cluster 4)

The graphical representation of *spa*-CC 015 showed that *spa* type t465, t2623 and t4576 evolved from *spa* type t015. Motif 08-16-02-16 occurred in 75% (3/4) of the *spa* types towards the beginning, followed 50% (2/4) of *spa* types in motif 34-13-17-34-16-34 (Appendix E, Table E4). *Spa* type t015 shows that repeat unit r16 mutated to repeat unit r23 in *spa* type t465 through a point mutation (T - C) at the last nucleotide position (Appendix F, Table F6). Additionally, a deletion of repeat unit r02 occurred in *spa* type t465. *Spa* type t2623 reveals a deletion of repeat unit r34, followed by a point mutation of repeat unit r34 to r13. The point mutation took place at the fifteenth nucleotide position (A - C) (Appendix F, Table F7). Lastly, a deletion of motif 13-17 occurred in *spa* type t4576, followed by a point mutation that resulted in repeat unit r34 becoming repeat unit r13 (Appendix F, Table F7).
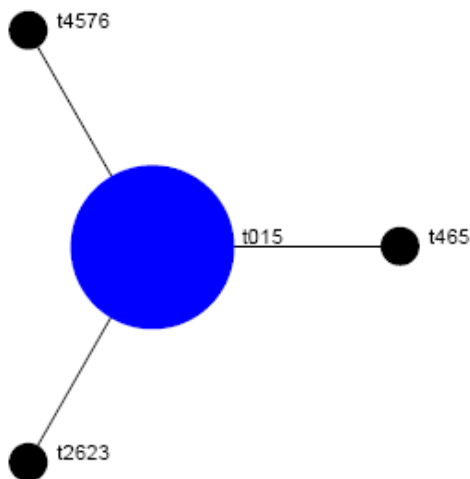
Figure 4.7: The relatedness of *spa*-types grouped into *spa* clonal complex 015 (Cluster 4)

4.1.3.5.5 *Spa*-Clonal Complex 174 (Cluster 5)

The alignment of repeat patterns within *spa*-CC 174 showed that *spa* type t5471 and t127 evolved from *spa* type t174. Motif 21-16-34-33-13 is identified in all of the *spa* types (Appendix E, Table E5). The *spa* types within *spa*-CC 174 differ by deletions and point mutations leading to new repeats.

Point mutations occurred in s*pa* type's t127 with repeat unit r07; and in *spa* type t5471 with repeat unit r35 instead of r14 as in *spa* type t174. Alignment of repeat units r14 and r07 (Appendix F, Table F8) showed a mutation at the last nucleotide (C – T), whereas alignment of repeat units r14 and r35 showed a mutation at the fifteenth nucleotide (C – A) (Appendix F, Table F9). Furthermore, an insertion of repeat unit r23 occurred in *spa* type t127.
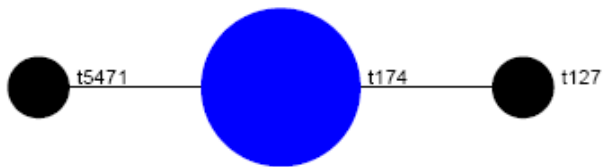
Figure 4.8: The relatedness of *spa*-types grouped into *spa* clonal complex 174 (Cluster 5)

4.1.3.5.6 *Spa*-Clonal Complex 346/085 (Cluster 6)

Spa types t346 and t085 were the two founders that arose within this clonal complex. Both of these *spa* types are closely related to t5396 (Novel *spa* type). When comparing *spa* type t346 to t085, a deletion of repeat unit r34 and a duplication of repeat unit r12 occurred. *Spa* type t5396 is related to t346 and t085 by an insertion of motif 13-23-12 and by deletions and duplications (Appendix E, Table E6).
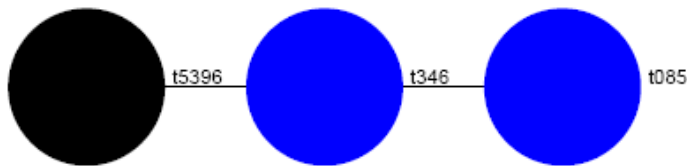
Figure 4.9: The relatedness of *spa*-types grouped into *spa* clonal complex 346/085 (Cluster 6)

4.1.3.5.7 No Founder (Cluster 7)

The evolution of these *spa* types cannot be predicted as this clonal complex has no founder strain. The only difference detected between *spa* type t521 and t267 was that a duplication of r34 occurred in *spa* type t521 (Appendix E, Table E7).
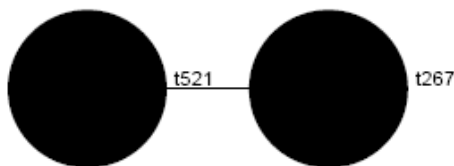
Figure 4.10: The relatedness of *spa*-types grouped into Cluster 7, No founder
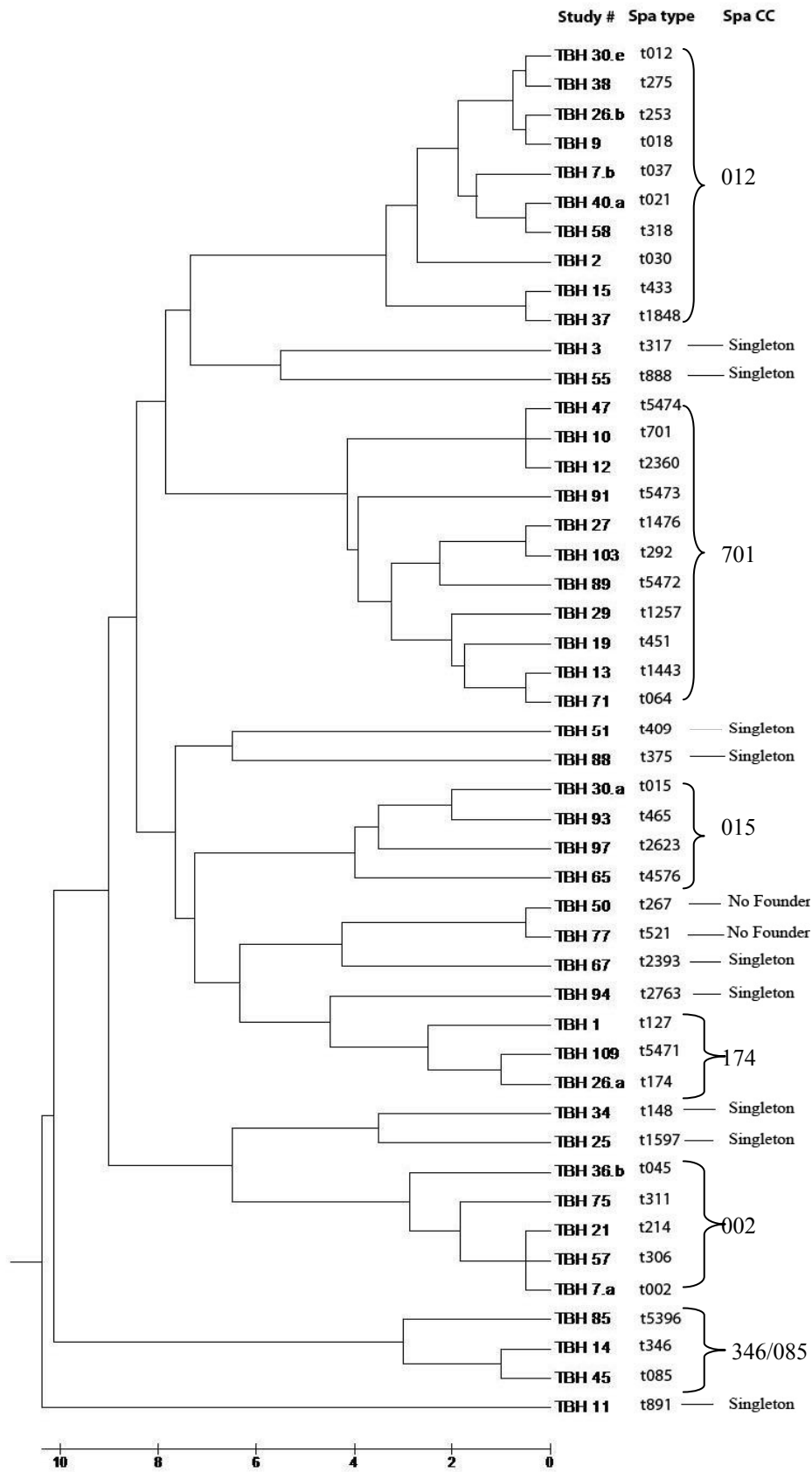
Only one representative strain per *spa* type is shown.

Figure 4.11: UPGMA dendogram of Tygerberg isolates determined by *spa* typing.

4.2 <u>Clinical data</u>

The clinical outcome data (discharged from hospital, transferred to a secondary hospital or death) of 102 patients were available. Of the 102 patients, 62% (63) of the patients were discharged from hospital, 26% (27) of the patients demised and 12% (12) of the patients were transferred to other hospitals. The clinical data of patients from which strains were included in this study can be viewed in appendix B, table B1. In this study, catheter and prosthetic-related sepsis (CSI) mainly constituted CVP catheter infection. Others catheter infections included peritoneal, hemodialysis, broviac, hickman and tenkoff catheters. A few cases of peripheral line sepsis, VP shunt sepsis and vascular graft sepsis also occurred.

4.2.1 HIV status in relation to staphylococcal bacteraemia

Five percent of strains in this study were from HIV positive patients and 47% from HIV negative patients. The HIV status of patients for the remaining 48% strains, were unknown (Figure 4.12). The descriptive (phenotypic/genotypic) data of the strains from the 5 HIV positive patients together with the clinical data on these patients were further analysed (Table 4.7). The majority of the strains (4/5) were MRSA and caused hospital-acquired infections. The clinical diagnoses included skin and soft tissue infections, catheter-related sepsis and primary bacteraemia (unknown source). Three of the strains were *spa* type t1257 (from 3 paediatric patients), with the remaining two strains, t064 and t1476 from adult patients. Interestingly, all of these strains were PVL negative and *spa* types from *spa*-CC 701 [Cluster 1] (Table 4.7).

HIV status

HIV +
5%

Unknown
48%

HIV -
47%

- ■ HIV +
- ■ HIV -
- ■ Unknown
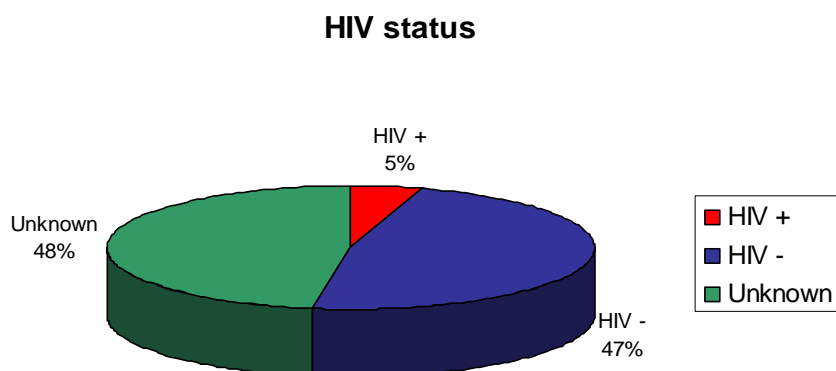
Figure 4.12: HIV positive, negative and unknown

Table 4.7: Descriptive and clinical data of HIV positive patients

HIV POSITIVE PATIENTS									
Study #	MRSA/ MSSA	Gender	Age	Clinical Diagnosis	HCA/ HA/CA	PVL	*spa-*type	Major/ Minor	*spa-CC*
TBH 27	MSSA	Female	22 years	PB	a	neg.	t1476	*	701
TBH 29	**MRSA**	Male	10 months	SSTI	HA	neg.	t1257	Major	701
TBH 48	**MRSA**	Female	10 months	CSI	HA	neg.	t1257	Major	701
TBH 59	**MRSA**	Male	2 years	CSI	HA	neg.	t1257	Major	701
TBH 71	**MRSA**	Female	34 years	SSTI	HA	neg.	t064	Minor	701

(**PB**): Primary bacteraemia; (**SSTI**): Skin & Soft tissue infection; (**CSI**): Catheter and prosthetic-device related sepsis

[a] PB excluded from analysis for HA/HCA/CA category due to unknown source

* <3 occurrences of *spa* type in study

4.2.2 Analysis of MRSA/MSSA association with hospital-acquired, healthcare associated and community-acquired bloodstream infections

The clinical data of study patients were analysed to determine if any community-acquired MRSA infections occurred during the study period. Infections were classified as health-care associated (HCA), hospital-acquired (HA) or community-acquired (CA) for 86 of the 113 strains (Figure 4.13). As mentioned previously, isolate TBH 43 was excluded from the analysis comparing MSSA to MRSA strains. Also patients in whom the source for the bacteraemia was unknown (15 isolates) or where the isolate was considered as a possible contaminant (11 isolates) were not evaluated. All episodes of recurrent bacteraemia were analysed as these were considered to be different episodes based on the available clinical information and consideration of the time periods between episodes. The analysis show 13 isolates (22% of 60 MSSA isolates) as CA-MSSA strains and no CA-MRSA strains (Table 4.8). This finding was statistically significant ($p = 0.00009$) (Figure 4.13). Twenty MSSA isolates (33% of MSSA) were HCA- strains, whereas only 3 MRSA isolates (12% of MRSA) were HCA- strains. The majority of MRSA strains were hospital-acquired (23/26; 88%). Also 27 HA-MSSA strains (45%) were isolated (Table 4.8).

Table 4.8: MRSA and MSSA isolates causing hospital acquired (HA), community-acquired (CA) and health-care associated (HCA) infections at Tygerberg Hospital.

	HA	CA	HCA	Total
MSSA	27	13	20	60
MRSA	23	0	3	26
Total	50	13	23	86
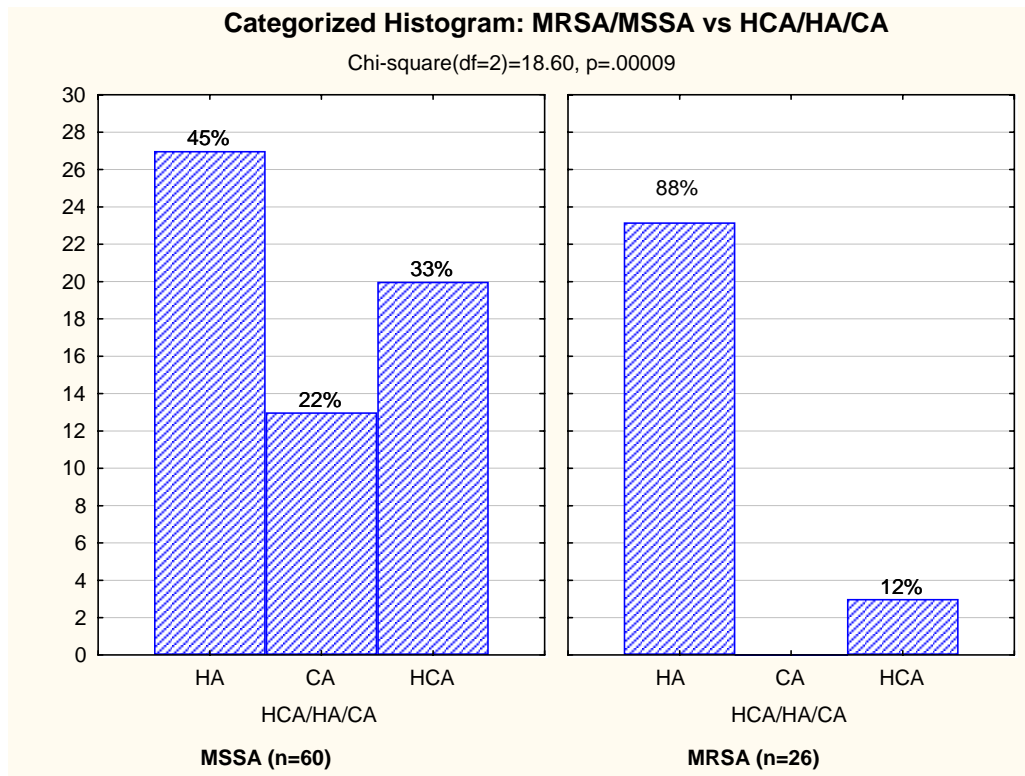
Figure 4.13: A histogram comparing MRSA and MSSA strains causing hospital acquired (HA), community-acquired (CA) and health-care associated (HCA) infections at Tygerberg Hospital.

4.2.3 Source of staphylococcal bloodstream infections according to clinical diagnosis

A high number of 44 (39%) of the 113 isolates represented catheter or prosthetic related sepsis, followed by skin and soft tissue infections (20/18%) and primary bacteraemia where the source was unknown (15/13%) [Figure 4.14]. This was followed by the unknown group, which included possible contaminants or where the clinical significance of isolate was not clear (11/10%), pneumonia (12/11%), septic arthritis (including osteomyelitis) (5/4%) and infective endocarditis (3/3%). Other diagnoses included pericarditis and urinary tract infection (2/2%).

Clinical Diagnosis
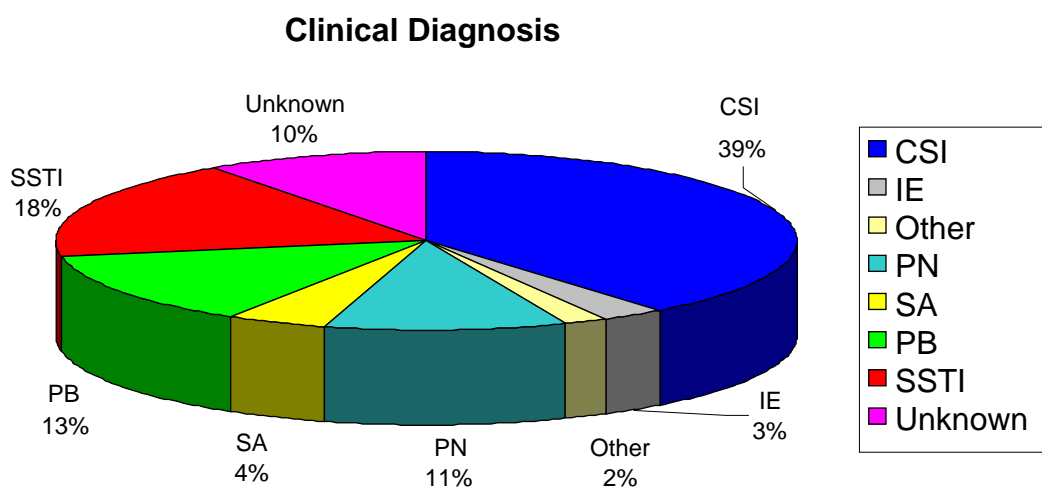
Catheter and prosthetic device-related sepsis (CSI), Infective endocarditis (IE), Pneumonia (PN), Septic arthritis and Osteomyelitis (SA), Skin and soft tissue infection (SSTI), Pericarditis & UTI (Other), Primary bacteraemia(PB), Unknown.

Figure 4.14: Distribution of the clinical diagnoses groups.

The number of MRSA and MSSA isolates for each diagnosis group is presented in Table 4.9. Septic arthritis (including osteomyelitis) (SA), infective endocarditis (IE) and other clinical diagnoses cases were excluded from further analysis as the numbers were too small for statistical analysis. No statistical significant association was found between the type of clinical diagnosis and MRSA/MSSA as the P-value was more than 0.05 (Figure 4.15). However, the highest number of strains was found in the catheter sepsis group for both MSSA and MRSA strains (Figure 4.15).

Table 4.9: MRSA and MSSA isolates compared to all clinical diagnoses in patients at Tygerberg hospital.

	SSTI	PN	CSI	PB	Unknown	SA	IE	Other	Total
MSSA	12	11	29	13	6	3	3	2	79
MRSA	8	1	15	2	5	2	0	0	33
Total	20	12	44	15	11	5	3	2	112
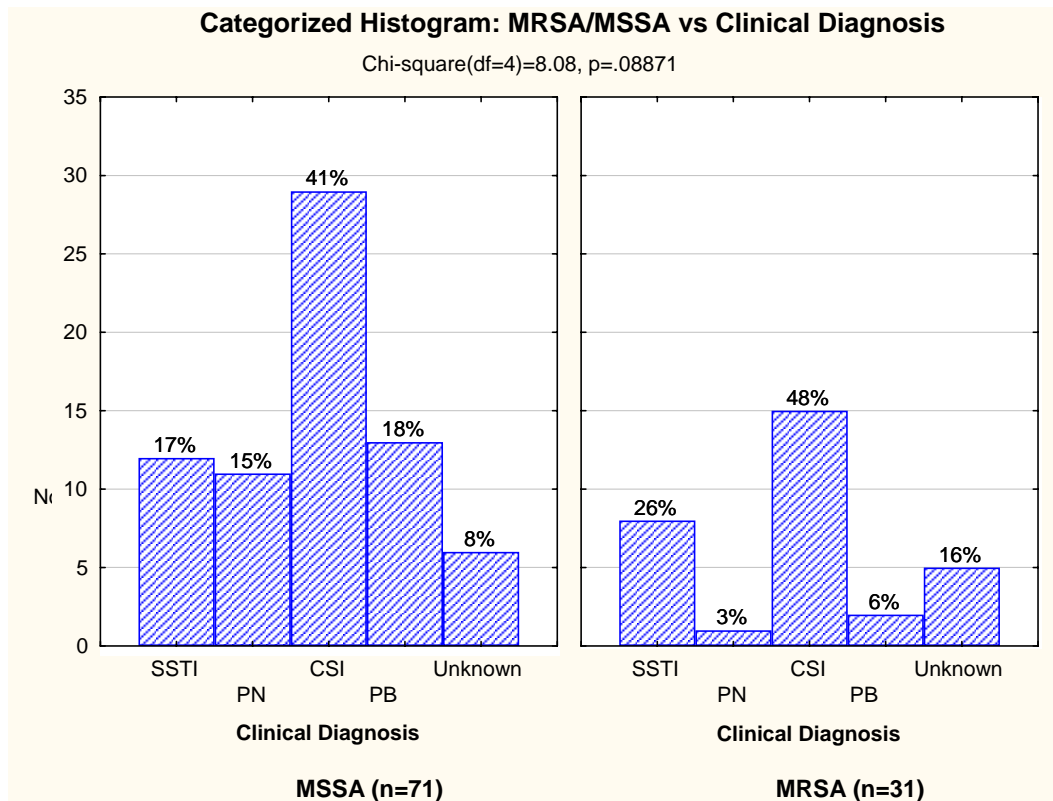
Figure 4.15: Comparison of MRSA and MSSA strains to the clinical diagnosis in patients at Tygerberg Hospital.

Hospital acquired (HA), Community acquired (CA) and Health-care associated (HCA) isolates versus clinical diagnosis is presented in Table 4.10. Again, septic arthritis (SA), infective endocarditis (IE) and other clinical diagnoses cases were excluded from further analysis as the numbers were too small for statistical analysis. The primary bacteraemia (PB) and unknown diagnosis group were also excluded from the analysis due to unknown source and uncertainty of clinical significance, respectively. Catheter sepsis was the most common clinical diagnosis associated with hospital-acquired and health-care acquired strains compared to community-acquired infections (P= 0.00003; Figure 4.16). HCA –strains were mostly isolated from chronic renal failure patients on haemo- or peritoneal dialysis with catheter sepsis. CA strains were associated with pneumonia and skin and soft tissue infections (Figure 4.16).

Table 4.10: The number of HA, CA and HCA isolates versus clinical diagnosis in patients at Tygerberg hospital.

	SSTI	PN	CSI	SA	IE	Other	Total
HA	16	6	23	3	0	2	50
CA	2	6	0	2	3	0	13
HCA	2	0	21	0	0	0	23
Total	20	12	44	5	3	2	86
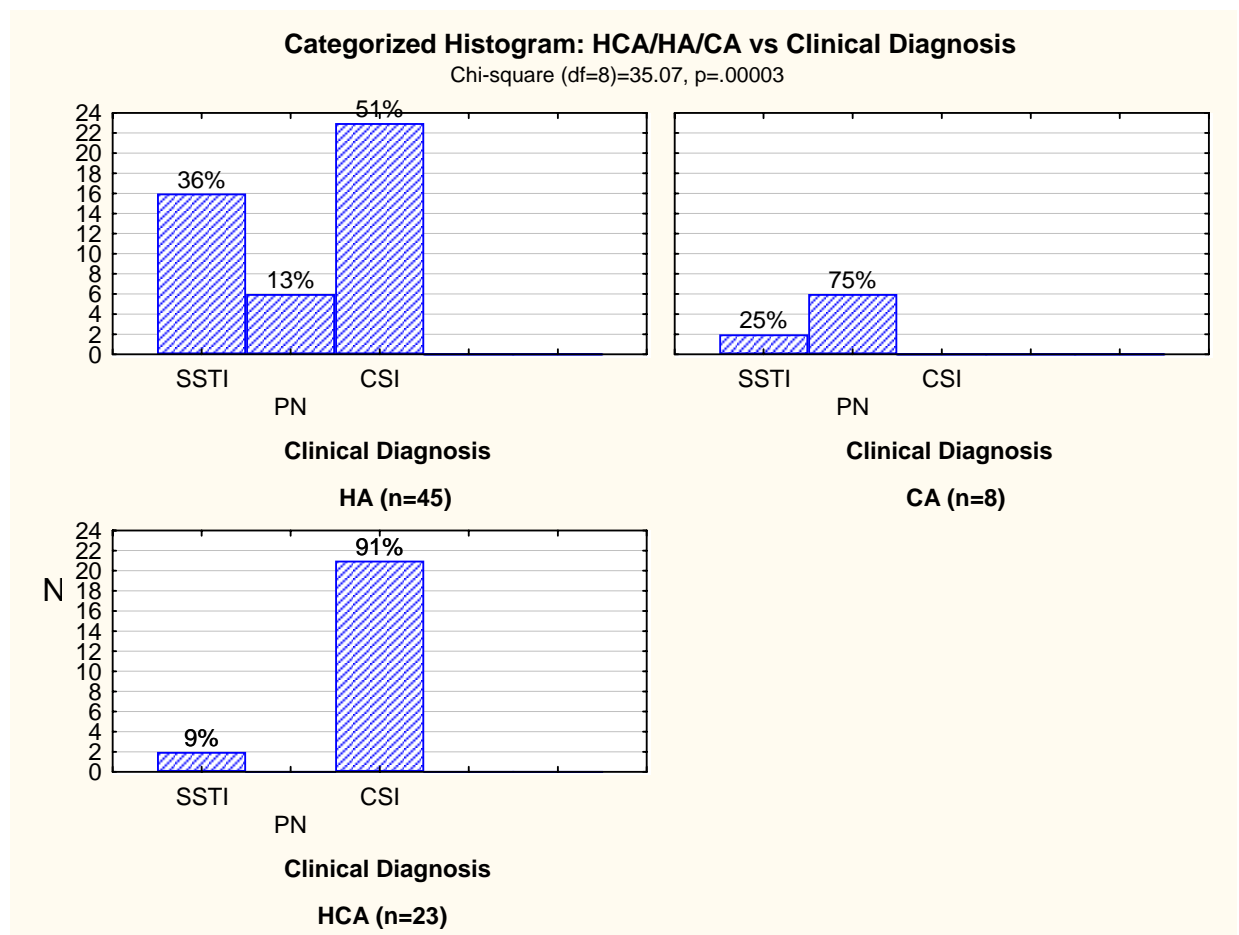
Strains due to primary bacteraemia (PB) and clinical diagnosis that were unknown were excluded from analysis.

Figure 4.16: Comparison of Health-care associated (HCA), Hospital acquired (HA) and Community acquired (CA) strains to the clinical diagnosis in patients at Tygerberg Hospital.

4.2.3.1 Analysis of *spa* types in cluster groups compared to clinical diagnosis

Due to the diverse number of *spa* types in this study, no clear association could be determined for each *spa* type in relation to clinical diagnosis. Therefore, *spa* cluster groups compared to clinical diagnosis were analysed. The most predominant source of bacteraemia in all cluster groups was catheter and prosthetic device - related sepsis (CSI) (Figure 4.17). This is because of the high numbers of CSI (44; 39%) detected in this study (Table 4.11). No clear trend was observed between cluster groups and clinical diagnosis, probably due to the low numbers of strains in each cluster group.

Clinical diagnosis of *spa* type clusters
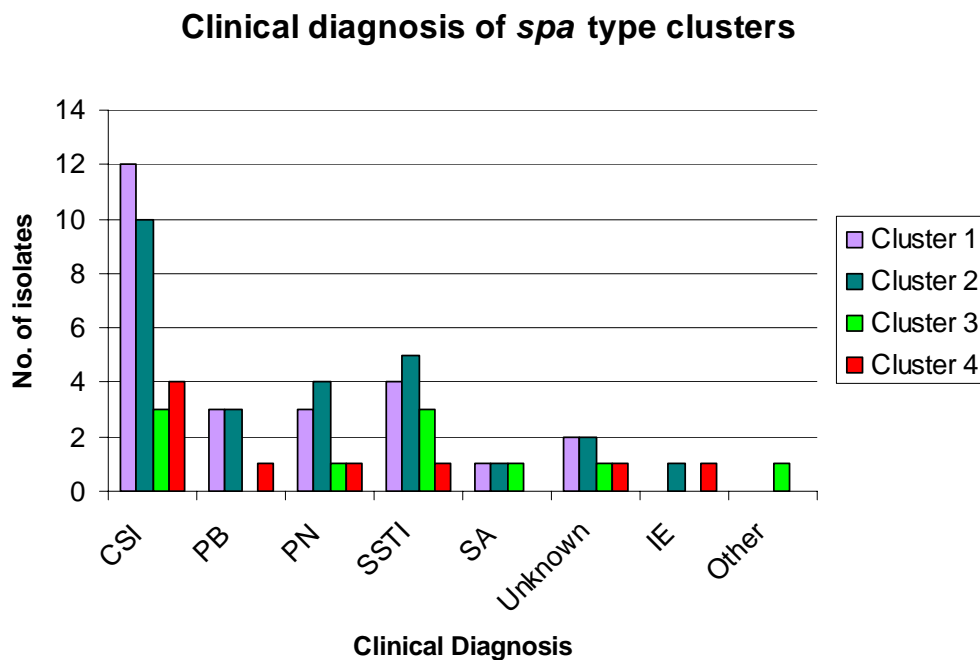
Cluster groups 5-7, singletons, excluded *spa* types and non-typeable strains were not analysed due to small numbers.

Figure 4.17: Comparison of clinical diagnosis and *spa* type cluster groups.

Table 4.11: Summary of the number of strains in each clinical diagnosis group, for the different cluster groups, as well as designation of singletons and excluded *spa* types by BURP analysis. Non-typeable (NT) strains were also included.

Spa Clusters	CSI	PB	PN	SSTI	SA	Unknown	IE	Other	Total
Cluster 1	12	3	3	4	1	2	0	0	25
Cluster 2	10	3	4	5	1	2	1	0	26
Cluster 3	3	0	1	3	1	1	0	1	10
Cluster 4	4	1	1	1	0	1	1	0	9
Cluster 5	2	1	0	1	0	1	0	0	5
Cluster 6	0	1	0	0	0	1	1	0	3
Cluster 7	0	1	1	0	0	0	0	0	2
Singletons	8	3	0	5	2	0	0	1	19
Excluded	2	0	0	0	0	0	0	0	2
NT	3	2	2	1	0	3	0	0	11
Total	44	15	12	20	5	11	3	2	**112**

Cluster 1 (*spa*-CC 701); Cluster 2 (*spa*-CC 012); Cluster 3 (*spa*-CC 002); Cluster 4 (*spa*-CC 015); Cluster 5 (*spa*-CC 174); Cluster 6 (*spa*-CC 345/085); Cluster 7 (No founder); NT (non-typeable strains)

4.2.3.2 Analysis of origin and clinical diagnosis of PVL positive strains

Of the 18 PVL positive strains (all MSSA), 5 were CA, 6 were HA and 4 were HCA. HA/CA/HCA could not be determined with the remaining 3 strains due to unknown source in the primary bacteraemia group (PB) and were not further analysed due to limited clinical data (Table 4.12). The majority of strains (6 isolates) were associated with catheter sepsis (Figure 4.18). There were no fatal cases associated with infections (with known source) due to PVL-positive-strains. All patients recovered on appropriate treatment. One case of osteomyelitis was chronic with persistent positive cultures from the site of infection but C-reactive protein values decreased over time indicating clinical response. Of the *spa* types in the PVL positive strains, t891 was the most predominant (7 strains) (Figure 4.19).

Table 4.12: A summary of all PVL positive MSSA strains in comparison with clinical data and *spa* typing results

PVL positive strains (All MSSA)					
Study #	**Clinical Diagnosis**	**HCA/ HA/CA**	***spa* type**	**Major/ Minor**	***spa-CC***
TBH 3	SSTI	CA	t317	*	Singleton
TBH 8	CSI	HA	NT	*	#
TBH 11	CSI	HA	t891	Major	Singleton
TBH 15	PN	CA	t433	*	012
TBH 25	Urinary Tract Infection	HA	t1597	Minor	Singleton
TBH 26.a	CSI	HCA	t174	Minor	174
TBH 30.d	CSI	HCA	t891	Major	Singleton
TBH 39	PB	a	t891	Major	Singleton
TBH 41	CSI	HCA	t891	Major	Singleton
TBH 51	CSI	HA	t409	*	Singleton
TBH 60	SSTI	HCA	t891	Major	Singleton
TBH 68	SA	CA	t891	Major	Singleton
TBH 73	SA	CA	t002	Major	002
TBH 75	Pericarditis	HA	t311	*	002
TBH 101	PN	HA	NT	*	#
TBH 102	PN	CA	NT	*	#
TBH 105	PB	a	t891	Major	Singleton
TBH 109	PB	a	t5471	*	174

NT Non-typeable strains

* <3 occurrences of *spa* type in study

Non-typeable strains that were excluded from BURP analysis

a PB (primary bacteraemia) excluded from analysis for HA/HCA/CA category due to unknown source
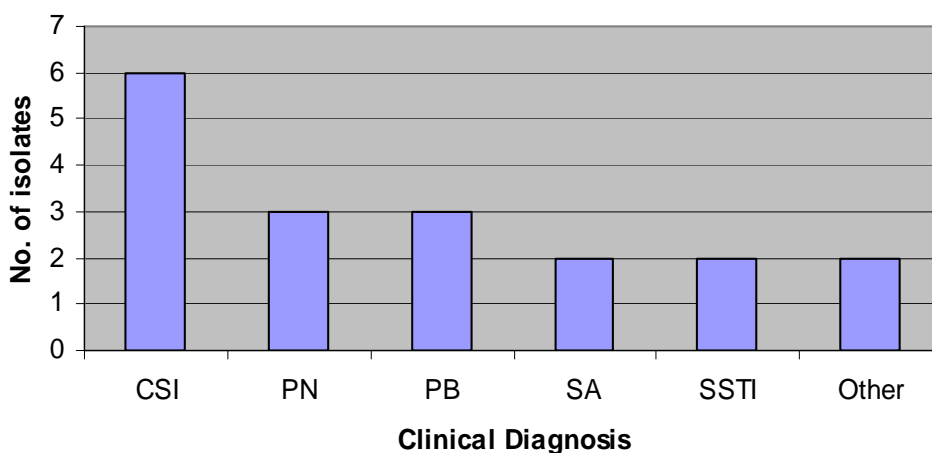
Figure 4.18: Clinical diagnosis in PVL positive strains.

PVL (+) vs. PVL (-) spa types
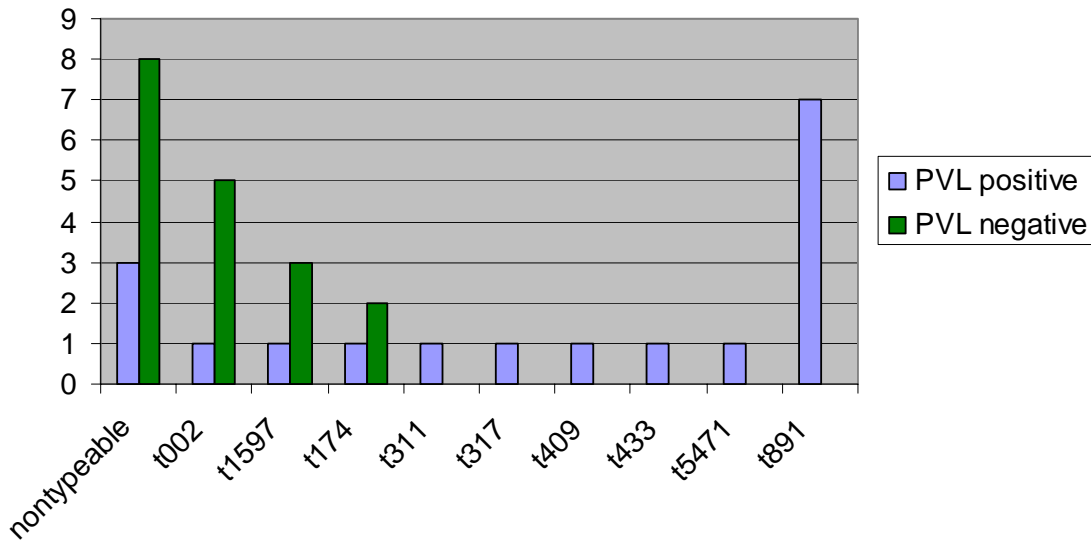
Figure 4.19: Illustration of *spa* types of all PVL positive and PVL negative strains.

4.2.4 Analysis of *spa* cluster groups to determine the association with MRSA/MSSA and HA/HCA/CA categories.

The numbers of MRSA and MSSA strains in the different *spa* cluster groups were evaluated. Statistical significance was found in differences between the cluster groups ($p = 0.00016$, figure 4.20). The majority of the MRSA isolates were found in cluster 2 (16 isolates, 53%) and cluster 1 (13 isolates, 43%) [Table 4.13]. The MSSA strains were more evenly distributed between clusters 1-4. Cluster 3 only contained MSSA strains. The association between HCA, HA, CA strains and *spa* cluster groups were also evaluated. Similar to the MRSA distribution, a high number of HA strains was found in clusters 1 and 2 (Table 4.14) No trend was found in HCA and CA strains (Figure 4.21).

Table 4.13: Summary of the number of MRSA and MSSA strains in different cluster groups, as well as MRSA/MSSA designation of singletons and excluded *spa*-types from BURP analysis.

spa-CC 701 Cluster 1	*spa*-CC 012 Cluster 2	*spa*-CC 002 Cluster 3	*spa*-CC 015 Cluster 4	*spa*-CC 174 Cluster 5
13 MRSA	16 MRSA	None	1 MRSA	none
12 MSSA	10 MSSA	10 MSSA	8 MSSA	5 MSSA

spa-CC 345/085 Cluster 6	No founder Cluster 7	Singletons	Excluded
None	none	None	1 MRSA
3 MSSA	2 MSSA	19 MSSA	1 MSSA

Cluster groups 5 – 7, singletons and excluded *spa* type numbers were too small for statistical analysis.

Figure 4.20: Histogram of *spa* cluster groups 1 – 4 with the number of MSSA and MRSA strains in each group.

Table 4.14: Summary of the number of hospital-acquired (HA), health-care associated (HCA) and community-acquired (CA) strains in all cluster groups, singletons and excluded *spa*-types from BURP analysis.

spa-CC 701 Cluster 1	*spa*-CC 012 Cluster 2	*spa*-CC 002 Cluster 3	*spa*-CC 015 Cluster 4	*spa*-CC 174 Cluster 5
14 HA	15 HA	3 HA	2 HA	1 HA
4 HCA	4 HCA	3 HCA	3 HCA	2 HCA
2 CA	2 CA	3 CA	2 CA	0 CA
5 (NA)	5 (NA)	-	2 (NA)	2 (NA)

spa-CC 345/085 Cluster 6	No founder Cluster 7	Singletons	Excluded *Spa* types
0 HA	1 HA	9 HA	0 HA
0 HCA	0 HCA	5 HCA	2 HCA
1 CA	0 CA	2 CA	0 CA
2 (NA)	1 (NA)	3 (NA)	-

(NA) = not applicable, refers to strains excluded from the analysis due to unknown source of bacteraemia (PB) or uncertain clinical significance (unknown clinical diagnosis group).

Categorized Histogram: HCA/HA/CA x spa-type Clusters
Chi-square(df=6)=7.95, p=.24161

HA (n=34)

CA (n=9)

HCA (n= 14)
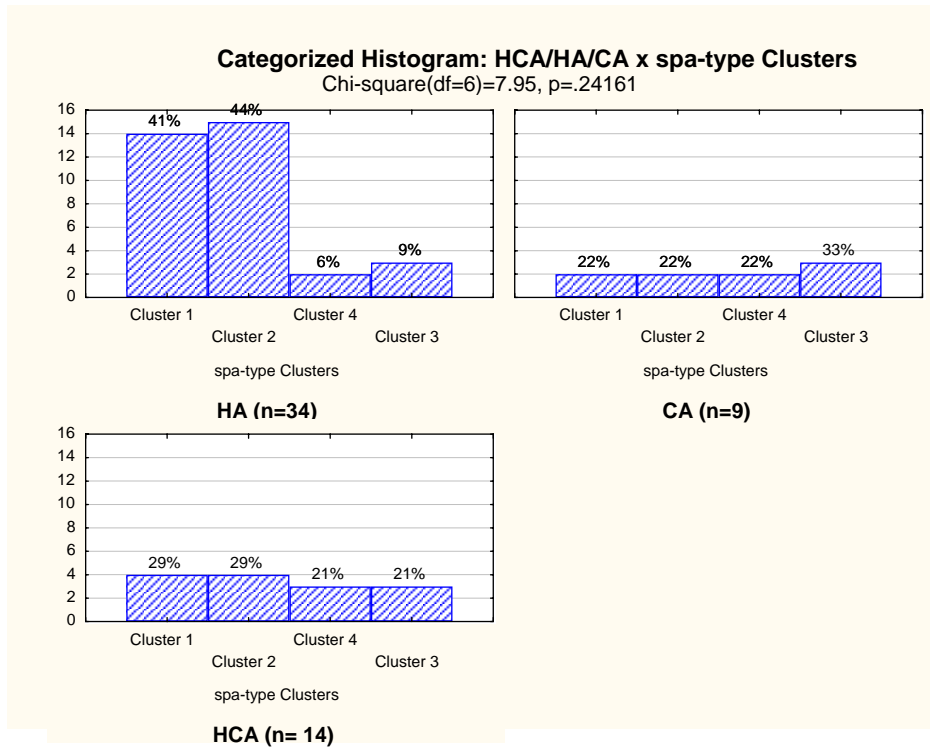
Cluster groups 5 – 7, singletons and excluded *spa* types were excluded as numbers are too low for statistical analysis.

Figure 4.21: Histgram of number of HA/HCA/CA strains in spa cluster groups 1-4.

4.2.5 Analysis of *spa* types to determine association with MRSA/MSSA and HA/HCA/CA categories

In this study a diverse number of *spa* types were detected. Due to the low number of isolates for each *spa* type, no statistical significance could be determined for *spa* types in various categories. Therefore, only the most frequent *spa* types in this study were further evaluated in relation to MRSA/MSSA and HCA/HA/CA categories. Notably, the most frequent *spa* types for HA-MRSA strains were t037, t1257 & t021 (Table 4.15). MSSA strains most frequently typed as t891 (also predominant PVL+ strain type), t002, t015 & t1597. Furthermore, t1597 was a frequent *spa* type for HA-MSSA strains, whereas t891, t002 & t015 were isolated from HA, HCA & CA-infections (Table 4.15).

Table 4.15: Summary of trends observed with analysis of most frequent *spa* types for MRSA/MSSA (phenotypic & genotypic data) and HA/HCA/CA (clinical data) categories.

Categories	*Spa* types
HA-MRSA	t037 (cluster 2)
	t1257 (cluster 1)
	t021 (cluster 2)
HA-MSSA	t1597 (singleton)
HA/HCA/CA-MSSA	t891 (PVL+; singleton)
	t002 (cluster 3)
	t015 (cluster 4)

Most frequent *spa* types of MRSA/MSSA
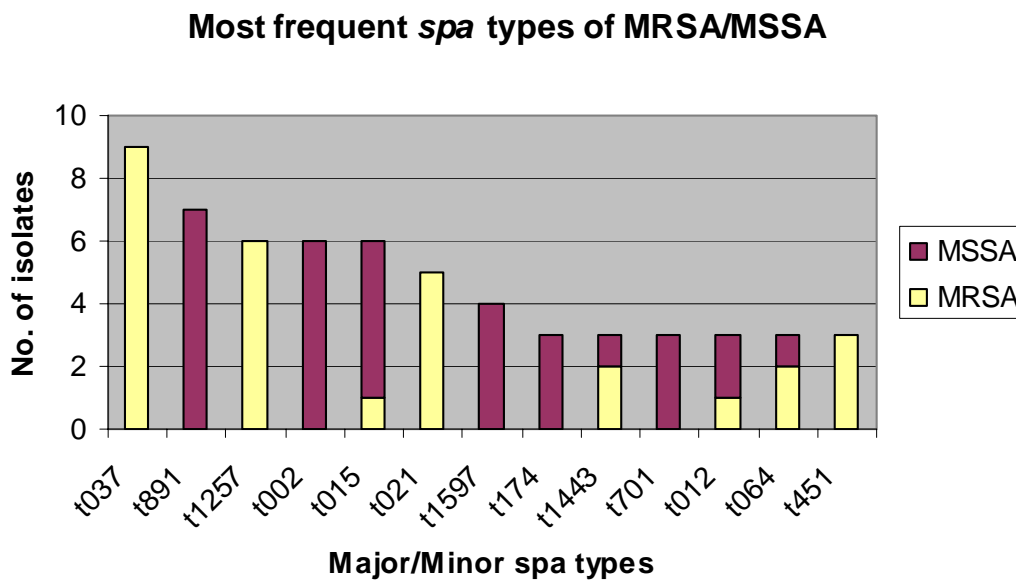
Figure 4.22: Most frequent (major/minor) *spa* types of MRSA and MSSA strains.

Most frequent *spa* types vs. HCA/HA/CA
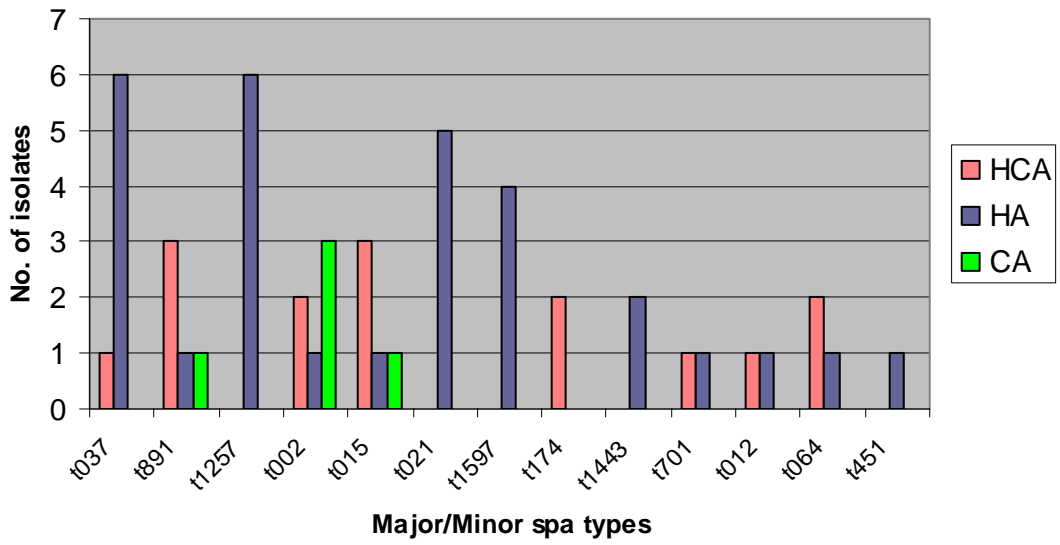
Figure 4.23: Most frequent (major/minor) *spa* types of health-care associated (HCA), hospital-acquired (HA) and community-acquired (CA) strains in this study

4.3 Persistent / Recurrent bacteraemia

After strain typing, the results of 5 patients with repeat strains (refer to table 4.1) were analysed for persistent or recurrent bacteraemia. This was achieved by comparing the *spa* type of the repeat strain with the initial strain for each patient. Persistent bacteraemia was defined as a positive blood culture ≥3 days after appropriate therapy was initiated. Recurrent bacteraemia was defined as the return of *S. aureus* bacteraemia after documenting a negative blood culture and/or clinical improvement after the completion of a course of appropriate antibiotics (Chang *et al.*, 2003). Furthermore, recurrent bacteraemia was subdivided into re-infection (different *spa* strain types) and relapse (the same *spa* strain type). The descriptive and clinical data of persistent and recurrent bacteraemia is presented in tables 4.16 to 4.18. No h-GISAs were detected in any of these strains.

Persistence was noted in patient TBH 40. This 7-day old infant presented with a hospital-acquired skin infection with subsequent abscess formation (Table 4.16). Both the initial and the repeat blood culture isolate was identified as MRSA, typed as *spa* -type t021 (*spa* CC 012) and were PVL negative.

Relapse was identified in 2 patients (TBH 30, 26). Both of these patients had underlying chronic renal failure and received renal dialysis. Bacteraemia was due to catheter-related sepsis with MSSA (Table 4.17). Both patients initially responded after the dialysis catheters were removed, but in both cases patients were treated with vancomycin although cloxacillin was advised by the resident microbiologist. *Spa* types t015 (*spa* CC 015) and t253 (*spa* CC 012) was identified for the respective cases.

Re-infection was noted in 4 patients, predominantly due to intravascular catheter infections (TBH 7, 26, 30, 110) (Table 4.18). The first blood culture isolate collected from TBH 7.a was a MSSA, whereas the second isolate (TBH 7.b) was a MRSA. The same pattern occurred for TBH 110.a and TBH 110.b. Both these patients were admitted to the burns unit. In patient 7 the initial MSSA bacteraemia was due to burn wound infection, and the subsequent bacteraemia due to catheter sepsis of the CVP. Patient 110 initially acquired CVP-sepsis, but the source for the subsequent

bacteraemia was unknown (categorized as primary bacteraemia). This patient had one temperature spike which resolved on removal of the intravascular catheter, but *S. aureus* was not cultured on the CVP-tip. The other two patients (TBH 26 & TBH 30) developed episodes of relapse (table 4.17) and re-infection (table 4.18) which occurs frequently in these chronic renal failure patients on dialysis due to invasive procedures (dialysis catheter) and frequent hospital exposure. Interestingly, these HCA-infections were due to MSSA-strains.

Table 4.16: Descriptive data of *S. aureus* strain and clinical data for the one persistent bacteraemia case

PERSISTENT									
Study #	Time apart from previous strain	MRSA/ MSSA	PVL	*spa*-type	Gender	Age	Clin. Diag	HIV	HCA/ HA/CA
TBH 40.a	-	MRSA	neg.	t021	Male	7days	SSTI	Unknown	HA
TBH 40.b	9 days	MRSA	neg.	t021			SSTI		

Table 4.17: Cases of recurrent bacteraemia due to relapse - descriptive and clinical data

RELAPSE									
Study #	Time apart from previous strain	MRSA/ MSSA	PVL	*spa*-type	Gender	Age	Clin. Diag	HIV	HCA/ HA/CA
TBH 30.a	-	MSSA	neg.	t015	Male	20 yrs	CSI	Negative	HCA
TBH 30.b	2 months & 12days	MSSA	neg.	t015			CSI		HCA
TBH 30.c	1 month & 23 days	MSSA	neg.	t015			CSI		HCA
TBH 26.b	3 months & 13days	MSSA	neg.	t253	Male	36 yrs	CSI	Negative	HCA
TBH 26.c	1 month & 28 days	MSSA	neg.	t253			CSI		HCA

Table 4.18: Cases of recurrent bacteraemia due to re-infection - descriptive and clinical data

Study #	Time apart from previous strain	MRSA/ MSSA	PVL	*spa*-type	Gender	Age	Clin. Diag	HIV	HCA/ HA/CA
				RE-INFECTION					
TBH 7.a	-	MSSA	neg.	t002	Male	37 yrs	SSTI	Unknown	HA
TBH 7.b	1 month	**MRSA**	neg.	t037			CSI		HA
TBH 26.a	-	MSSA	**pos.**	t174	Male	36 yrs	CSI	Negative	HCA
TBH 26.b	3 months & 13days	MSSA	neg.	t253			CSI		HCA
TBH 30.c	1 months & 23days	MSSA	neg.	t015	Male	20 yrs	CSI	Negative	HCA
TBH 30.d	4 months & 27days	MSSA	**pos.**	t891			CSI		HCA
TBH 30.e	3 months & 7 days	MSSA	neg.	t012			CSI		HCA
TBH 110.a	-	MSSA	neg.	t1476	Male	35 yrs	CSI	Unknown	HA
TBH 110.b	12 days	**MRSA**	neg.	t037			PB		-

CHAPTER FIVE

5 DISCUSSION

In the first part of this study, a multiplex PCR was performed on staphylococcal (MRSA/MSSA) isolates to determine the presence of the *mecA* and *pvl* genes, as well as to strain type these isolates using *spa* typing. Clonal cluster complexes were determined by the BURP algorithm installed in Ridom StaphType software program, where *spa*-types were compared to international important clones. The genotypic profiles of staphylococcal isolates were then further analysed in context of the clinical data (clinical diagnosis and HA/HCA/CA categories) for epidemiological purposes. Lastly, persistent and recurrent bacteraemia cases were analysed for possible association to organism related factors at our hospital.

5.1 Analysis of descriptive data

5.1.1 Phenotypic characteristics of strains

Phenotypically, a high prevalence rate of MRSA (30%) at Tygerberg Hospital was reported in this study. *S. aureus* methicillin susceptible strains amounted to 69.9%. In hospitals, high prevalence rates of MRSA have been found to be associated with increased patient mortality and higher healthcare costs (Deurenberg *et al.*, 2007). In Europe, the MRSA prevalence rates vary, as the Netherlands and Scandinavian countries present with lower MRSA rates (0.6%) compared to other European countries (up to 44.7%). Low prevalence rates (0.6%) were due to a low antibiotic selection pressure and screening of 'high risk' patients in hospitals for MRSA before or at the time of admission (Tiemersma *et al.*, 2004). In order to decrease high prevalence MRSA rates in nosocomial settings, more effective disinfection procedures and hand hygiene guidelines could perhaps reduce the spread of MRSA. Furthermore, only one MRSA isolate (TBH 107) was reported with heteroresistance to glycopeptides (h-GISA) in this present study.

5.1.2 Patient demographics

The distribution of gender was fairly equal in this study with 51% females and 49% males. Thirty-eight percent of staphylococcal isolates were found in paediatric patients, 26% were less than a year old and 12% were between the ages 1 to 13 (Figure 4.1). Generally, infants and the elderly are more prone to infections than other age groups. In the current study, the proportion of paediatric patients (38%) was predominantly higher than the elderly group (11%). This may be due to colonisation of *S. aureus* in the nose and skin, which can occur in newborn babies through transmission from their nursing mothers. Staphylococcal strains could spread around the newborn babies' nursery if healthcare workers become colonized. Thus, this leads to the babies becoming colonized and may develop skin infections within a few days after birth, for example skin infection of the umbilicus or at intravascular catheter sites (Tinelli *et al.*, 2009).

CA-MRSA infections have been reported from predominantly young and healthy individuals in community settings such as sports facilities, the military and prisons. On the other hand, HA-MRSA has been associated with immunocompromised patients in hospital settings (Jappe *et al.*, 2008). As no CA-MRSA was detected in the current study, the association between HA-MRSA versus CA-MRSA could not be analysed.

MRSA colonization could occur due to previous exposure to antibiotics, prolonged duration of antimicrobial therapy, hospitalization in an intensive care or burns unit, severe underlying illness, invasive procedures, surgical wounds or burns and contact with patients colonized with MRSA (Boyce *et al.*, 1981; Boyce *et al.*, 1983; Crossley *et al.*, 1979; Deurenberg *et al.*, 2007; Jernigan *et al.*, 1996; Peacock *et al.*, 1980; Ward *et al.*, 1981). In the present study, the ward with the highest percentage of MRSA isolates compared to MSSA was seen in the surgical ICU when compared to the burns and renal unit (44%; 4/9). The wards with the highest number of *S. aureus* isolates were the burns unit (12 isolates), renal unit (11 isolates) and the surgical ICU (9 isolates). After categorising the wards into ward groups, the paediatric ward group had the highest number of MRSA isolates. These were due to intravascular catheter-related sepsis in the majority of cases.

5.2 Genotypic characteristics of strains in relation to Clinical data

The following section will include a discussion on the *mecA, pvl* and *spa* genes. Each of these sub-sections will feature the results obtained using the clinical data (section 4.2) that is applicable to each gene.

5.2.1 The *mecA* gene

The phenotypic detection of methicillin resistance correlated very well with the genotypic detection of the *mecA* gene using the multiplex PCR method. Only one discrepancy was found amongst the *mecA* gene results (Table 4.3). Isolate, TBH 43 was phenotypically reported as MRSA, but no *mecA* gene was detected in this strain. Upon re-testing this strain tested sensitive to methicillin. This could have arisen due to initial incorrect readings in the laboratory or because of an initial mixed culture.

S. aureus is an important cause of hospital-acquired (HA), health-care associated (HCA) and community acquired (CA) infections. CA-MRSA has rapidly emerged in many communities, but has also been reported to spread to hospitals causing hospital outbreaks (Regev-Yochay *et al.*, 2005). In the USA, at Texas children hospital, figures as high as 70% for CA-MRSA isolates have been reported from 2001 to 2004 (Kaplan *et al.*, 2005). The rate of increase in CA-MRSA isolates was significantly higher compared to an insignificant increase of CA-MSSA isolates in the study (Kaplan *et al.*, 2005). In 2001, the percentage of community-acquired *S. aureus* isolates that were methicillin resistant was 71.5% (551/771) and increased to 76.4% (1193/1562) in 2004 (p=.008) (Kaplan *et al.*, 2005). It has also been proven that some CA-MRSA strains are closely related directly or indirectly to the health-care setting (Aires-de-Sousa & de Lencastre, 2003). Characteristically, the PVL gene is associated with CA-MRSA infections, primarily with skin and soft tissue infections and necrotizing pneumonia. These associations are further discussed in section 5.2.2 (The PVL gene). The prevalence rate of HA-MRSA infections differs among various countries. In Israel, *S. aureus* bloodstream isolates that were HA-MRSA were reported as 44% according to the European antimicrobial resistance surveillance system (EARSS) (Chmelnitsky *et al.*, 2008). In the present study, the clinical data of study patients were analysed to determine the occurrence of CA-MRSA infections at Tygerberg Hospital. No CA-MRSA was detected compared to 22% of MSSA isolates

that were CA-MSSA (Figure 4.13). This finding was found to be highly significant (p = 0.00009, Figure 4.13). Furthermore, 88% of MRSA were HA-infections and 12% HCA-infections. MSSA was also most commonly isolated in the hospital-setting (45%) and health-care associated setting (33%) (Section 4.2.2: Analysis of MRSA/MSSA to determine the association with HA/HCA/CA categories).

The majority of bacteraemia cases were related to catheter sepsis (CSI) for both MRSA (48%) and MSSA (41%) strains. The second commonest source for bacteraemia was skin and soft tissue infections (SSTI) (Figure 4.15). No clear trends were observed between MRSA and MSSA strains in relation to clinical diagnosis as numbers were relatively low (Section 4.2.3: Source of staphylococcal bloodstream infections according to clinical diagnosis).

5.2.2 The *pvl* gene

PVL positive MSSA strains were detected in 15.9% (18/113) of the total number of *S. aureus* strains in this study. These PVL-positive strains were all MSSA, constituting 22.7% (18/79) of total number of MSSA strains. The number of PVL negative strains amounted to 95/113 (84%). All patients with PVL positive MSSA in this study were HIV negative (Section 4.2.1: HIV status and staphylococcal bacteraemia in this patient group).

It has been reported that PVL is more commonly found in MRSA isolates than in MSSA isolates, specifically amongst CA-MRSA strains (Aires-de-Sousa *et al.*, 2006; Tristan *et al.*, 2009). However, even though CA-MRSA infections are of great concern, MSSA infections may present with the same epidemiological and clinical characteristics (Miller *et al.*, 2007; Mongkolrattanothai *et al.*, 2003). Thus, PVL genes are not only found in MRSA isolates (Kim, 2009; Tinelli *et al.*, 2009). This is not only a recent finding, as historical MSSA isolates that carried the PVL genes, were pandemic in the 1950's and 1960's. These strains include the "Oxford Staphylococcus" and the phage type 80/81 (Kearns *et al.,* 2006; Robinson *et al.,* 2005). Our findings correlate with these observations, as only MSSA isolates were found harboring the PVL gene (Table 4.11). Moreover, no CA-MRSA infections were detected in our study (Figure 4.13). It was found that only 5/18 (28%) PVL positive MSSA isolates caused community-acquired infections in our study (CA-MSSA)

(Table 4.11). These included two cases of pneumonia, two septic arthritis cases and one skin and soft tissue infection. On the other hand, 6/18 (33%) PVL positive MSSA isolates caused hospital-acquired infection and 4/18 (22%) HCA infection, mainly intravascular catheter-related sepsis (sections 4.2.3 and 4.2.5). A study conducted by Perez-Vazquez *et al* (2009), detected PVL genes in 42/203 (20.7%) *S. aureus* blood culture isolates from 21 Spanish hospitals. PVL positive strains were detected in 41/113 MSSA isolates and in only one MRSA isolate (1/90 of all MRSA). In contrast to our study, a high percentage of 48.8% (20/41) PVL positive MSSA isolates were found in the community and 36.6% (15/41) of the isolates were found in the hospital setting (details of 6 isolates were not available) (Pérez-Vázquez *et al.*, 2009).

Results of a study performed in Northern Italy showed that a PVL positive MSSA strain associated with prolonged community and hospital outbreaks of skin infections closely resembled a CA-MRSA strain (Tinelli *et al.*, 2009). The PVL positive MSSA strain was identified as *spa* type t005 and sequence type ST22. This clone (ST22) is related to one of the predominant MRSA clones spreading in hospitals in the United Kingdom, namely EMRSA-15 (Johnson *et al.*, 2001). Both MRSA and MSSA isolates, as well as PVL genes can be found in clonal group ST22 (Enright *et al.*, 2002). ST22-IV is also found carrying SCC*mec* type IV and is emerging in Portugal and Australia, with rare findings in the United States (Tinelli *et al.*, 2009). It was suggested that the MSSA clone (ST22) found in Northern Italy, could correspond to the ancestor of a hospital MRSA clone, or, less likely, to have been derived from a hospital MRSA clone with deletion of the SCC*mec* gene, in order to emerge into a MSSA clone (Tinelli *et al.*, 2009).

It is uncertain whether PVL positive CA-MRSA clones were acquired through the PVL phage by strains with a methicillin resistance background or, on the contrary, by the acquisition of a *SCCmec* element of strains that were PVL positive (Strommenger *et al.*, 2008b; Tristan *et al.*, 2007). CA-MRSA can be differentiated from HA-MRSA in that these strains retain susceptibility to non-beta-lactam antibiotics and are associated with SCC*mec* type IV (Miller *et al.*, 2005; Vandenesch *et al.*, 2003). However, these distinctions are conflicting to the evidence that suggest that hospital and community acquired MRSA strains share a common ancestor (Robinson *et al.*, 2005). Goering *et al* (2008) reported a study on the molecular characterization of *S.*

aureus isolates, acquired from uncomplicated skin infections from 10 different countries, during five phase III global clinical trials of retapamulin, a recent topical antibiotic agent. The most common PVL-positive MRSA clone was isolated in the United States, with a SCC*mec* type IV, multilocus sequence type 8 and a pulsed-field type USA300. Interestingly, the most common PVL-positive MSSA clone was found in South Africa and the Russian Federation with a multilocus sequence type 121 and pulsed-field type USA1200. In addition, PVL positive MSSA isolates from South Africa and India displayed pulsed field types related to EMRSA-15.

In another study by Campbell *et al* (2008), the genotypic differences between strains from different geographic regions is illustrated. A collection of *S. aureus* strains collected for an international clinical trial (FAST II) evaluating telavancin for treatment of complicated skin and skin structure infections was analyzed. The trial included 99 *S. aureus* isolates from 11 sites in the United States (56 isolates) and 7 sites in South Africa (34 isolates). No MRSA were collected from South African isolates, whereas more than three-quarters of the U.S isolates were MRSA. Genotyping consisted of SCC*mec, agr* and PFGE to determine the presence of 31 virulence genes. Briefly, results from South Africa showed that it's more common for MSSA isolates to carry certain virulence genes which include *sdrD* (p=0.01)*, sea* (p<0.01) and *pvl* (p=0.01). In contrast, MRSA isolates from the United States more frequently carried these virulence genes than MSSA isolates. Furthermore, PFGE patterns of South African and U.S isolates were distinctively different. The outcome was that virulence genes are distributed differently within various geographic regions.

Lina *et al* (1999) suggested that necrotizing pneumonia and primary skin and soft tissue disease are associated with *S. aureus* strains producing PVL toxin. They did not detect PVL-producing *S. aureus* strains in infective endocarditis, mediastinitis, urinary tract infections or enterocolitis infections (Lina *et al.*, 1999). Similarly, Holmes *et al* (2005) did not detect invasive infections, such as endocarditis and osteomyelitis, nor toxic shock syndrome or food poisoning associated with *S. aureus* isolates harbouring PVL. Furthermore, a high prevalence of PVL positive strains (38.9%) associated with arthritis and skin abscess were detected by (Melles *et al.*, 2006). A relationship was found between skin and soft tissue infections and PVL isolates, but not for bacteraemia (Melles *et al.*, 2006). This suggests that

staphylococcal bacteraemia is not a common factor of PVL-positive strains, which is in agreement to a study by (Johnsson *et al.*, 2004).

Moreover, Ellington *et al* (2007) detected a very low incidence of 1.6% (4/244) PVL positive MSSA strains, with no PVL positive MRSA from 244 bacteraemic patients from the UK and Ireland. The source for bacteraemia in these four patients included skin and soft tissue infection, indwelling line-related sepsis, surgical site infection and one unknown source. In another study, Holmes *et al* (2005) noted *S. aureus* bacteraemia in 2% of all PVL positive strains in England and Wales. It was also found that CA-MRSA strains in patients presenting with pneumonia, did not develop an associated bacteraemia (Etienne, 2005). On the contrary, Seybold *et al* (2006) detected up to 20% bloodstream infections that were due to the PVL positive CA-MRSA strain, USA300. This clone is currently not prevalent in the UK or in Europe, as only isolated cases are being found.

Due to the low incidence rates of PVL in *S. aureus* bacteraemia, it has been suggested that a complex combination of host and/or pathogen related factors play an important role in *S. aureus* bacteraemia (Ellington *et al.*, 2007). Studies have noted that the most likely reservoir of PVL-positive strains are found in skin infections (Holmes *et al.*, 2005; Lina *et al.*, 1999). Holmes *et al* (2005) detected 8/515 (1.6%) PVL positive *S. aureus* isolates from various sites of infection from patients in the UK. Skin and soft tissue infections (especially abscesses) were the most predominant, followed by community acquired pneumonia, burn wound infections, bacteraemia and scalded skin syndrome. Of the PVL positive MSSA strains, most belonged to sequence types ST22 and ST30, which is associated with the most prevalent MRSA clones in the United Kingdom, EMRSA-15 and EMRSA-16.

Overall, our findings are in agreement with these published reports. PVL positive CA-MSSA strains were associated with pneumonia, soft tissue infections and septic arthritis (Table 4.10). However, in our study catheter sepsis was more common in HA-infections and HCA-infections due to PVL-positive isolates. The incidence of staphylococcal bacteraemia due to PVL-positive stains was also higher (15.9%) compared to the UK study (1.6%, Ellington study) and more in line with the incidence of 20% reported in the American study (Seybold *et al.*, 2006).

Of the six major *spa* types detected in this study (Table 4.5), all seven isolates of *spa* type t891 (singleton) and one isolate from *spa* type t002 (*spa*-CC 002) were positive for PVL. Other major *spa* types were all negative for PVL. Of the remaining PVL positive strains, *spa* types t311 (*spa*-CC 002), t433 (*spa*-CC 012) and three other singletons (t317, t1597, t409) were detected. From *spa*-CC 174, *spa* types t174 and t5471 (novel type) were also PVL positive as well as three non-typeable *S. aureus* strains (Table 4.10). It was noted that the strains of six *spa* types (t891, t311, t317, t409, t433, t5471) were all PVL positive (Figure 4.18). This may indicate that the PVL positive strains in our study are associated with certain *spa* types. However, as the number of PVL positive MSSA strains in our study was low, this cannot be concluded (Section 4.2.5). A Spanish study by Pérez-Vázquez *et al* (2009), also observed a broad distribution of *spa* clonal complexes and singletons for MSSA that were PVL positive. Of the PVL positive MSSA strains found in their study, only *spa* types t002 and t311, belonging to *spa*-CC 002, were also detected in our study.

As only a low number of *S. aureus* strains are PVL-positive, it has been suggested that only a few *S. aureus* strains are susceptible to infection with PVL-converting phages (Holmes *et al.*, 2005). This was noted in a study by Narita *et al* (2001), where only 3% of clinical PVL negative *S. aureus* strains were infected with the temperate phage φSLT, thus converting into a PVL positive *S. aureus* strain. It has also been reported that various *S. aureus* strains harbor various PVL-carrying phages (Kaneko *et al.*, 1997; Kaneko *et al.*, 1998; Narita *et al.*, 2001; Zou *et al.*, 2000). Nonetheless, it is unclear whether these phages are able to infect PVL negative strains, leading to the expression of PVL toxin (Holmes *et al.*, 2005).

A few studies from European countries report a low prevalence of PVL positive *S. aureus* infections. In the Netherlands, the PVL gene was identified in 0.6% (5/829) of colonizing MSSA strains and in 2.1% (3/146) of blood culture isolates (Melles *et al.*, 2006). Another study from Germany also reported low PVL positive *S. aureus* rates for colonizing and blood culture isolates (von Eiff *et al.*, 2004). On the other hand, a study from Spain reports a high prevalence rate (36.4%) of invasive PVL positive *S. aureus* infections (Pérez-Vázquez *et al.*, 2009). The authors stated that the prevalence of PVL positive MSSA infections were increasing rapidly in Spain, since the prevalence was 1.6% in 2006 (Cuevas *et al.*, 2006). Other studies in which high rates

of PVL were detected include a study from Argentina where 61.3% (19/31) of MSSA isolates produced PVL (Sola *et al.*, 2007) and from the Cape Verde region, 35% of nosocomial MSSA isolates were PVL positive (Aires-de-Sousa *et al.*, 2006). A study conducted in Singapore presented with a high incidence of MRSA (43%) with no PVL detection in these MRSA strains (Hsu *et al.*, 2005). Of all the *S. aureus* strains, PVL was found in 11.6% MSSA strains only. The majority of the MSSA strains (27.2%) were detected in skin and soft tissue infections (Hsu *et al.*, 2005). These findings were similar to our current study, as PVL positive strains were only detected in MSSA and not in MRSA strains (Table 4.11).

In summary, infections caused by PVL positive MSSA isolates have been reported since the 1930's (Melles *et al.*, 2006). However there has been a vast increase in these infections from certain geographic areas, particularly Spain and Argentina. PVL-positive CA-MRSA has emerged in the United States, but this USA300 strain, is still rare elsewhere. PVL is found predominantly in MSSA strains in our setting. Further epidemiological studies are required to monitor if infections associated with these strains are increasing and to monitor for the emergence of CA-MRSA.

5.2.3 The *spa* gene

In this study, 49 different spa types were identified in 89.3% (101/113) of staphylococcal isolates. Non-typeable strains that failed to yield the *spa* gene constituted 9.7% (11/113) of the strain collection.

A study conducted by Larsen *et al* (2008) revealed 9/759 (1.2%) isolates that failed to yield the *spa* gene. These isolates were identified as *S. aureus* by coagulase tests and PCR detection of the *nuc* and *femA* gene. Similarly, a study by Forsgren (1970) identified 8/700 (1.2%) isolates lacking the *spa* gene. These eight isolates were assigned as protein A negative strains. Guzman *et al* (1992) identified 5% (10/196) of their *S. aureus* strains as protein A negative. Interestingly, it was also found that two organisms namely, *S. hyicus* and *S. intermedius*, yielded negative results (Guzman *et al.*, 1992). These organisms were misidentified as *S. aureus* as they also produced staphylocoagulase (Guzman *et al.*, 1992).

Therefore, there are various reasons for absence of the *spa* gene. This could be due to either isolates not being *S. aureus*, or due to the fact that these strains are protein A

negative. To confirm that these isolates are indeed *S. aureus* strains, PCR should be performed for the detection of either the *nuc* or *femA* genes (Fenner *et al.*, 2008; Larsen *et al.*, 2008). The reason is that phenotypic tests alone is not sufficient to identify these non-typeable isolates, as various studies have found that up to 10% of *S. aureus* strains lacking protein A (Forsgren, 1970; Kronvall *et al.*, 1971; Lindmark *et al.*, 1977; Maxim *et al.*, 1976; Winbald & Ericson, 1973). In our study, these strains were only re-tested phenotypically (refer to section 3.2) and not confirmed genotypically via PCR detection of the *nuc* or *femA* genes.

5.2.4 *Spa* Typing

Spa typing, a PCR and DNA sequencing based technique, was performed to compare the *spa* types with profiles of international important clones. Furthermore, the BURP (Based Upon Repeat Patterns) algorithm installed in the Ridom StaphType software program, was used to determine various clonal cluster complexes in this study. In the following section, *spa* types within each *spa*-CC and its association to important clones from various countries will be discussed. In addition, the relevant genotypic and clinical data correlated to each *spa*-CC will be discussed.

5.2.4.1 *Spa*-Clonal Complex 701 (Cluster 1)

The most frequent (major) *spa* type observed in *spa*-CC 701 was t1257, followed by minor types t1443, t701, t451 and t064. These *spa* types were associated with hospital or health-care associated infections. As mentioned previously, t1257 was one of the major HA-MRSA strain types in our study (Table 4.14), whereas t1443 was detected in both MRSA and MSSA hospital acquired strains. *Spa* type t701 was only found in HA and HCA- MSSA strains and t064 in MRSA and MSSA hospital acquired and health-care associated strains. *Spa*-CC 701 (Cluster 1) and *Spa*-CC 012 (cluster 2) contained the majority of MRSA strains (96.6%) [Figure 4.19]. Cluster 1 also contained 30% (12 isolates) of MSSA strains (Figure 4.19).

From *spa*-CC 701, three novel *spa* types (t5472, t5473, t5474) were detected from Tygerberg Hospital. Also, *spa* types, t2360 and t1443 were only identified in South Africa thus far. All the remaining *spa* types in this clonal complex are geographically distributed in various parts of the world (Appendix D).

A recent South African study identified t037 as the most prevalent *spa* type, followed by t012 as the second most prevalent (Oosthuysen *et al.*, 2007). In the present study, *spa* type t037 was the most prevalent, whereas t012 was a minor *spa* type. Oosthuysen *et al* (2007) identified *spa* type t064 as the third most prevalent type in 20.3% of *S. aureus* isolates from all provinces, which belonged to SCC*mec* type I-*pls* (Oosthuysen *et al.*, 2007). In contrast, the present study identified *spa* type t1257 (*spa*-CC 701) in 6% (6/101) of the isolates, being the third most prevalent *spa* type at Tygerberg Hospital, whereas t064 was a minor *spa* type in our study (Table 4.5).

Oosthuysen *et al* (2007), also found *spa* type t1257 in 8.9% of their isolates. Besides South Africa, *spa* type t1257 has also been detected in Denmark, Germany and Norway (Appendix D). Interestingly, all five strains of the HIV positive patients in this study were identified as *spa*-CC 701. Three of the *spa* types were t1257, one t1476 and one t064. All of these *spa* types were hospital-acquired MRSA strains, except for t1476. It has been previously reported that *spa* type t064 has been associated with persons living with AIDS (Gordan *et al.*, 2005). However, to our knowledge, no studies have reported HIV in association with *spa* types t1257 and t1476.

Chmelnitsky *et al* (2008) identified *spa* type t064, possessing SCC*mec* type IVc and PFGE clone G. Also, s*pa* type t064 was designated ST8 ORSA I in the USA. Shittu *et al* (2009) identified *spa* type t064 with different sequence types ST1173/SCC*mec* IV and ST1338/SCC*mec* IV. Multilocus sequence types ST1173 and ST1338 are single-locus and double-locus variants of ST8. The clone ST8 has been previously reported in Europe, Australia and North America (Shittu *et al.*, 2009).

5.2.4.2 *Spa*-Clonal Complex 012 (Cluster 2)

In the present study, t037 (major), t021 (major) and t012 (minor) were the most frequent *spa* types observed in *spa-CC* 012. These types were associated with hospital and healthcare associated infections. The majority of MRSA isolates (16; 53%) were detected in *spa*-CC 012 (Cluster 2) (Figure 4.19). Ten (25%) of MSSA strains were also detected in this cluster group (Figure 4.19). *Spa* type t037 was also the most frequent *spa* type in this study (9/101, 9%).

Spa type t433 was revealed as the only PVL positive MSSA strain in this cluster group (Figure 4.18). To our knowledge, no studies have detected an association between PVL and *spa* type t433. *Spa* type t1848 was only identified in South Africa and France (Appendix D). All the other *spa* types in this cluster group were found worldwide (Appendix D).

Studies have reported that divergent *spa* types t037 and t030 were found in MRSA isolates of ST239 (CC8), clustering with *spa* types t012 and t018, which is associated with *spa*-CC 012 (corresponds to MLST CC30) (Koreen *et al.*, 2004; Malachowa *et al.*, 2005; Strommenger *et al.*, 2006). It was shown that these isolates were all grouped together in one clonal complex by BURP analysis, even though they belonged to different clonal complexes by MLST, CC8 and CC30, respectively. This has been described previously by chromosomal DNA replacement in the MRSA isolates of CC8 with a large genetic element originating from CC30, which included the *spa* gene (Robinson & Enright, 2004). Similarly, a South African study found that 95% of strains were associated with a single SCC*mec* type within a *spa*-CC (Oosthuysen *et al.*, 2007). However, this was not the case in *spa*-CC 012, as the clonal complex was associated to both the Brazilian/Hungarian clone (n=79; *spa* type t037; SCC*mec* type III) and EMRSA-16 (prevalent MRSA clone in UK), or a single-locus variant (SLV) thereof (n=80; *spa* types t018/t012; SCC*mec* type II). We suggest that the same phenomenon occurred in the present study, as *spa* types t012, t018, t037 and t030 were all grouped into *spa*-CC 012 (Figure 4.5). Nonetheless, SCC*mec* typing and MLST should be performed in order to confirm this.

Spa type t037 has been reported from the United States in MRSA isolates of lineages ST8 and ST239 (Koreen *et al.*, 2004). The Brazilian/Hungarian clone with SCC*mec* type III and ST 239 was identified in *spa* types t037 and t030 (Deurenberg *et al.*, 2007). Fenner *et al* (2008) found that *spa* type t030 corresponded to hospital acquired MRSA strains. In contrast, our study detected two isolates from *spa* type t030 that were hospital acquired MSSA strains. *Spa* types t030 and t037 was also detected in Germany and other parts of Europe in a study by von Eiff *et al* (2004). Khandavilli *et al* (2009) detected both *spa* types t012 and t018 as EMRSA-16 (ST 36), with the majority of strains (92%) from *spa* type t018 (Khandavilli *et al.*, 2009).

A recent study conducted from all cities in China, identified *spa* type t030 as the most predominant type (Liu *et al.*, 2009). The *spa* type was detected in 52% of the isolates, with PFGE types A to E. *Spa* type t037 was established as the second most common type, constituting 25% of all isolates, with PFGE types F to G. Both *spa* types t030 and t037 were designated PFGE types A to K when associated with ST239, belonging to CC239 (distinct branch within CC8). The Brazilian/Hungarian (ST239; SCC*mec* type III) and the New York/Japan (ST5; SCC*mec* type II) clones were found to be the most dominant in Asia (Liu *et al.*, 2009).

Similarly, a South African study from hospitals in the KwaZulu-Natal province, revealed *spa* type t037 as the second most common type (Shittu *et al.*, 2009). The *spa* type was designated as a Brazilian/Hungarian clone with ST239, SCC*mec* type III and PFGE type F. This clone has been widely distributed throughout Brazilian hospitals (Teixeira *et al.*, 1995), countries in South America (Corso *et al.*, 1998), Europe (Aires-de-Sousa *et al.*, 1998) and Asia (Feil *et al.*, 2008; Liu *et al.*, 2009). Furthermore, Smyth *et al* (2008) reported the geographical stratification of the Brazilian/Hungarian clone. According to our present study, *spa* type t037 was identified as the most predominant type at Tygerberg Hospital. This *spa* type was only found in MRSA strains in hospital and health-care associated infections (Figure 4.21 and 4.22). This corresponds with a study by Oosthuysen *et al* (2007), indicating that *spa* type t037 is rapidly circulating throughout various provinces in South Africa.

Spa type t021 has been previously identified as a major hospital pathogen in MSSA strains, containing the *lukS-lukF* determinant for PVL, in the 1960's and 1980's

(Parker *et al.*, 1974). However, of the PVL positive MSSA strains in this present study, *spa* type t021 was not found (Table 4.18). All strains from *spa* type t021 were HA-MRSA. In a recent study, the *spa* type t021 was identified in MRSA and MSSA isolates containing *tst* but not the *lukS-lukF* determinant for PVL (Strommenger *et al.*, 2006). *Spa* types t012, t018 and t021 are commonly found in MSSA isolates of MLST CC30 from the United States and Poland (Koreen *et al.*, 2004; Koreen *et al.*, 2005; Malachowa *et al.*, 2005). These three *spa* types were also found in Australian and South American isolates, designated ST30 (Deurenberg *et al.*, 2007). ST30 was also discovered as a major clone in Asia and Oceania, which was referred to as the South West Pacific Clone (Tristan *et al.*, 2007). *Spa* types t012 and t021 were also reported from a University hospital, Switzerland in a study conducted by Fenner *et al* (2008).

5.2.4.3 *Spa*-Clonal Complex 002 (Cluster 3)

In the present study, t002 was the major *spa* type observed in *spa-CC* 002 and the fourth most prevalent type (6/101, 6%). This *spa* type was found predominantly in PVL-negative CA-MSSA strains, but HA-MSSA and HCA-MSSA strains and one PVL-positive strain were also detected. Other types detected in this cluster included t045, t214, t306 and t311. These strains were all MSSA (25% of MSSA strains in study) [figure 4.19].

Spa type t045 and t002 was detected in Germany and central Europe in a study (Strommenger *et al.*, 2006). Previous studies have reported that t002 clustered with t045 in MRSA isolates of lineage ST5 from Central Europe, Japan, South Korea and the United States (Chmelnitsky *et al.*, 2008; Harmsen *et al.*, 2003; Koreen *et al.*, 2004; Koreen *et al.*, 2005). *Spa* type t002 was the most frequent type amongst isolates in Israel (Chmelnitsky *et al.*, 2008). PFGE clones (B, D, H, J and K) with SCC*mec* types II or V, was identified within *spa* type t002. PFGE clone H exhibited SCC*mec* type IIIA variant which has been reported to exhibit amplicons of loci E and F in Taiwan (Lu *et al.*, 2005). Moreover, *spa* types t002 and t045 has been found in the Paediatric clone (SCC*mec* IV; ST5) and in the UK EMRSA-3 (SCC*mec* I; ST5) strain (Deurenberg *et al.*, 2007). In 2005, a PVL positive MRSA strain with ST5 (*spa* type t002) was described in an outbreak among members of a football team from Slovenia (Muller-Premru *et al.*, 2005). PVL positive strains were also detected in *spa* type t002

in southern Germany (Fenner *et al.*, 2008). Liu *et al* (2009) found *spa* type t002 with PFGE types L to T and ST5 (CC5) to be the third most prevalent type in cities among China.

A study conducted in the KwaZulu Natal province, South Africa, found *spa* type t045 (MRSA) to be the third major clone presenting with ST5, PFGE type G and SCC*mec* type III (Shittu *et al.*, 2009). This clone is similar to the New York/Japan clone (ST5; SCC*mec* II) and the Paediatric clone (ST5-SCC*mec* IV). As mentioned in previous studies, *spa* type t002 is found in ST5, as well as in ST125 and ST231 (Pérez-Vázquez *et al.*, 2009). Nübel *et al* (2008) discovered at least six types of SCC*mec* types in association to the ST5 clone. It was revealed that ST5 MRSA clones emerged frequently in various regions of the world with an addition of SCC*mec* into a methicillin susceptible ST5 (Nübel *et al.*, 2008). It was identified that MRSA in ST5 from South Africa and Kenya developed a sublineage (ST5-D) which was not closely related to MRSA *spa* type t045 from other continents. Moreover, PVL positive MSSA strains from Kenya were found to be the closest relatives of MRSA from South Africa (Shittu *et al.*, 2009). These observations indicate that MSSA clones are distributed worldwide and could become resistant through multiple independent imports of SCC*mec* in Africa.

Perez-Vazquez *et al* (2009) found *spa* type t002 as the second most dominant type in Spain, which has also been detected in Germany (Harmsen *et al.*, 2003), Austria (Ruppitsch *et al.*, 2006) and the USA (Shittu *et al.*, 2009). Forty one PVL positive MSSA strains were identified, including *spa* types t002 and t311 (*spa*-CC 002) (Pérez-Vázquez *et al.*, 2009). In our study, one t002 and one t311 strain from this cluster were identified as PVL positive MSSA strains (Table 4.10). Perez-Vazquez *et al* (2009) also identified *spa* type t002 and ST5 in both MRSA and MSSA isolates. The study identified nine isolates of the ST5 clone as PVL positive MSSA. Authors suggest that the acquisition of the *mecA* gene by ST5 could possibly lead to the prevalence of PVL in MRSA isolates (Pérez-Vázquez *et al.*, 2009).

A study by Otter & French (2008) from a London teaching hospital, identified *spa* type t002 in a range of health-care associated (HCA) strains as well as in hospital

(HA) and community acquired (CA) infections (Otter & French, 2008). The findings of this study is similar to our study, as HCA, HA and CA strains were identified in *spa* types t002 (Figure 4.22).

5.2.4.4 *Spa*-Clonal Complex 015 (Cluster 4)

Spa type t015 was found to be associated with ST45 and SCC*mec* type IV (Deurenberg *et al.*, 2007) and also found to be one of the major *spa* types in our study (6/101, 6%) and the most frequent *spa* type in cluster 4. The majority of strains in this *spa* type were MSSA, and found in HA, HCA and CA infections (Figure 4.21 and 4.22). Furthermore, this s*pa* type is broadly distributed throughout the world (Appendix D), reported mainly from the United States (Koreen *et al.*, 2004) and Poland (Malachowa *et al.*, 2005). Other *spa* types detected in cluster 4 include t465, t2623 and t4576. *Spa* type t4576 has been identified only in Germany and South Africa, whereas type t465 was found in Germany, South Africa and Denmark (Appendix D). The remaining *spa* type 2623 from *spa*-CC 015 has been detected in the Netherlands, Austria and South Africa.

5.2.4.5 *Spa*-Clonal Complex 174 (Cluster 5)

It has been reported in USA isolates, that *spa* type t127 and t174 both are associated with multi-locus sequence type 1 (ST1). Although CA-MRSA strains are commonly associated with SCC*mec* type IV, these strains have been found in the variant SCC*mec* type IVa (ST1) (Witte *et al.*, 2004). CA-MRSA strains with SCC*mec* type V in ST1 have been described in Singapore (Deurenberg *et al.*, 2007). *Spa* type 127 (ST1) is currently one of the most predominant CA-MRSA clones circulating within Germany and Central Europe (Strommenger *et al.*, 2008b).

In our study, all 5 isolates from *spa*-CC 174 (t127; t174; t5471) were found to be MSSA strains (Table 4.12) from hospital and health-care associated infections (Table 4.13). None of the *spa* types from this clonal complex were found among community acquired strains. Otter & French (2008) most commonly identified *spa* type t127 as ST1 and PVL negative. We identified only one isolate from t127 which was a PVL negative strain. Furthermore, two PVL-positive isolates from t174 and novel *spa* type t5471 were isolated in this cluster.

5.2.4.6 *Spa*-Clonal Complex 346/085 (Cluster 6)

Interestingly, two founders arose within this clonal complex, namely *spa* types t346 and t085. They are both closely related to *spa* type t5396, which is a novel strain detected at Tygerberg Hospital and in Poland (Appendix D). All three of *spa* types in this cluster group were found to be MSSA strains (Table 4.12) with one community acquired strain (Table 4.13). The HA/HCA/CA category could not be determined for the remaining two isolates (Table 4.13).

In *spa* type t085, a duplication of repeat unit r34 and a deletion of repeat unit r12 occurred. We suggested that *spa* types t346 and t085 are both founders due to the same SCC*mec* type and MLST. Only further studies involving other *S. aureus* genotyping techniques on both of these strains would enlighten this statement, as to our knowledge, no evidence is currently found. A study by Mellman *et al* (2008) also identified two founder strains in two of their *spa*-CC's namely, *spa*-CC 382/399 and *spa*-CC 084/346. However, the findings of these founder strains were not further described.

5.2.4.7 No founder (Cluster 7)

As this clonal complex has no founder, the evolution of these strains cannot be deduced. The only difference detected between these two strains was that a duplication of r34 occurred in *spa* type t521. It was shown that s*pa* types t267 and t521 were both MSSA strains (Table 4.12), These *spa* types are distributed in many countries throughout the world (Appendix D).

5.2.4.8 Singleton *spa* types

All the *spa* types grouped together as singletons by BURP analysis were MSSA strains (Table 4.12) that were not related to any other *spa* types or could not be categorized into a clonal complex. *Spa* type t891 was the most common singleton and also the second most prevalent *spa* type in our study (7/101; 7%). All isolates from this *spa* type were MSSA and were positive for PVL. The majority of the strains from this *spa* type were associated with health-care associated infections (Figure 4.22). *Spa* type t1597, which was also found frequently in this study, was associated with HA-MSSA strains. The remaining singletons that were positive for PVL included *spa*

types t317 and t409. All of these *spa* types that are singletons are observed widely throughout many countries of the world (Appendix D).

5.3 <u>Persistent/Recurrent bacteraemia</u>

Persistent bacteraemia, commonly found among hospitalized patients, was defined in the current study as a repeat positive blood culture ≥3 days after appropriate therapy was initiated. This definition was also used in a study by Hawkins *et al* (2007). However, the literature regarding persistent *S. aureus* bacteraemia, poorly define persistence and vary in study design and study population. For instance, Fowler *et al* (2004) used a definition of persistence as bacteraemia for longer than 7 days in patients presenting with MRSA infections only (Fowler *et al.*, 2004). Khatib *et al* (2006) defined persistence as bacteraemia for ≥3 days, which was observed in 38.4% of the patients with both MRSA and MSSA isolated from January to December 2002. In 2009, the same authors used a more stringent definition of persistence of ≥7 days (Khatib *et al.*, 2009). This study occurred from January 2002 to June 2006 and November 2005 to December 2006. The authors reported that the frequency of persistent *S. aureus* bacteraemia was higher than their previous study published in 2006 (Khatib *et al.*, 2009). Furthermore, Chang *et al* (2003) subclassified persistence as a positive blood culture at ≥3 days or ≥7 days after appropriate antibiotic therapy in order to compare nafcillin therapy with vancomycin at these two time periods. Nafcillin was superior to vancomycin in preventing bacteriological failure (persistent bacteraemia >7d and/or relapse; 0% versus 19%, p=0.058).

5.3.1 Persistent bacteraemia:

In the current study, persistence according to our definition was proved in one patient only (1/104, 0.9%) (Table 4.15). This 7 day-old infant presented with MRSA bacteraemia due to a hospital-acquired MRSA skin infection of the umbilicus and cellulitis of the hand with subsequent abscess formation. MRSA was cultured from pus swabs from both skin sites. The patient was treated with vancomycin for 11 days. No surgical abscess drainage was done. *Spa* type t021 (*spa* CC012/Cluster 2) was detected in both the initial and subsequent blood culture strain (9 days later) with no

detection of *pvl*. Phenotypic testing for heteroresistance to the glycopeptides using the E-test macromethod was negative.

Yardena *et al* (2005) found that most of the patients in their study presented with a secondary focus of infection serving as the site of persistence, in addition to the primary focus (or portal of entry). It has been reported that MRSA infections in neonates may vary from either superficial skin infections to severe, invasive diseases such as bacteraemia, bone infections and meningitis (Chuang *et al.*, 2004). Chuang *et al* (2004) found that infants with persistent bacteraemia were commonly due to undrained pus or abscesses or a delay in catheter removal. Their study concluded that catheter-related infections were predominantly seen in neonates with MRSA bacteraemia. The findings in a study conducted by Khatib *et al* (2009), suggested that MRSA and the presence of risk factors were associated with persistent *S. aureus* bacteraemia. It was reported that factors related to persistence included endovasular sources, cardiovascular prosthesis, metastatic infections, diabetes and vancomycin treatment (Khatib *et al.*, 2006). Hawkins *et al* (2007) associated persistence with methicillin resistance, intravascular catheter or other foreign body use, chronic renal failure, more than two sites of infection and infective endocarditis. In another study, persistent and recurrent *S. aureus* bacteraemia has been primarily found to be associated with endocarditis, osteomyelitis or abscess formation (Yardena *et al.*, 2005).

Another factor contributing to the persistence of MRSA bacteraemia is the possibility of reduced susceptibility to vancomycin in subpopulations. In the current study, the one persisting *S. aureus* strain did not exhibit reduced susceptibility or heteroresistance to vancomycin. Heteroresistance to vancomycin is associated with cell wall thickening due to overproduction of cell wall precursors in the peptidoglycan layer, the cell wall of staphylococci become less permeable to vancomycin and other antibiotics. This results in an increase in MICs after initial vancomycin therapy is given (Cui *et al.*, 2003; Cunha *et al.*, 2009). A study by Jang *et al* (2009), evaluated the effectiveness of linezolid with or without carbapenem in salvage treatment for persistent MRSA bacteraemia over a 3 year period. It was found that patients had a higher salvage success rate for linezolid therapy than for vancomycin-based combination therapy (with aminoglycosides or rifampicin) (Jang *et al.*, 2009).

Yardena *et al* (2005), examined the possible association of vancomycin therapy to persistent *S. aureus* bacteraemia in Israel. The first study occurred during a 2 year period, and included patients who completed ≥10 days of appropriate anti-staphylococcal therapy. It was found that MRSA and MSSA persistent *S. aureus* bacteraemia, after >3 days of therapy, occurred in none of the 52 cloxacillin recipients and in 11/55 (20%) vancomycin recipients. The second study was done over a 4 year period and included all patients with persistence and/or relapse of *S. aureus* bacteraemia on anti-staphylococcal therapy. Persistence after more than 3 days of vancomycin therapy occurred in 94% (32/34) of the patients. In general, persistent *S. aureus* bacteraemia leads to significantly longer hospitalization of patients and high mortality rates (Hawkins *et al.*, 2007).

5.3.2 Recurrent bacteraemia:

Recurrent bacteraemia was defined in our study as the return of *S. aureus* bacteraemia after documenting a negative blood culture and/or clinical improvement after the completion of a course of appropriate antibiotics.

Reports on recurrence of *S. aureus* bacteraemia cite endocarditis, distal septic complications and a short duration (<10 days) of parenteral antibiotic treatment for catheter-related bacteraemia as risk factors (Chang *et al.*, 2003). In order to distinguish between relapse of the same infection and re-infection, molecular typing techniques for *S. aureus* should be performed. Chang *et al* (2003) used PFGE to differentiate between relapse and re-infection of *S. aureus* bacteraemia in patients,that were followed up for 6 months after completion of therapy. Recurrence was detected in 9.4% (42/448) of the patients. Fowler *et al* (1999) also used PFGE to identify relapses where isolates were identical in recurrent episodes of *S. aureus* bacteraemia.

5.3.2.1 Recurrent bacteraemia due to relapse

In our current study, *spa* typing revealed identical *spa* types of recurrent and initial isolates (t015/*spa*-CC 015/cluster 4; t253/*spa*-CC 012/cluster 2) respectively in two patients (2/104, 2%) [Table 4.16]. These recurrent episodes were considered to be

relapses, although the possibility of re-infection with the same colonizing strain cannot be excluded. Both patients had underlying chronic renal failure and received haemodialysis. HCA-MSSA was identified in both these patients, with bacteraemia due to catheter-related sepsis. The patients initially responded after removal of dialysis catheters, but both patients were treated with vancomycin although cloxacilin was advised by the resident microbiologist, at Tygerberg Hospital. These findings are in agreement to those of Fowler *et al* (1999), as relapses in patients were commonly related to a indwelling foreign body, patients receiving vancomycin therapy and in patients undergoing haemodialysis. The population of haemodialysis patients have a high prevalence of *S. aureus* nasal carriage (35-62%) which may be the cause of higher recurrent bacteraemia rates with the same colonizing strain (Mokrzycki *et al.*, 2006). Chang *et al* (2003) also found that patients on haemodialysis were significantly more likely to receive vancomycin for MSSA bacteraemia (46% on vancomycin versus 12% on nafcillin). Furthermore, multivariate analysis (adjusting for hemodialysis) showed that endocarditis and vancomycin therapy were predictive of relapse (Chang *et al.*, 2003). Patients that presented with catheter-related bacteraemia had the catheter removed in 90% of the cases in that study.

Hartstein *et al* (1992) used a typing technique by restriction endonuclease analysis of plasmid DNA (REAP DNA fingerprinting) and immunoblotting to differentiate between strains of recurrent *S. aureus* bacteraemia. The study included eight patients with ten episodes of recurrent bacteraemia. It was found that relapsing infections were related to the presence of intravascular foreign bodies, as well as vancomycin therapy of preceding episodes. Other studies have also used antimicrobial susceptibility patterns, phage typing and plasmid profiles to differentiate between strains of recurrent bacteraemia cases. Generally, relapses were commonly associated with intravascular devices that did not appear to be clinically infected (Hartstein *et al.*, 1992).

Beta-lactam agents are preferred above vancomycin to treat methicillin-susceptible *S. aureus* infections. Various studies have found that patients treated with vancomycin for MSSA bacteraemia present with worse outcomes (Chang *et al.*, 2003; Gottlieb *et al.*, 2000; Johnson *et al.*, 2003). For instance, Chang *et al* (2003) provided evidence that nafcillin was superior to vancomycin in treating *S. aureus* infection due to MSSA.

Vancomycin therapy should therefore be restricted to patients who are infected with methicillin resistant strains or patients who are allergic to beta-lactam antibiotics (Hartstein *et al.*, 1992). However, haemodialysis patients were more likely to receive vancomycin with significant association to relapse identified in a statistical model. The reason why vancomycin is given is that it has a long half-life in renal failure and can therefore be given once weekly as out-patient therapy (Chang *et al.*, 2003). A study by Walker *et al* (2009) reported a significant association between recurrence of MSSA bacteraemia and prior glycopeptide treatment in cases of central venous catheter sepsis where the catheter was removed (Walker *et al.*, 2009).

5.3.2.2 Recurrent bacteraemia due to re-infection:

In our current study, *spa* types were different between the initial and recurrent bloodculture isolates of 4 patients (4/104, 4%) and were therefore defined as re-infection with new strains. The majority of these re-infection cases were due to intravascular catheter infections. Two of the patients presented with a HA-MSSA infection (t002/*spa*-CC 002/cluster 3 and t1476/*spa*-CC 701/cluster 1) of the initial blood culture isolate, followed by a subsequent MRSA isolate (t037/*spa*-CC 012/cluster 2 in both patients) [Table 4.17]. Both patients were admitted to the burns ICU unit. The initial MSSA bacteraemia in one of the patients (TBH 7) was due to burn wound infection, whereas the subsequent bacteraemia was due to catheter sepsis CVP. The other patient (TBH 110) initially acquired CVP sepsis, and then developed a subsequent primary bacteraemia of unknown source. This patient had one temperature spike which resolved on the removal of the intravascular catheter. However, only *Proteus mirabilis* was cultured on the CVP tip. A survey of the North American National Nosocomial Infection Surveillance System, reported catheter-related infections at a high rate of 30.2 (in the burns ICU) and 2.1 (in the respiratory ICU) cases per 1000 central catheter days between 1986 and 1990 (Jarvis *et al.*, 1991).

Steinberg *et al* (1996) detected high rates of hospital acquired *S. aureus* bacteraemia due to infected intravascular related devices (Steinberg *et al.*, 1996). Yet, the skin is the most common site of infection. The infected skin area could serve as a portal of entry for the development of more invasive infections. If MRSA infection is endemic

in a setting, MRSA bacteraemia is likely to arise from localized skin infections and colonized indwelling intravascular devices (Chuang *et al.*, 2004). The outermost surface of catheters may become colonised with organisms that originate from the skin, whereas the inner surface of the catheter could become colonised with the introduction of organisms through the catheter hub (Crump & Collignon, 2000). Crump and Collognon (2000) give evidence on the number of organisms found on catheters that develop into local and bloodstream infections. Organisms were cultured from the catheter tip using the semi-quantitative roll-plate and sonication methods (Crump & Collignon, 2000). Biofilms also play an important part in catheter related infections, as they adhere to these medical devices. Appropriate antimicrobial therapy is often not effective in eliminating these infections. Therefore, the removal of catheter related devices is of utmost importance for successful patient outcome.

The remaining two patients developed relapse (table 4.16) and re-infection (table 4.17) episodes with HCA-MSSA infections. This phenomenon frequently occurs in chronic renal failure patients on haemodialysis, due to invasive procedures (dialysis catheter) and frequent hospital exposure. It was reported that catheter-related bacteraemia is commonly associated with patients undergoing haemodialysis (Allon, 2004). The frequency of these cases are more likely to increase, as haemodialysis patients are dependent on using catheters (Maya *et al.*, 2007). The majority (76.3%) of MRSA bacteraemia cases were found to be HCA in patients admitted to the emergency department of a medical centre in Taiwan (Liao *et al.*, 2008). Catheter-related sepsis (22.6%) was commonly found, followed by skin and soft tissue infection (20.9%), primary bacteraemia (15.3%) and infective endocarditis (6.8%) (Liao *et al.*, 2008). Robinson *et al* (2009) associated HCA bacteraemia predominantly to device related infections, or to surgical procedures in the elderly (Robinson *et al.*, 2009).

To our knowledge, no studies have been reported using specifically *spa* typing to differentiate between strains causing persistent or recurrent *S. aureus* bacteraemia. Thus, no association of *spa* types with persistent and recurrent *S. aureus* bacteraemia in comparison to other studies could be deduced. However, the discriminatory power of *spa* typing is similar to PFGE and this method has been validated for outbreak investigation (Hallin *et al.*, 2007).

It is important that cases of persistent/recurrent bacteraemia should be identified at an early stage and followed-up by infectious diseases specialists to ensure optimal management of these patients. A limitation to the current study is that the numbers of patients with persistent/recurrent bacteraemia were too small for meaningful statistical analysis. Prospective studies with larger patient numbers are therefore needed in order to investigate persistent and recurrent *S. aureus* bacteraemia.

CHAPTER SIX

6 CONCLUSION

Data on *S. aureus* epidemiology from South Africa is limited. This study provides the first data on *S. aureus* genotypes linked to prospective clinical data in the setting of a large academic hospital (Tygerberg hospital in the Western Cape Province, South Africa). Blood culture isolates collected over a period of 14 months were included in this study.

The multiplex PCR method for the detection of the three important genes used for MRSA surveillance: *mecA, pvl* and *spa* gene, performed well in our setting with a typeability of 89.3%. Our multiplex PCR method successfully detected *spa* genes and *spa* typing proved to be a useful method to determine strain relatedness. A considerable variety of *spa* types were detected at Tygerberg hospital. Many of these *spa* types are found worldwide, but 5 novel *spa* types have also been found. The most predominant *spa* type found in our setting was t037 (only in MRSA) and according to the literature is associated to the Brazilian/Hungarian clone (*SCCmec* type III; ST 239). South African studies indicate that t037 has been identified in clinical strains from numerous provinces in South Africa. Interestingly, all strains from *spa* type t891 (second most prevalent) were found to be PVL positive MSSA strains.

The information provided in this study contributes to our understanding of the local epidemiology of *S. aureus* and the prevalence rate of different strains. A major finding is that no CA-MRSA isolates were detected in this study. This is in contrast to the emergence of CA-MRSA reported from other countries. Furthermore, although PVL has been associated with CA-MRSA, PVL was only detected in MSSA isolates in this study. PVL positive strains constituted 15.9% of *S. aureus* isolates. PVL positive CA-MSSA strains were associated with pneumonia, soft tissue infections and septic arthritis. However, in our study catheter-related sepsis was more common in HA-infections and HCA-infections, and often caused by PVL-positive isolates.

The majority of bacteraemia cases were associated with intravascular catheter infections (for both MRSA and MSSA), followed by skin and soft tissue infections. MRSA strains were mainly found to cluster in *spa* CC 701 and CC-012 (cluster 1 & 2), whereas CC-002 (cluster 3) consisted of MSSA strains only. Furthermore, the majority of HA-strains were found in clusters 1 & 2. This information indicates that *spa* types in clusters 1 and 2 are closely associated with HA-MRSA strains at Tygerberg hospital.

The number of persistent and recurrent bacteraemia episodes were low in this study (9/113, 8%). Persistent bacteraemia was found in one patient only, presenting with a hospital-acquired skin and soft tissue infection. Recurrent bacteraemia cases were found in patients on dialysis for chronic renal failure and in burns patients related to catheter infections. These insights are useful for optimal diagnostic and therapeutic measures.

The techniques and *spa* typing software set up in our molecular laboratory through this study can be used in future for MRSA surveillance, outbreak detection and determining strain relatedness in persistent and recurrent infections. In future, *spa* typing may replace MLST as the typing method of choice, since this sequence-based method is much more affordable and quicker to perform. This may facilitate comparison of our strains with international epidemic clones.

Further studies are needed for the surveillance of *S. aureus* strains using a combination of molecular techniques (SCC*mec* typing, PFGE, *spa* typing, MLST) to relate our strains to international epidemic clones and to provide a much larger view of molecular epidemiology of MRSA and MSSA isolates in South Africa.

CHAPTER SEVEN

7 REFERENCES

Adem, P. V., Montgomery, C. P., Husain, A. N. & Others (2005). *Staphylococcus aureus* sepsis and the Waterhouse-Friderichsen syndrome in children. *N Engl J Med* **353**, 1245-1251.

Adesida, S. A., Likhoshvay, Y., Eisner, W., Coker, A. O., Abioye, O. A., Ogunsola, F. T. & Kreiswirth, B. N. (2006). Repeats in the 3' region of the protein A gene is unique in a strain of *Staphylococcus aureus* recovered from wound infections in Lagos, Nigeria. *African Journal of Biotechnology* **5**, 1858-1863.

Afroz, S., Kobayashi, N., Nagashima, S. & other authors (2008). Genetic Characterization of *Staphylococcus aureus* Isolates Carrying Panton-Valentine Leukocidin Genes in Bangladesh. *Jpn J Infect Dis* **61**, 393-396.

Aires-de-Sousa, M., Santos Sanches, I., Ferro, M. L., Vaz, M. J., Saraiva, Z., Tendeiro, T., Serra, J. & de Lencastre, H. (1998). Intercontinental spread of a multidrug-resistant methicillin-resistant *Staphylococcus aureus* clone. *Journal of Clinical Microbiology* **36**, 2590-2596.

Aires-de-Sousa, M. & de Lencastre, H. (2003). Evolution of sporadic isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) in hospitals and their similarities to isolates of community-acquired MRSA. *Journal of Clinical Microbiology* **41**, 3806-3815.

Aires-de-Sousa, M., Conceição, T. & de Lencastre, H. (2006). Unusually high prevalence of nonsocomial Panton-Valentine leukocidin-positive *Staphylococcus aureus* isolates in Cape Verde Islands. *Journal of Clinical Microbiology* **44**, 3790-3793.

AL-Haj, N. A., Mashan, N. I., Shamsudin, M. N., Mohamad, H., Vairappan, C. S. & Sekawi, Z. (2009). Antibacterial Effect of *Gracilaria changii* and *Euchema denticulatum* on Molecular Properties of *Staphylococcus aureus* Genes *mecA, mecR1* and *mecI*. *Research Journal of Biological Sciences* **4**, 580-584.

Allon, M. (2004). Dialysis catheter-related bacteremia: Treatment and prophylaxis. *Am J Kidney Dis* **44**, 779-791.

Amod, F., Moodley, I., Peer, A. K. C., Sunderland, J., Lovering, A., Wootton, M., Nadvi, S. & Vawda, F. (2005). Ventriculitis due to a hetero strain of vancomycin intermediate *Staphylococcus aureus* (hVISA): successful treatment with linezolid in combination with intraventricular vancomycin. *Journal of Infection* **50**, 252-257.

Arakere, G., Nadig, S., Swedberg, G., Macaden, R., Amarnath, S. K. & Raghunath, D. (2005). Genotyping of methicillin-resistant *Staphylococcus aureus* strains from two hospitals in Bangalore, South India. *Journal of Clinical Microbiology* **43**, 3198-3202.

Archer, G. L., Thanassi, J. A., Niemeyer, D. M. & Pucci, M. J. (1996). Characterization of IS*1272,* an Insertion sequence-like element from *Staphylococcus haemolyticus*. *Antimicrobial agents and Chemotherapy* **40**, 924-929.

Archer, G. L. (1998). *Staphylococcus aureus:* A Well-Armed Pathogen. *Clinical Infectious Diseases* **26**, 1179-1181.

Bannerman, T. L., Hancock, G. A., Tenover, F. C. & Miller, L. G. (1995). Pulsed-Field gel eletrophoresis as a replacement for bacteriophage typing of *Staphylococcus aureus*. *journal of Clinical Microbiology* **33**, 551-555.

Bouchillon, S. K., Johnson, B. M., Hoban, D. J., Johnson, J. L., Dowzicky, M. J., Wu, D. H., Visalli, M. A. & Bradford, P. A. (2004). Determining incidence of extended spectrum B-lactamase producing Enterobacteriaceae, vancomycin-resistant *Enterococcus faecium* and methicillin-resistant *Staphylococcus aureus* in 38 centres from 17 countries: the PEARLS study 2001-2002. *International Journal of Antimicrobial Agents* **24**, 119-124.

Boyce, J., Landry, M., Deetz, T. R. & DuPont, H. L. (1981). Epidemiologic studies of an outbreak of nonsomomial methicillin-resistant *Staphylococcus aureus* infections. *Infect Control* **2**, 110-116.

Boyce, J., White, R. L., Causey, W. A. & Lockwood, W. R. (1983). Burn units as a source of methicillin-resistant *Staphylococcus aureus* infections. *JAMA* **1983**, 2803-2807.

Boyle-Vavra, S. & Daum, R. S. (2007). Community-acquired methicillin-resistant *Staphylococcus aureus*: the role of Panton-Valentine leukocidin. *Laboratory Investigation* **87**, 3-9.

Callegan, M. C., Engel, L. S., Hill, J. M. & O'Callaghan, R. J. (1994). Corneal virulence of *Staphylococcus aureus*: Roles of alpha-toxin and protein A in pathogenesis. *Infection and Immunity* **62**, 2478-2482.

Campbell, S. J., Deshmukh, H. S., Nelson, C. L. & other authors (2008). Genotypic characteristics of *Staphylococcus aureus* isolates from a mulinational trial of complicated skin and skin structure infections. *Journal of Clinical Microbiology* **46**, 678-684.

Chang, F.-Y., Peacock, J. E., Musher, J., Daniel M, Triplett, P., MacDonald, B. B., Mylotte, J. M., O'Donnell, A., Wagener, M. M. & Yu, V. L. (2003). *Staphylococcus aureus* Bacteremia: Recurrence and the Impact of Antibiotic Treatment in a Prospective Multicenter Study. *Medicine* **82**, 333-339.

Chmelnitsky, S., Navon-Venezia, S., Leavitt, A., Somekh, E., Regev-Yochay, G., Chowers, M., Shirit, P. & Carmeli, Y. (2008). SCC*mec* and *spa* types of methicillin-resistant *Staphylococcus aureus* strains in Israel. Detection of SCC*mec* type V. *Eur J Clin Microbiol Infect Dis* **27**, 385-390.

Chongtrakool, P., Ito, T., Ma, X. X. & other authors (2006). Staphylococcal cassette chromosome mec (SCC*mec*) typing of methicillin-resistant *Staphylococcus aureus* strains isolated in 11 Asian countries: a proposal for new nomeclature for SCC*mec* elements. *Antimicrobial agents and Chemotherapy* **50**, 1001-1012.

Chuang, Y.-Y., Huang, Y.-C., Lee, C.-Y., Lin, T.-Y., Lien, R. & Chou, Y.-H. (2004). Methicillin-resistant *Staphylococcus aureus* bacteraemia in neonatal intensive care units: an analysis of 90 episodes. *Acta Paediatr* **93**, 786-790.

Cookson, B. D., Ashley, R., Monk, A. B. & other authors (2007). Evaluation of molecular typing methods in characterizing a European collection of epidemic methicillin-resistant *Staphylococcus aureus* strains: the HARMONY collection. *journal of Clinical Microbiology* **45**, 1830-1837.

Corso, A., Santos Sanches, I., Aires-de-Sousa, M., Rossi, A. & De Lencastre, H. (1998). Spread of methicillin-resistant and multi-resistant epidemic clone of *Staphylococcus aureus* in Argentina. *Microb Drug Resis* **4**, 277-288.

Crossley, K., Loesch, D., Landesman, B., Mead, K., Chern, M. & Strate, R. (1979). An outbreak of infections caused by strains of *Staphylococcus aureus* resistant to methicillin and aminoglycosides. I.Clinical studies. *Journal of infect Dis* **139**, 273-279.

Crump, J. A. & Collignon (2000). Intravascular catheter-associated infections. *Eur J Clin Microbiol Infect Dis* **19**, 1-8.

Cuevas, O., Cercenado, E., Vindel, A., Castellares, C., Guinea, J. & Bouza, E. (2006). Molecular characterization of *Staphylococcus aureus* isolated in a nationwide prevalence study in Spain. In *Abstracs of the Sixteenth European Congress Clinical Microbiology and Infectious Diseases*. Nice, France Abstract P465: European Journal of Clinical Microbiology Infectious Disease.

Cui, L., Ma, X., Sato, K. & other authors (2003). Cell wall thickening is a common feature of vancomycin resistance in Staphylococcus *S. aureus*. *Journal of Clinical Microbiology* **41**, 5-14.

Cunha, B. A., Nausheen, S. & Schoch, P. (2009). Persistent methicillin-sensitive *Staphylococcus aureus* (MSSA) bacteremia due to linezolid "tolerant" strain unresponsive due to daptomycin. *Heart and Lung*, 1-3.

de Bentzmann, S., Tristan, A., Etienne, J., Brousse, N., Vandenesch, F. & Lina, G. (2004). *Staphylococcus aureus* isolates associated with necrotizing pnemonia bind to basement membrane type I and IV collagens and lamini. *Journal of infectious Disease* **190**, 1506-1515.

Deurenberg, R. H., Vink, C., Kalenic, S., Friedrich, A. W., Bruggerman, C. A. & E.E, S. (2007). The molecular evolution of methicillin-resistant *Staphylococcus aureus*. *Clin Microbiol Infect* **13**, 222-235.

Dinges, M. M., Orwin, P. M. & Schlievert, P. M. (2000). Exotoxins of *Staphylococcus aureus*. *Clin Microbiol Rev* **13**, 16-34.

Dossett, J. H., Kronvall, G., Williams, R. C. J. & Quie, P. G. (1969). Antiphagocytic effects of staphylococcal protein A *The Journal of Immunology* **103**, 1405-1410.

Duckworth, G. (2003). Controlling methicillin-resistant *Staphylococcus aureus*. *British Medical Journal* **327**, 1177-1178.

Dumitrescu, O., Boisset, S., Badiou, C., Bes, M., Benito, Y., Reverdy, M.-E., Vandenesch, F., Etienne, J. & Lina, G. (2007). Effect of Antibiotics on *Staphylococcus aureus* producing Panton-Valentine Leukocidin. *Antimicrobial agents and Chemotherapy* **51**, 1515-1519.

Durack, D. T., Lukes, A. S. & Bright, D. K. (1994). New Criteria for diagnosis of infective endocarditis: utilization of specific echocardiographic findings. *The American Journal of Medicine* **96**, 200-209.

El-Sayed, A., Alber, J., Lämmler, C., Abdulmawjood, A., Zschöck, M. & Castaneda, V. H. (2006). Comparative sequence analysis of *spa* gene of *Staphylococcus aureus* isolated from bovine mastitis: characterization of an unusual *spa* gene variant. *Journal of Daily Research* **73**, 322-327.

Ellington, M. J., Hope, R., Ganner, M., Ganner, M., East, C., Brink, G. & Kearns, A. M. (2007). Is Panton-Valentine leukocidin associated with the pathogenesis of *Staphylococcus aureus* bacteraemia in the UK? *Journal of Antimicrobial Chemotherapy*, 1-4.

Enright, M. C., Nicholas, D. P. J., Catrin, D. E., Sharon, P. J. & Spratt, B. G. (2000). Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. *Journal of Clinical Microbiology* **38**, 1008-1015.

Enright, M. C., Robinson, D. A., Randle, G., Feil, E. J., Grundmann, H. & Spratt, B. G. (2002). The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). *PNAS:National Academy of Science of the USA* **99**, 7987-7692.

Essa, Z., Connolly, C. & Essack, S. (2009). *Staphylococcus aureus* from public hospitals in KwaZulu-Natal, South Africa-infection detection and strain-typing. *South Afr J Epidemiol Infect* **24**, 04-07.

Etienne, J. (2005). Panton-Valentine leukocidin: a marker of severity for *Staphylococcus aureus* infection? *Clin Infect Dis* **41**, 591-593.

Faria, N. A., Carrico, J. A., Oliveria, D. C., Ramirez, M. & Lencastre, H. (2008). Analysis of typing methods for epidemiological surrveillance of both methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* strains. *Journal of Clinical Microbiology* **46**, 136-144.

Feil, E. J., Nickerson, E. K., Chantratita, N., Wuthuekanun, V., Srisomang, P., Cousins, R., Pan, W., Zhang, G. & Xu, B. (2008). Rapid detection of the pandemic methicillin-resistant *Staphylococcus aureus* clone ST239 and its dominance in Asian hospitals. *Journal of Clinical Microbiology* **46**, 1520-1522.

Feng, Y., Chen, C.-J., Su, L.-H., Hu, S., Yu, J. & Chiu, C.-H. (2008). Evolution and pathogenesis of *Staphylococcus aureus*: lessons learned from genotyping and comparitive genomics. *FEMS MIcrobiolol Rev* **32**, 23-37.

Fenner, L., Widmer, F. A., Dangel, M. & Frei, R. (2008). Distribution of *spa* types among meticillin-resistant *Staphylococcus aureus* isolates during a 6 year period at a low-prevalence university hospital. *Journal of Medical Microbiology* **57**, 612-616.

File, T. M. (2008). Methicillin-resistant *Staphylococcus aureus* (MRSA): focus on community-associated MRSA. *South Afr J Epidemiol Infect* **23**, 13-15.

Fink-Barbancon, V., Prevost, G. & Piemont, Y. (1991). Improved purification o leukocidin from *Staphylococcus aureus* and toxin distribution among hospital strains. *Re Micrbiol* **142**, 75-85.

Fischetti, V. A., Novick, R. P., Ferretti, J. J., Portnoy, D. A. & Rood, J. I. R. (2006). *Gram-Positive Pathogens: Section III, The Staphylococcus*, Second Edition edn. U.S.A.

Forsgren, A. (1970). Significance of protein A production by Staphylococci. *Infection and Immunity* **2**, 672-673.

Foster, T. J. (2004). The *Staphylococcus aureus* "superbug". *J Clin Invest* **114**, 1693-1696.

Fournier, B. & Klier, A. (2004). Protein A gene expression is regulated by DNA supercoiling which is modified by the ArIS-ArIR two component system of *Staphylococcus aureus*. *Microbiology* **150**, 3807-3819.

Fowler, V. G., Sakoulas, G., McIntyre, L. M. & other authors (2004). Persistent bacteremia due to methicillin-resistant *Staphylococcus aureus* infection is associated with *agr* dysfunction and low-level in vitro resistance to thrombin-induced platelet microbicidial protein. *Journal of infect Dis* **190**, 1140-1149.

Francois, P., Renzi, G., Pittet, D. & Others (2004). A novel multiplex real-time PCR assays for rapid typing of major staphylococcal cassette chromosome *mec* elements. *Journal of Clinical Microbiology* **42**, 3309-3312.

Frenay, H. M. E., Theelen, P. G., Schouls, M., Vandenbroucke-Grauls, M. J. E., Verhoef, J., van Leeuwen, W. J. & Mooi, F. R. (1994). Discrimination of Epidemic

and Nonepidemic methicillin-resistant *Staphylococcus aureus* strains on the basis of Protein A gene polymorphism. *Journal of Clinical Microbiology* **32**, 846-847.

Friedman, N. D., Kaye, K. S., Stout, J. E. & other authors (2002). Health Care-Associated Bloodstream Infections in Adults: A reason to change the accepted definition of community-acquired infections. *Ann Intern Med* **137**, 791-797.

Genovese, A., Bouvet, J. P., Florio, G., Lamparter-Schummert, B., Bjorck, L. & Marone, G. (2000). Bacterial immunoglobulin superantigen proteins A and L activate human heart mast cells by interacting with imunoglogulin E. *infection and Immunity* **68**, 5517-5524.

Gillet, Y., Issartel, B., Vanhems, P. & other authors (2002). Association between *Staphylococcus aureus* strains carrying gene for Panton-Valentine leukocidin and highly lethal necrotising pneumonia in young immunocompetent patients. *Lancet* **359**, 753-759.

Goering, R. V., Shawar, R. M., Scangarella, N. E. & other authors (2008). Molecular epidemiology of methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* isolates from global clinical trials. *American Society for Microbiology* **46**, 2842-2847.

Gordan, R. J., Quagliarello, B., Cespedes, C., Chung, M., de Lencastre, H., Vavagiakis, P., Miller, M., Zeller, B. & Lowy, F. D. (2005). A molecular epidemiological analysis of two *Staphylococcus aureus* clonal types colonizing and infecting patients with AIDS. *Clinical Infectious Diseases* **40**, 1028-1036.

Gottlieb, G. S., Fowler, V. G., Kong, L. K. & other authors (2000). *Staphylococcus aureus* bacteremia in the surgical patient: A prospective analysis of 73 postoperative patients who developed *Staphylococcus aureus* bacteremia at a tertiary care facility. *American College of Surgeons* **190**, 50-57.

Graille, M., Stura, E. A., Corper, A. L., Sutton, B. J., Taussig, M. J., Charbonnier, J. & Silverman, G. (2000). Crystal structure of a *Staphylococcus aureus* protein A domain complexed with the Fab fragment of a human IgM antibody: Structural basis for recognition of B-cell receptors and superantigen activity. *PNAS:National Academy of Science of the USA* **97**, 5399-5404.

Grundmann, H., Aires-de-Sousa, M., Boyce, J. & Tiemersma, E. (2006). Emergence and resurgence of meticillin-resistant *Staphylococcus aureus* as a public-health threat. *Lancet* **368**, 874-885.

Guss, B., Leander, K., Hellman, U., Ulhlén, U., Sjöquist, J. & Lindberg, M. (1985). Analysis of protein A encoded by a mutated gene of *Staphylococcus aureus* Cowan I. *Eur J Biochem* **153**, 579-585.

Guzman, C. A., Guardati, M. C., Fenoglio, D., Coratza, G., Pruzzo, C. & Satta, G. (1992). Novel immunoenzymatic assay for identification of coagulase and protein A-negative *Staphylococcus aureus* strains. *Journal of Clinical Microbiology* **30**, 1194-1197.

Hallin, M., Deplano, A., Denis, O., De Mendonca, R., De Ryck, R. & Struelens, M. J. (2007). Validation of Pulsed-Field Gel Electrophoresis and *spa* Typing for Long-term, Nationwide Epidemiological Surveillance Studies of *Staphylococcus aureus* Infections. *Journal of Clinical Microbiology* **45**, 127-133.

Harbarth, S., Rutschmann, O., Sudre, P. & Others (1998). Impact of methicillin resistance on the outcome of patients with bacteremia caused by *Staphylococcus aureus*. *Archives of Internal Medicine* **158**, 182-189.

Harmsen, D., Claus, H., Witte, W., Rothganger, J., Claus, H., Turnwald, D. & Vogel, U. (2003). Typing of Methicillin-Resistant *Staphylococcus aureus* in a University Hospital Setting by Using Novel Software for *spa* Repeat Determination and Database Management. *Journal of Clinical Microbiology* **41**, 5442-5448.

Hartlieb, J., Köhler, N., Dickson, R. B. & other authors (2000). Protein A is the von Willebrand factor binding protein on *Staphylococcus aureus*. *American Society of Hematology* **96**, 2149-2156.

Hartstein, A. I., Mulligan, M. E., Morthland, V. H. & Kwok, R. Y. Y. (1992). Recurrent *Staphylococcus aureus* bacteremia. *Journal of Clinical Microbiology* **30**, 670-674.

Hawkins, C., Huang, J., Jin, N., Noskin, G. A., Zembower, T. R. & Bolon, M. (2007). Persistent *Staphylococcus aureus* bacteremia. *Archives of Internal Medicine* **167**, 1861-1867.

Heubner, J. & Goldmann, D. A. (1999). Coagulase-negative staphylococci: Role as Pathogens. *Annu Rev Med* **50**, 223-236.

Hiramatsu, K. (1995). Molecular evolution of MRSA. *Microbiol Immunol* **39**, 531-543.

Hiramatsu, K., Cui, L., Kuroda, M. & Ito, T. (2001). The emergence and evolution of methicillin-resistant *Staphylococcus aureus*. *Trends in Microbiology* **9**, 486-493.

Holmes, A., Ganner, M., McGuane, S., Pitt, T. L., Cookson, B. D. & Kearns, A. M. (2005). *Staphylococcus aureus* Isolates carrying Panton-Valentine Leukocidin genes in England and Wales: Frequency, Characterization, and association with Clinical Disease. *Journal of Clinical Microbiology* **43**, 2384-2390.

Hsu, L. Y., Koh, T. H., Kurup, A., Low, J., Chlebicki, M. P. & Tan, B. H. (2005). High incidence of Panton-Valentine leukocidin-producing *Staphylococcus aureus* in a tertiary care public hospital in Singapore. *Clinical Infectious Diseases* **40**, 486-489.

Ito, T., Katayama, Y. & Hiramatsu, K. (1999). Cloning and neuclotide sequence determination of the entire mec DNA of pre-methicillin-resistant *Staphylococcus aureus* N315. *Antimicrobial agents and Chemotherapy* **43**, 1449-1458.

Jang, H.-C., Kim, S.-H., Kim, K. H. & other authors (2009). Salvage treatment for persistent methicillin-resistant *Staphylococcus aureus* bacteremia: Efficacy of linezolid with or without carbopenum. *Clinical Infectious Diseases* **49**, 395-401.

Jansen van Rensburg, M., Madikane, E. V., Whitelaw, A. & Elisha, G. (2009). Molecular characterisation of methicillin-resistant *Staphylococcus aureus* in hospitals in Cape Town. In *Abstracts: 3rd Fidssa Congress 2009: Laboratory: anti-infective and pathogen surveillance*, pp. 3: South African Journal of Epidemiology Infection.

Jappe, U., Heuck, D., Strommenger, B., Wendt, C., Altmann, D. & Witte, W. (2008). *Staphylococcus aureus* in dermatology outpatients with special emphasis on community-associated methicillin-resistant strains. *Journal of Investigative Dermatology* **128**, 2655-2664.

Jarraud, S., Mougel, C., Jean, T., Lina, G., Meugnier, H., Forey, F., Nesme, X., Etienne, J. & Vandenesch, F. (2002). Relationships between *Staphylococcus aureus* Genetic Background, Virulence Factors, *agr* Groups (Alleles) and human disease. *Infection and Immunity* **70**, 631-641.

Jarvis, W. R., Edwards, J. R., Culver, D. H. & other authors (1991). Nonsocomial infection rates in adult and paediatric intensive care units in the United States. In *National Nonsocomial Infections Surveillance System*, pp. 185-191. United States American Journal of Medicine.

Jernigan, J. A., Titus, M. G., Groschel, D. H., Getchell-White, S. & Far, B. M. (1996). Effectiveness of contact isolation during a hospital outbreak of methicillin-resistant *Staphylococcus aureus*. *American Journal of Epidemiology* **1996**, 496-504.

Johnson, A. P., Aucken, H. M., Cavendish, S., Ganner, M., Wale, M. C., Warner, M., Livermore, D. M. & Cookson, B. D. (2001). Dominance of EMRSA-15 and 16 among MRSA causing nonsocomial bacteraemia in the UK: analysis of isolates from the European Antimicrobial Resistance Surveillance System (EARSS). *Journal of Antimicrobial Chemotherapy* **48**, 143-144.

Johnson, L. B., Almoujahed, M. O., Llg, K., Maolood, L. & Khatib, R. (2003). *Staphylococcus aureus* bacteremia: compliance with standard treatment, long-term outcome and predictors of relapse. *Scand J Infect Dis* **35**, 782-789.

Johnsson, D., Molling, P., Stralin, K. & Soderquist, B. (2004). Detection of Panton-Valentine leukocidin gene in *Staphylococcus aureus* by LightCycler PCR: clinical and epidemiological aspects. *Clin Microbiol Infect* **10**, 884-889.

Kahl, B. C., Mellmann, A., Deiwick, S., Peters, G. & Harmsen, D. (2005). Variation of the polymorphic region X of the protein A gene during persistent airway infection of cystic fibrosis patients reflects two independent mechanisms of genetic change. *journal of Clinical Microbiology* **43**, 502-505.

Kaneko, J., Kimura, T., Kawakami, Y., Tomita, T. & Kamio, Y. (1997). Panton-valentine leukocidin genes in a phage-like particle isolated from mitomycin C-treated

Staphylococcus aureus V8 (ATCC 49775). *Biosci Biotechnol Biochem* **61**, 1960-1962.

Kaneko, J., Kimura, T., Narita, S., Tomita, T. & Kamio, Y. (1998). Complete neuclotide sequence and a molecular characterization of the temperature staphylococcal bacteriophage phiPVL carrying Panton-Valentine leukocidin genes. *Gene* **215**, 57-67.

Kaneko, J. & Kamio, Y. (2004). Bacterial two-component and hetro-heptameric pore-forming cytolytic toxins: structures, pore-forming mechanism, and organization of the genes. *Biosci Biotechnol Biochem* **68**, 981-1003.

Kaplan, S. L., Hulten, K. G., Gonzalez, B. E., Hammerman, W. A., Lamberth, L., Versalovic, J. & Mason Jr, E. O. (2005). Three-year surveillance of community-acquired *Staphylococcus aureus* infections in children. *Clin Infect Dis* **40**, 1785-1791.

Karynski, M., Sabat, A., Empel, J. & Hryniewicz (2008). Molecular surveillance of methicillin-resistant *Staphylococcus aureus* by multiple-locus variable number tandem repeat fingerprinting (formerly multiple-locus variable number tandem repeat analysis) and *spa* typing in a hierarchic approach. *Diagn Microbiol Infect Dis* **62**, 255-262.

Katayama, Y., Ito, T. & Hiramatsu, K. (2001). Genetic Organisation of the Chromosome Region Surrounding *mecA* in Clinical Staphylococcal strains: Role of IS*431*-Mediated *mecI* Deletion in Expression of Resistance in *MecA*-Carrying, Low-Level Methicillin-Resistant Staphylococcus haemolyticus. *Antimicrobial agents and Chemotherapy* **45**, 1955-1963.

Kearns, A. M., Ganner, M. & Holmes, A. (2006). The 'Oxford *Staphylococcus*': a note of caution. *Journal of Antimicrobial Chemotherapy* **58**, 480-481.

Khandavilli, S., Wilson, P., Cookson, B. D., Cepeda, J., Bellingan, G. & Brown, J. (2009). Utility so *spa* typing for investigating the local epidemiology of MRSA on a UK intensive care ward. *Journal of Hospital Infections* **71**, 29-35.

Khatib, R., Johnson, L. B., Fakih, M. G., Riederer, K., Khosrovaneh, A., Shamse Tabriz, M., Sharma, M. & Saeed, S. (2006). Persistance in Staphylococcus aureus bacteremia: incidence, characteristics of patients and outcome. *Scand J Infect Dis* **38**, 7-14.

Khatib, R., Johnson, L. B., Sharma, M., Fakih, M. G., Ganga, R. & Riederer, K. (2009). Persistent *Staphylococcus aureus* bacteremia: Incidence and outcome trends over time. *Scand J Infect Dis* **41**, 4-9.

Kielian, T., Cheung, A. & Hickey, W. F. (2001). Diminished Virulence of an Alpha-Toxin Mutant of *Staphylococcus aureus* in Experimental Brain Abscesses. *Infection and Immunity* **69**, 6902-6911.

Kim, J. (2009). Understanding the evolution of methicillin-resistant *Staphylococcus aureus*. *Clinical Microbiology Newsletter* **31**, 17-23.

Koreen, L., Ramaswamy, S. V., Graviss, E. A., Naidich, S., Musser, J. M. & Kreiswirth, B. N. (2004). *Spa* typing method for discriminating among *Staphylococcus aureus* isolates: implications for use of a single marker to detect genetic micro and macrovariation. *Journal of Clinical Microbiology* **42**, 792-799.

Koreen, L., Ramaswamy, S. V., Naidich, S., Koreen, I. V., Graff, G. R., Graviss, E. A. & Kreiswirth, B. N. (2005). Comparative sequencing of the scrincasparate repeat-encoding region of the clumping factor B gene (*clfB*) for resolution within clonal groups of *Staphylococcus aureus*. *Journal of Clinical Microbiology* **43**, 3985-3994.

Korem, M., Sheoran, A. S., Gov, Y., Tzipori, S., Borovok, I. & Balaban, N. (2003). Characterization of RAP, a quorum sensing activator of *Staphylococcus aureus*. *FEMS Microbiology Letters* **223**, 167-175.

Kronvall, G., Dossett, J. H., G, Q. P. & Williams, R. C. J. (1971). Occurrence of protein A in staphylococcal strains: quantitavie aspects and correlation to antigens and bacteriophage types. *Infection and Immunity* **3**, 10-15.

Krut, O., Ultermöhlem, O., Schlossher, X. & Krönke, M. (2003). Strain-Specific Association of Cytotoxic Activity and Virulence of Clinical *Stphylococcus aureus* Isolates. *Infection and Immunity* **71**, 2716-2723.

Kuzma, K., Malinowski, E., Lassa, H. & Klossowska, A. (2005). Analysis of protein A gene polymorphism in *Staphylococcus aureus* isolates from bovine mastitis. *Bull Vet Int Pulawy* **49**, 41-44.

Labandeira-Rey, M., Couzon, F., Boisset, S. & other authors (2007). *Staphylococcus aureus* Panton-Valentine Leukocidin causes necrotizing pneumonia. *Science* **315**.

Larsen, A. R., Bocher, S., Stegger, M., Goering, R. V., Pallesen, L. V. & Skov, R. (2008). Epidmiology of European community-associated methicillin-resistant *Staphylococcus aureus* clonal complex 80 type IV strains isolated in Denmark from 1993 to 2004. *Journal of Clinical Microbiology* **46**, 62-68.

Liao, C. H., Chen, S. Y., Huang, Y. T. & Hsueh, P. R. (2008). Outcome of patients with methicillin-resistant *Staphylococcus aureus* bacteraemia at an emergency department of a medical centre in Taiwan. *International Journal of Antimicrobial Agents* **32**, 326-332.

Lina, G., Piemont, Y., Godail-Gamot, F., Bes, M., Peter, M.-O., Gauduchon, V., Vandenesch, F. & Etienne, J. (1999). Involvement of Panton-Valentine Leukocidin-Producing *Staphylococcus aureus* in Primary Skin infections and pneumonia. *Clinical Infectious Diseases* **29**, 1128-1132.

Lindmark, R., Movitz, J. & Sjoquist, J. (1977). Extracellular protein A from a methicillin resistant *Staphylococcus aureus*. *Eur J Biochem* **74**, 623-628.

Liu, Y., Wang, H., Du, N., Shen, E., Chen, H., Niu, J., Ye, H. & Chen, M. (2009). Molecular evidence for spread of two major methicillin-resistant *staphylococcus aureus* clones with a unique geographic distribution in Chinese hospitals. *Antimicrobial agents and Chemotherapy* **53**, 512-518.

Löfdahl, S., Guss, B., Uhlén, M., Philipson, L. & Lindberg, M. (1982). Gene for staphylococcal protein A. *Proc Natl Acad Sci USA* **80**, 697-701.

Lowy, F. D. (1998). *Staphylococcus aureus* Infections. *The New England Journal of Medicine* **339**, 520-532.

Lowy, F. D. (2000). Is *Staphylococcus aureus* an intracellular pathogen? *Trends in Microbiology* **8**, 341-343.

Lu, P. L., Chin, L. C., Peng, C. F., Chiang, Y. H., Chen, T. P., Ma, L. & Siu, L. K. (2005). Risk factors and molecular analysis of community methicillin-resistant *Staphylococcus aureus* carriage. *Journal of Clinical Microbiology* **43**, 132-139.

Maiden, M. C., Bygraves, J. A., Feil, E. J. & other authors (1998). Multilocus sequence typing: a portable approach to the infection of clones within populations of pathogenic microorganisms. *Proc Natl Acad Sci USA* **95**, 3140-3145.

Malachowa, N., Sabat, A., Gniadkowski, M., Krzyszton-Russjan, J., Empel, J., Miedzobrodzki, J., Kosowska-Shick, K., Appelbaum, P. C. & Hyniewicz, W. (2005). Comparison of multiple-locus variable-number tandem-repeat analysis with pulsed-field gel electrophoresis, *spa* typing, an multilocus typing for clonal characterization of *Staphylococcus aureus* isolates. *Journal of Clinical Microbiology* **43**, 3095-3100.

Marais, E., Aithma, N., Perovic, O., Oosthuysen, W. F., Musenge, E. & Duse, A. G. (2009). Antimicrobial susceptibility of methicillin-resistant *Staphylococcus aureus* isolates from South Africa. *South African Medical Journal* **99**, 170-173.

Maree, C., Daum, R. S., Boyle-Vavra, S. & Others (2006). Rapid temporal increase in community-acquired methicillin-resistant *Staphylococcus aureus* strains causing nonsocomial infections. In *Interscsience Conference Antimcrobial Agents and Chemotherapy*. Washington, DC: Antimcrobial Agents and Chemotherapy.

Marone, G., Tamburini, M., Giudizi, M. G., Biagiotti, R., Almerigogna, F. & Romagnani, S. (1987). Mechanism of activation of human basophils by *Staphylococcus aureus* Cowan 1. *Infection and Immunity* **55**, 803-809.

Maxim, P. E., Matheus, H. L. & Mengoli, H. F. (1976). Single-tube mixed agglutination tests for the detection of stapylococcal protein. *A J Clin Microbiol* **4**, 418-422.

Maya, I. D., Carlton, D., Estrada, E. & Allon, M. (2007). Treatment of dialysis catheter-related *Staphylococcus aureus* bacteremia with an antibiotic lock: A quality improvement report. *American Journal of Kidney Diseases* **50**, 289-295.

McClure, J.-A., Conly, J., Lau, V., Elsayed, S., Louie, T., Hutchins, W. & Zhang, K. (2006). Novel Multiplex PCR Assay for Detection of the Staphylococcal Virulence Marker Panton-Valentine Leukocidin Genes and Simultaneous Discrimination of Methicillin-Susceptible from -Resistant Staphylococci. *Journal of Clinical Microbiology* **44**, 1141-1144.

McDonald, R. R., Antonishyn, N. A., Hansen, T., Snook, L. A., Nagle, E., Mulvey, M. R., Levett, P. N. & Hornsman, G. B. (2005). Development of a Triplex Real-Time PCR Assay for detection of Panton-Valentine Leukocidin Toxin Genes in Clinical isolates of Methicillin-Resistant *Staphylococcus aureus*. *Journal of Clinical Microbiology* **43**, 6147-6149.

McKinney, T. K., Sharma, V. K., Craig, W. A. & Archer, G. L. (2001). Transportation of the Gene Mediating Methicillin Resistance in *Staphylococcus aureus* (*mecA*) Is Coreporessed but Not Coinduced by Cognate *mecA* and β-Lactamase Regulators. *Journal of Bacteriology* **183**, 6862-6868.

Melles, D. C., Gorkink, R. F., Boelens, H. A., Snijders, S. V., Peerters, J. K., Moorhouse, M. J. & Others (2004). Natural population dynamics and expansion of pathogenic clones of *Staphylococcus aureus*. *J Clin Invest* **114**, 1732-1740.

Melles, D. C., Leeuwen, W. V., Boelens, H. A., Peeters, J. K., Verbrugh, H. A. & Belkum, A. V. (2006). Panton-Valentine leukocidin genes in *Staphylococcus aureus*. *Emerging Infectious Diseases* **12**, 1174-1175.

Mellmann, A., Weniger, T., Berssenbrugge, C., Keckevoet, U., Friedrich, A. W., Harmsen, D. & Grundmann, H. (2008). Characterization of clonal relatedness among the natural population of *Staphylococcus aureus* strains by using *spa* sequence typing and the BURP (Based upon repeat patterns) algorithm. *Journal of Clinical Microbiology* **46**, 2805-2808.

Milheirico, C., Oliveira, D. C. & Lencastre, H. (2007). Update to the Multiplex PCR Strategy for assignment of *mec* Element types in *Staphylococcus aureus*. *Antimicrobial agents and Chemotherapy* **51**, 3374-3377.

Miller, L. G., Perdreau-Remmington, F., Rieg, G., Mehdi, S., Perlroth, J., Bayer, A. S., Tang, A. W., Phung, T. O. & Spellberg, B. (2005). Necrotizing fasciits caused by community-associate methicillin-resistant *Staphylococcus aureus* in Los Angeles. *N Engl J* **352**, 1445-1453.

Miller, L. G., Perdreau-Remmington, F., Bayer, A. S., Diep, B., Tan, N., Bharadwa, K. & Others (2007). Clinical and epidemiologic characteristics cannot distinguish community-associated methicillin-resistant *Staphylococcus aureus* from methicillin-susceptible *Staphylococcus aureus* infection: a prospective investigation. *Clin Infect Dis* **44**, 471-482.

Mims, C., Dockrell, H. M., Goering, R. V., Roitt, I., Wakelin, D. & Zuckerman, M. (2004). *Medical Microbiology 3rd Edition*: Elsevier Mosby.

Mitani, N., Ohnishi, M., Masutani, T., Murakawa, K. & Okamoto, Y. (2002). Molecular typing of methicillin-resistant *Staphylococcus aureus* by protein A gene sequencing. *Jpn J Infect Dis* **55**, 179-180.

Mokrzycki, M. H., Zhang, M., Cohen, H., Golestaneh, L., Laut, J. M. & Rosenberg, S. O. (2006). Tunnelled haemodialysis catheter bacteraemia: risk factors for bacteraemia recurrence, infectious complications and mortality. *Nephrol Dial Transplant* **21**, 1024-1031.

Mongkolrattanothai, K., Boyle, S., Kahana, M. D. & Daum, R. S. (2003). Severe *Staphylococcus aureus* infections caused by clonally related community-acquired methicillin-susceptible and methicillin-resistant isolates. *Clin Infect Dis* **37**, 1050-1058.

Moodley, A., Stegger, M., Bagcigil, A. F. & other authors (2006). *spa* typing of methicillin-resistant *Staphylococcus aureus* isolated from domestic animals and veterinary staff in the UK and Ireland. *Journal of Antimicrobial Chemotherapy* **58**, 1118-1123.

Muller-Premru, M., Strommenger, B., Alikadic, N. & Others (2005). New strains of community-acquired methicillin-resistant *Staphylococcus aureus* with Panton-Valentine leukocidin causing an outbreak of severe soft tissue infection in a football team. *Eur J Clin Microbiol Infect Dis* **24**, 848-850.

Naas, T., Fortineau, N., Spicq, C., Robert, J., Jarlier, V. & Nordmann, P. (2005). Three-year survey of community-acquired methicillin-resistant *Staphylococcus aureus* producing Panton-Valentinc leukocidin in a French university hospital. *Journal of Hospital Infections* **61**, 321-329.

Naimi, T. S., LeDell, K. H., Como-Sabetti, K., Borchardt, S. M., Boxrud, D. J., Etienne, J., Johnson, S. K., Vandenesch, F. & Fridkin, S. (2003). Comparison of community and health care associated methicillin-resistant *Staphylococcus aureus* infection. *JAMA* **290**, 2976-2984.

Narita, S., Kaneko, J., Chiba, J., Piemont, Y., Jarraud, S., Etienne, J. & KAmio, Y. (2001). Phage conversion of Panton-Valentine leukocidin in *Staphylococcus aureus*: molecular analysis of PVL-converting phage, phiSLT. *Gene* **268**, 195-206.

Nashev, D., Toshkova, K., Salasia, S. I. O., Hassan, A. A., Lammler, C. & Zschock, M. (2004). Distribution of virulence genes of *Staphylococcus aureus* isolates from stable nasal carriers. *FEMS Microbiology Letters 233*, 45-52.

Nübel, U., Roumagnac, P., Feldkamp, M. & other authors (2008). Frequent emergence and limited geographic dispersal of methicillin-resistant *Staphylococcus aureus*. *PNAS:National Academy of Science of the USA* **105**, 14130-14135.

Nunes, A. P. F., Teixeira, L., Bastos, C. C. R. & Others (2002). Susceptibility of Brazilian Staphylococcal Strains to Glycopeptides Evaluated by Different Testing Methods. *Current Microbiology* **44**, 385 – 390.

Nunes, A. P. F., Teixeira, L. M., Iorio, N. L. P. & Others (2006). Heterogeneous resistance to vancomycin in *Staphylococcus epidermidis*, *Staphylococcus haemolyticus* and *Staphylococcus warneri* clinical strains: characterisation of glycopeptide susceptibility profiles and cell wall thickening. *International Journal of Antimicrobial Agents* **27**, 307-315.

Okuma, K., Iwakawa, K., Turnidge, J. D. & Others (2002). Dissemination of new methicillin-resistant *Staphylococcus aureus* clones in the community. *Journal of Clinical Microbiology* **40**, 4289-4294.

Oliveira, D. C., Tomasz, A. & Lencastre, H. (2002). Secrets of success of a human pathogen: molecular evolution of pandemic clones of methicillin-resistant *Staphylococcus aureus*. *Infectious Diseases* **2**, 180-189.

Oosthuizen, D., Walsh, T. R. & Weldhagen, G. F. (2005). Detection of Staphylococcus aureus with reduced susceptibility to vancomycin in a South African teaching hospital. *The Southern African Journal of Epidemiology and Infection* **20**, 127-129.

Oosthuysen, W. F., Duse, A. G. & Marais, E. (2007). Molecular characterisation of methicillin-resistant *Staphylococcus aureus* in South Africa. In *17th European Congress of Clinical Microbiology and Infectious Diseases*. ICC, Munich, Germany.

Orrett, F. A. & Land, M. (2006). Methicillin-resistant *Staphylococcus aureus* prevalence: Current susceptibility patterns in Trinidad. *BMC Infectious Diseases* **6:83**.

Otter, J. A. & French, G. L. (2008). The ermergence of community-associated methicillin-resistant *Staphylococcus aureus* at a London teaching hospital, 2000-2006. *Clin Microbiol Infect* **14**, 670-676.

Palmqvist, N., Foster, T. J., Tarkowski, A. & Josefsson, E. (2002). Protein A is a virulence factor in *Staphylococcus aureus* arthritis and septic death. *Micobial Pathogenesis* **33**, 239-249.

Palvecino, E. (2007). Clinical, epidemiological, and laboratory aspects of methicillin-resistant *Staphylococcus aureus* (MRSA) infections. *Methods in Molecular Biology* **391**, 1-19.

Parker, M. T., Asheshow, E. H., Hewitt, J. H., Nakhla, L. S. & Brock, B. M. (1974). Endemic staphylococcal infections in hospitals. *Ann N Y Acad Sci* **236**, 466-484.

Pathirage, H. (2008). Panton Valentine Leucocidin (PVL) positive staphylococcal infection: an emerging infection across the world *Sri Lanka Journal of Child Health* **37**, 109-111.

Peacock, J. E., Marsik, F. J. & Wenzel, R. P. (1980). Methicillin-resistant *Staphylococcus aureus*: introduction and spread within a hospital. *Ann Intern Med* **93**, 526-532.

Peddie, E. F., Donald, P. R., Burger, P. J. & Sadler, C. A. (1988). Methicillin-resistant *Staphylococcus aureus. South African Medical Journal* **74**, 223-224.

Pérez-Vázquez, Vindel, A., Marcos, C., Oteo, J., Cuevas, O., Trincado, P., Bautista, V., Grundmann, H. & Campos, J. (2009). Spread of invasive *Staphylococcus aureus spa*-type t067 associated with a high prevalence of the aminoglycoside-modifying enzyme gene *ant(4')-Ia* and the efflux pump genes *msrA/msrB. Journal of Antimicrobial Chemotherapy* **63**, 21-31.

Que, Y.-A., Haefliger, J.-A., Piroth, L. & other authors (2005). Fibrinogen and fibronectin binding cooperate for valve infection and invasion in *Staphylococcus aurues* experimental endocarditis. *Journal of Experimental Medicine* **201**, 1627-1635.

Randrianirina, F., Soares, J.-L., Ratsima, E., Carod, J.-F., Combe, P., Grosjean, P., Richard, V. & Talarmin, A. (2007). In vitro activities of 18 antimicrobial agents against *Staphylococcus aureus* isolates from the Institut Pasteur of Madagascar. *Annals of Clinical Microbiology and Antimicrobials* **6:5**.

Regev-Yochay, G., Rubinstein, E., Barzilai, A. & other authors (2005). Methicillin-resistant *Staphylococcus aureus* in neonatal intensive care unit. *Emerging Infectious Diseases* **11**, 453-456.

Robinson, D. A. & Enright, M. C. (2003). Evolutionary Models of the Emergence of Methicillin-Resistant *Staphylococcus aureus. Antimicrobial agents and Chemotherapy* **47**, 3926-3934.

Robinson, D. A. & Enright, M. (2004). Evolution of *Staphylococcus aureus* by large chromosomal replacements. *J Bacteriol* **186**, 1060-1064.

Robinson, D. A., Kearns, A. M., Holmes, A. & other authors (2005). Re-emergence of early pandemic *Staphylococcus aureus* as a community-acquired meticillin-resistant clone. *Lancet* **365**, 1256-1258.

Robinson, J. O., Pearson, J. C., Christiansen, K. J., Coombs, G. W. & Murray, R. J. (2009). Community-associated versus healthcare-associated methicillin-resistant *Staphylococcus aureus* bacteraemia: a 10-year retrospective review. *Eur J Clin Microbiol Infect Dis* **28**, 353-361.

Ruppitsch, W., Indra, A., Stoger, A. & Others (2006). Classifying *spa* types in complexes improves interpretation of typing results for methicillin-resistant *Staphylococcus aureus. Journal of Clinical Microbiology* **44**, 2442-2448.

Ryan, K. J. & George Ray, C. (2004). *Sherris Medical Microbiology: An Introduction to Infectious Diseases*, Fourth Edition edn: Elsevier Science.

Salaam, Z. (2007). Detection of Staphylococci with reduced susceptibility to glycopeptides at Tygerberg Hospital. In *Unpublished Honours Thesis*. Cape Town: University of Stellenbosch.

Seybold, U., Kourbatova, E. V., Johnson, J. G. & Others (2006). Emergence of community-assiciated methicillin-resistant *Staphylococcus aureus* USA300 genotype as a major cause of healthcare-associated blood stream infections. *Clin Infect Dis* **42**, 647-656.

Shittu, A. O. & Lin, J. (2006). Antimicrobial susceptibility patterns and characterization of clinical isolates of *Staphylococcus aureus* in Kwazulu-Natal province, South Africa. *BMC Infectious Diseases* **6:125**.

Shittu, A. O., Nubel, U., Udo, E., Lin, J. & Gaogakwe, S. (2009). Characterization of methicillin-resistant *Stapylococcus aureus* isolates from hospitals in KwaZulu-Natal province, Republic of South Africa. *Journal of Medical Microbiology* **58**, 1219-1226.

Shopsin, B., Gomez, M., Montgomery, O. & other authors (1999). Evaluation of Protein A gene polymorphic region DNA sequencing for typing of *Staphylococcus aureus* strains. *Journal of Clinical Microbiology* **37**, 3556-3563.

Shopsin, B. (2001). Molecular epidemiology of methicillin-resistant *Staphylococcus aureus*. *Emerging Infectious Diseases* **7**.

Shukla, S. K., Stemper, M. E., Ramaswamy, S. V., Conradt, J. M., Reish, R., Graviss, E. A. & Reed, K. D. (2004). Molecular characteristics of nonsocomial and Native American community-associated methicillin-resistant *Staphylococcus aureus* clones from rural Wisconsin. *Journal of Clinical Microbiology* **42**, 3752-3757.

Sinha, B. & Herrmann, M. (2005). Mechanism and consequences of invasion of endothelial cells by *Staphylococcus aureus*. *Thromb Haemost* **94**, 266-277.

Skinner, D. & Keefer, C. S. (1941). Significance of bacteremia caused by *Staphylococcus aureus*. *Archives of Internal Medicine* **68**.

Sola, C., Saka, H. A., Vindel, A. & Others (2007). High frequency of Panton-Valentine leukocidin genes in invasive methicillin-susceptible *Staphylococcus aureus* strains and the relationship with methicillin-resistant *Stapylococcus aureus* in Cordoba, Argentina. *Eur J Clin Microbiol Infect Dis* **26**, 281-286.

Srinivasan, A., Dick, J. D. & Perl, T. M. (2002). Vancomycin Resistance in Staphylococci. *Clinical Microbiology Reviews* **15**, 430-438.

Steinberg, J. P., Clark, C. & Hackman, B. O. (1996). Nonsocomial and community-acquired *Staphylococcus aureus* bacteremia from 1980 to 1993: impact of intravascular devices and methicillin resistance. *Clin Infect Dis* **23**, 255-259.

Strommenger, B., Kettlitz, C., Weniger, T., Harmsen, D., Friedrich, A. W. & Witte, W. (2006). Assignment of *Staphylococcus aureus* isolates to groups by *spa* typing, smaI macrorestriction analysis, and multilocus sequence typing. *Journal of Clinical Microbiology* **44**, 2533-2540.

Strommenger, B., Braulke, C., Heuck, D., Schmidt, C., Pasemann, B., Nubel, U. & Witte, W. (2008a). *spa* typing of *Staphylococcus aureus* as a frontline tool in epidemiological typing. *Journal of Clinical Microbiology* **46**, 547-581.

Strommenger, B., Braulke, C., Pasemann, B., Schmidt, C. & Witte, W. (2008b). Multiplex PCR for rapid detection of *Staphylococcus aureus* isolates suspected to represent community-acquired strains. *Journal of Clinical Microbiology* **46**, 582-587.

Teixeira, L. A., Resende, C. A., Ormonde, L. R., Rosenbaum, R., Figueiredo, A. M., de Lencastre, H. & Tomasz, A. (1995). Geographic spread of epidemic multiresistant *Staphylococcus aureus* clone in Brazil. *Journal of Clinical Microbiology* **33**, 2400-2404.

Tenover, F. C., Arbeit, R. D. & Goering, R. V. (1997). How to select and interpret molecular strain typing methods for epidemiological studies of bacterial infections: A review of healthcare epidemiologists. *infect Control and Hospital Epidemiology* **18**, 426-438.

Tenover, F. C., M.V, L. & B.C, H. (2008). Characterization of Staphylococci with Reduced Susceptibilities to Vancomycin and Other Glycopeptides. *Journal of Clinical Microbiology* **36**, 1020-1027.

Tiemersma, E., Bronzwaer, S. L., Lyytikainen, O. & Others (2004). Methicillin-resistant *Staphylococcus aureus* in Europe, 1999-2002. *Emerging Infectious Diseases* **10**, 1627-1634.

Tinelli, M., Manaco, M., Vimercati, M., Ceraminiello, A. & Pantosti, A. (2009). Methicillin-Susceptible *Staphylococcus aureus* in skin and soft tissue infections, Northern Italy. *Emerging Infectious Diseases* **15**, 250-257.

Tiwari, H. K. & Sen, M. R. (2006). Emergence of vancomycin resistant Staphylococcus aureus (VRSA) from a tertiary care hospital from a northern part of India. *BMC Infectious Diseases* **6**, 156.

Todar, K. (2008). Todar's Online Textbook of Bacteriology: *Staphylococcus* and Staphylococcal Disease, pp. 1-6 http://www.textbookofbacteriology.net/staph.html [Accessed 06 March 2009].

Tolan, R. W., Baorto, E. P. & Baorto, D. (2009).*Staphylococcus aureus* Infection, pp. http://emedicine.medscape.com/article/971358-overview

Trindade, P. A., McCulloch, J. A., Oliveira, G. A. & Mamizuka, E. K. (2003). Molecular techniques for MRSA typing: Current issues and perspectives. *the Brazilian Journal of Infectious Diseases* **7**, 32-43.

Tristan, A., Bes, M., Meugnier, H. & other authors (2007). Global distribution of Panton-Valentine leukocidin-positive methicillin-resistant *Staphylococcus aureus*, 2006. *Emerging Infectious Diseases* **13**, 594-600.

Tristan, A., Benito, Y., Montserret, R. & other authors (2009). The signal peptide of *Staphylococcus aureus* panton valentine leukocidin LukS component mediates increased adhesion to heparan sulfates. *PLoS ONE* **4**.

van Belkumm, A., Tassios, P. T., Dijkshoorn, L. & other authors (2007). Guidelines for the validation and application of typing methods for use in bacterial epidemiology. *clinical microbiology and Infectious Diseases* **13**, 1-46.

Vandenesch, F., Naimi, T. S., Enright, M., Lina, G., Nimmo, G. R., Heffernan, H. & Others (2003). Community-acquired methicillin-resistant *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes: worldwide emergence. *Emerging Infectious Diseases* **9**, 978-984.

von Eiff, C., Friedrich, A. W., Peters, G. & Becker, K. (2004). Prevalence of genes encoding for members of the staphylococal leukotoxin family among clinical isolates of *Staphylococcus aureus*. *Diagn Microbiol Infect Dis* **49**, 157-162.

Walker, T. M., Bowler, I. C. J. W. & Bejon, P. (2009). Risk factors for recurrence after *Staphylococcus aureus* bacteraemia. A retrospective matched case-control study. *Journal of infection* **58**, 411-416.

Walsh, T. R., Bolmström, A., Qwärnström, A. & Others (2001). Evaluation of Current Methods for Detection of Staphylococci with Reduced Susceptibility to Glycopeptides. *Journal of Clinical Microbiology* **39**, 2439-2444.

Walsh, T. R. & Howe, R. A. (2002). The Prevalence and Mechanism of Vancomycin Resistance in *Staphylococcus aureus*. *Annu Rev Microbiol* **56**, 657-675.

Wann, E. R., Fehringer, A. P., Ezepchuk, Y. V. & Others (1999). *Staphylococcus aureus* isolates from patients with Kawasaki disease express high levels of protein A. *Infect Immun* **67**, 4737-4743.

Ward, T. T., Winn, R. E. & Hartstein, A. I. (1981). Observations relating to an inter-hospital outbreak of methicillin-resistant *Staphylococcus aureus*: role of antimicrobial therapy in infection control. *Infect Control* **2**, 453-459.

Winbald, S. & Ericson, C. (1973). Sensitized sheep red cells as reactants for *S. aureus* protein A. *Acta Pathol Microbiol Scand Sect B* **81**, 150-156.

Witte, W., Cunny, C., Strommenger, B., Braulke, C. & Heuck, D. (2004). Emergence of a new community-acquired MRSA strain found in Germany. *Euro Surveill* **9**, 16-18.

Yamasaki, O., Kaneko, J., Morizane, S., Akiyama, H., Arata, J., Narita, S., Chiba, J.-i., Kamio, Y. & Iwatsuki, K. (2005). The Association between Staphylococcus aureus Strains Carrying Panton-Valentine Leukocidin Genes and the development of Deep-Seated Follicular Infection. *Clin Infect Dis* **40**, 381-385.

Yardena, S., Prachia, R., Ruth, O., David, S. & Michael, G. (2005). The role of vancomycin in the persistence or recurrence of *Staphylococcus aureus* bacteraemia. *Scand J Infect Dis* **37**, 572-578.

Zhang, K., McClure, J.-A., Elsayed, S., Louie, T. & Conly, J. (2005). Novel Multiplex PCR Assay for characterization and concomitant subtyping of staphylococcal cassette chromosome *mec* types I to V in Methicillin-Resistant *Staphylococcus aureus*. *Journal of Clinical Microbiology* **43**, 5026-5033.

Zinn, C. S., Westh, H. & Rosdahl, V. T. (2004). An international multicenter study of antimicrobial resistance and typing of hospital *Staphylococcus aureus* isolates from 21 laboratories in 19 countries or states. *Microb Drug Resis* **10**, 160-168.

Zou, D., Kaneko, J., Narita, S. & Kamio, Y. (2000). Prohage, philPV83-pro, carrying Panton-Valentine leukocidin genes, on the *Staphylococcus aureus* P83 chromosome: comparative analysis of the genome structures of phiPV83-pro, phiPVL, phi11, and other phages. *Biosci Biotechnol Biochem* **64**, 2631-2643.

CHAPTER 8

8 APPENDICES

8.1 APPENDIX A: Microbiological and Clinical Forms

Staphyolcoccus aureus study- Microbiological Data (Date_____)		
Entry point:	All *S.aureus* positive blood cultures(MRSA&MSSA) isolated at the microbiology lab at Tygerberg Hospital during study period	
Exclusion criteria:polimicrobial blood cultures		
Name	_____	
Age	_____	
Date of birth	_____	
Hospital number	_____	
Ward	_____	
First positive blood culture(s)		
Specimen number: B Number	_____	Date: _____
STY Number	_____	
Organism (MRSA/MSSA)	_____	
Source of infection		
Negative blood cultures following treatment		
Document dates	_____	
	_____	
Positive blood culture on optimal therapy(after at least 3days therapy)		(same organism)
Specimen number		
Date	_____	
Vancomycin heteroresistance (MIC - macromethod)		
	_____	
Other positive culture with same organism		
Specimen types and dates	_____	
	_____	
Other Lab results		
WCC	_____	
Neutrophil count (if available)	_____	
CRP	_____	
Lab identification		
VITEK _____	Pasteurex _____	
MSA _____	Slide coagulase _____	
DNAse	Tube coagulase	
Antibiotic sensitivity		
Penicillin ▢	Rifampicin ▢	
Cloxacillin ▢	Vancomycin ▢	
Gentamicin ▢	Fusidic Acid ▢	
Erythromycin ▢	Cotrimoxazole ▢	
Clindamycin ▢	Teicoplanin ▢	
Response to treatment		
Complete symptom resolution (Y/N)	_____	
Time to symptom resolution	_____	
Persistent symptoms after 3 days optimal therapy	_____	
Specify:	_____	
Complications (e.g. metastatic abscess)	_____	
Specify:	_____	
Clinical Diagnosis		
Intravascular catheter sepsis	CSI	▢
Infective endocarditis	IE	▢
Pneumonia	PN	▢
Septic arthritis (including osteomyelitis)	SA	▢
Skin and soft tissue infection	SSTI	▢
Primary bacteraemia	PB	▢
Unknown	Unknown	▢
Other: Specify	_____	

Staphylococcus aureus study - Core clinical datasheet (Date_____)

Entry point:	All *S. aureus* positive blood cultures (MRSA & MSSA) isolated at the microbiology lab at Tygerberg Hospital during study period

Name _____

Gender _____

Date of birth _____

STY number _____

Specimen date _____

Hospital number _____

Date of hospital admission _____

Ward _____

Name of prior hosp./institution _____

Days at referring hosp./institution _____

Previous hospitalization in 12 months(Y/N): _____

If Y, date & diagnosis _____

_____

_____

Previous antibiotic therapy in last 3 months (Y/N): _____

If Y, specify _____

_____

_____

Symptoms on day of blood culture

Time since admission (in days): _____

Fever (Y/N): _____

If Y, specify: Duration in days _____

Temperature at presentation _____

Productive cough (Y/N): _____

If Y, specify: duration in days _____

Painful limb or joint (Y/N): _____

If Y, specify: duration in days & location _____

Skin/soft tissue lesion/inflammation/abscess (Y/N): _____

If Y, specify: location _____

duration in days _____

single/multiple _____

red & warm (Y/N) _____

Other major symptoms _____

Specify: _____

_____

_____

_____

Clinical diagnosis

Septicaemia _____

Infective endocarditis _____

Native valve _____

Prosthetic valve _____

Echo result _____

_____

Pneumonia _____

necrotizing (Y/N) _____

CXR findings _____

cavities (Y/N) _____

alveolar consolidation (Y/N) _____

Osteomyelitis _____

Septic Arthritis _____

Soft tissue infection/abscess _____

location _____

Clinical diagnosis continued

Deep seated abscess _____
location _____
Metastatic abscesses (Y/N) _____
location _____
Intravascular catheter sepsis (CVP, a-line etc.) _____

Other: Specify _____
_____
_____
_____
_____

Underlying disease/risk factors

Diabetic _____
Malignancy _____
Renal failure on hemodialysis/peritoneal dialysis _____
Liver cirrhosis _____
HIV status (Pos/Neg/unknown) _____
Other _____
_____
_____
_____

Treatment

Antibiotics given (specify dates & dosages) _____
_____
_____
_____
_____

Removal of catheter (Y/N) _____
If Y, date removed _____

Surgery _____
_____
_____

Drainage of abscess _____

Response to treatment

Complete symptom resolution (Y/N) _____
Time to symptom resolution _____
_____

Persistent symptoms after 3 days optimal therapy _____
Specify: _____
_____
_____

Complications (e.g. metastatic abscess) _____
Specify: _____
_____
_____

Outcome

Discharged _____

Demised _____

Transferred to another hospital _____

Other additional comments

_____
_____
_____

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8.2 APPENDIX B: Descriptive data and Clinical data

Table B1: Patient demographics and Clinical data obtained from all isolates

Patient details		Patient Demographics					Clinical Data			
# of strains	Study #	Gender	Age	All Wards	All Ward Groups	MRSA/MSSA	Clinical Diag.	HCA/HA/CA	HIV status	Patient outcome
1	**TBH 1**	Female	36 yrs	A8E	M	MSSA	SSTI	HA	Negative	Discharged
2	**TBH 2**	Female	47 yrs	A1W	A1W	MSSA	PN	HA	Unknown	Discharged
3	**TBH 3**	Male	6 yrs	G ground	Pd	MSSA	SSTI	CA	Unknown	Discharged
4	**TBH 5**	Female	3 mnths	G4	Pd	**MRSA**	CSI	HA	Negative	Demised
5	**TBH 6**	Female	45 yrs	A7	A7	**MRSA**	CSI	HCA	Negative	Demised
6	**TBH 7.a**	Male	37 yrs	A1B	A1B	MSSA	SSTI	HA	Unknown	Discharged
7	**TBH 7.b**					**MRSA**	CSI	HA		
8	**TBH 8**	Female	22 yrs	A7	A7	MSSA	CSI	HA	Negative	Discharged
9	**TBH 9**	Female	65 yrs	A1W	A1W	**MRSA**	SSTI	HA	Negative	Discharged
10	**TBH 10**	Male	75 yrs	A1W	A1W	MSSA	PB	a	Negative	Discharged
11	**TBH 11**	Male	8 days	G2	N	MSSA	CSI	HA	Unknown	Discharged
12	**TBH 12**	Female	1 mnth	G1	N	MSSA	PN	CA	Unknown	Discharged
13	**TBH 13**	Female	1 yr	G3	Pd	**MRSA**	CSI	HA	Negative	Discharged
14	**TBH 14**	Male	41 yrs	D9	M	MSSA	IE	CA	Negative	Demised
15	**TBH 15**	Male	37 days	A9E	A9E	MSSA	PN	CA	Negative	Discharged
16	**TBH 16**	Female	58 yrs	A1W	A1W	**MRSA**	CSI	HA	Negative	Demised
17	**TBH 17**	Male	1 yr	G7	Pd	**MRSA**	Unknown		Negative	Discharged
18	**TBH 18**	Male	57 yrs	A6	M	MSSA	CSI	HCA	Unknown	Discharged
19	**TBH 19**	Male	4 mnths	G ground	Pd	**MRSA**	Unknown		Negative	Discharged
20	**TBH 20**	Female	7 mnths	G10	Pd	MSSA	SSTI	HA	Negative	Discharged
21	**TBH 21**	Female	7 days	G2	N	MSSA	Unknown		Unknown	Discharged
22	**TBH 22**	Male	7 yrs	G3	Pd	**MRSA**	CSI	HCA	Negative	Discharged
23	**TBH 23**	Female	41 yrs	KHA	KHA	MSSA	SSTI	CA	Negative	Demised
24	**TBH 24**	Male	1 day	A9E	A9E	MSSA	Unknown		Unknown	Transferred to another HP
25	**TBH 25**	Female	43 yrs	A4W	S	MSSA	Other(UTI)	HA	Unknown	Transferred to another HP
26	**TBH 26.a**	Male	36 yrs	A7	A7	MSSA	CSI	HCA	Negative	Transferred to another HP
27	**TBH 26.b**					MSSA	CSI	HCA		
28	**TBH 26.c**					MSSA	CSI	HCA		

Patient details		Patient Demographics					Clinical Data			
29	**TBH 27**	Female	22 yrs	D9	M	MSSA	PB	[a]	Positive	Discharged
30	**TBH 28**	Male	13 mnths	G ground	Pd	MSSA	CSI	HCA	Unknown	Discharged
31	**TBH 29**	Male	10 mnths	G9	Pd	**MRSA**	SSTI	HA	Positive	Discharged
32	**TBH 30.a**	Male	20 yrs	A7	A7	MSSA	CSI	HCA	Negative	Discharged
33	**TBH 30.b**					MSSA	CSI	HCA		
34	**TBH 30.c**					MSSA	CSI	HCA		
35	**TBH 30.d**					MSSA	CSI	HCA		
36	**TBH 30.e**					MSSA	CSI	HCA		
37	**TBH 31**	Male	61 yrs	KHA	KHA	MSSA	SSTI	HA	Unknown	Transferred to another HP
38	**TBH 32**	Female	56 yrs	A7	A7	MSSA	CSI	HCA	Negative	Discharged
39	**TBH 33**	Female	6 days	G2	N	**MRSA**	CSI	HA	Unknown	Discharged
40	**TBH 34**	Male	59 yrs	D1	S	MSSA	CSI	HCA	Unknown	Demised
41	**TBH 35**	Male	40 yrs	A7	A7	MSSA	CSI	HCA	Negative	Discharged
42	**TBH 36**	Female	22 yrs	A7	A7	MSSA	CSI	HCA	Negative	
43	**TBH 37**	Female	74 yrs	F1	M	MSSA	IE	CA	Unknown	Demised
44	**TBH 38**	Female	63 yrs	KHA	KHA	MSSA	Unknown		Unknown	Discharged
45	**TBH 39**	Female	36 yrs	F1	M	MSSA	PB	[a]	Unknown	Demised
46	**TBH 40.a**	Male	7 days	G2	N	**MRSA**	SSTI	HA	Unknown	
47	**TBH 40.b**					**MRSA**	SSTI	HA		Discharged
48	**TBH 41**	Female	93 days	G9	Pd	MSSA	CSI	HCA	Unknown	Discharged
49	**TBH 42**	Male	49 yrs	D5	S	**MRSA**	CSI	HA	Unknown	Demised
50	**TBH 43**	Male	22 days	A9E	A9E	MRSA				
51	**TBH 44**	Male	63 yrs	A1B	A1B	**MRSA**	SSTI	HA	Unknown	Demised
52	**TBH 45**	Female	16 yrs	D8	M	MSSA	Unknown		Negative	Discharged
53	**TBH 46**	Female	34 yrs	D8	M	**MRSA**	Unknown		Negative	Discharged
54	**TBH 47**	Male	28 yrs	A1B	A1B	MSSA	CSI	HA	Unknown	Discharged
55	**TBH 48**	Female	10 mnths	G7	Pd	**MRSA**	CSI	HA	Positive	Discharged
56	**TBH 49**	Female	18 days	G2	N	**MRSA**	SA	HA	Unknown	Discharged
57	**TBH 50**	Male	9 yrs	A9E	A9E	MSSA	PN	HA	Unknown	Discharged
58	**TBH 51**	Female	76 yrs	D4	Other	MSSA	CSI	HA	Negative	Discharged
59	**TBH 52**	Male	4 mnths	G9	Pd	MSSA	PN	CA	Negative	Discharged
60	**TBH 53**	Female	7 days	A9E	A9E	**MRSA**	CSI	HA	Negative	Transferred to another HP

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Patient details		Patient Demographics					Clinical Data			
61	**TBH 55**	Male	65 yrs	A6	M	MSSA	SSTI	HCA	Unknown	Demised
62	**TBH 57**	Female	87 yrs	D5	S	MSSA	SSTI	HA	Unknown	Transferred to another HP
63	**TBH 58**	Female	24 yrs	C2A	Other	MSSA	PB	[a]	Unknown	Demised
64	**TBH 59**	Male	2yrs	G7	Pd	**MRSA**	CSI	HA	Positive	Discharged
65	**TBH 60**	Female	5yrs	G3	Pd	MSSA	SSTI	HCA	Negative	Discharged
66	**TBH 61**	Female	5 days	G2	N	**MRSA**	CSI	HA	Unknown	Demised
67	**TBH 62**	Male	23 yrs	A1B	A1B	MSSA	CSI	HA	Unknown	Demised
68	**TBH 63**	Male	47 yrs	D2	S	**MRSA**	Unknown		Negative	Discharged
69	**TBH 65**	Male	20 yrs	F1	M	MSSA	IE	CA	Negative	Discharged
70	**TBH 66**	Female	61 yrs	A2W	S	**MRSA**	CSI	HA	Unknown	Demised
71	**TBH 67**	Male	38 yrs	A1W	A1W	MSSA	CSI	HA	Unknown	Discharged
72	**TBH 68**	Male	9 yrs	KHA	KHA	MSSA	SA	CA	Unknown	Discharged
73	**TBH 69**	Female	31 yrs	A1B	A1B	**MRSA**	SSTI	HA	Unknown	Discharged
74	**TBH 71**	Female	34 yrs	A8H	M	**MRSA**	SSTI	HA	Positive	Demised
75	**TBH 72**	Male	23 yrs	A1B	A1B	MSSA	PN	HA	Negative	Discharged
76	**TBH 73**	Male	10 yrs	G6	Pd	MSSA	SA	CA	Unknown	Discharged
77	**TBH 75**	Male	31 yrs	A1B	A1B	MSSA	Other(Pericarditis)	HA	Negative	Demised
78	**TBH 76**	Female	33 yrs	A7	A7	**MRSA**	CSI	HCA	Negative	Discharged
79	**TBH 77**	Female	75 yrs	D10	M	MSSA	PB	[a]	Unknown	Transferred to another HP
80	**TBH 78**	Female	16 yrs	A1W	A1W	MSSA	PB	[a]	Negative	Discharged
81	**TBH 79**	Male	41 yrs	A7	A7	MSSA	CSI	HCA	Negative	Discharged
82	**TBH 80**	Male	46 yrs	A1B	A1B	MSSA	CSI	HA	Unknown	Demised
83	**TBH 81**	Female	22 days	G1	N	MSSA	SA	HA	Negative	Discharged
84	**TBH 82**	Female	10 days	G8	Pd	MSSA	CSI	HA	Unknown	Discharged
85	**TBH 83**	Female	67 yrs	A2W	S	**MRSA**	PB	[a]	Unknown	Demised
86	**TBH 84**	Male	39 yrs	F1	M	MSSA	PN	CA	Negative	Demised
87	**TBH 85**	Male	39 yrs	A1W	A1W	MSSA	PB	[a]	Unknown	Demised
88	**TBH 86**	Male	6 mnths	A9E	A9E	**MRSA**	CSI	HA	Unknown	Transferred to another HP
89	**TBH 87**	Male	28 yrs	A1B	A1B	MSSA	CSI	HA	Unknown	Discharged
90	**TBH 88**	Male	1 day	G1	N	MSSA	PB	[a]	Negative	Discharged
91	**TBH 89**	Male	66 yrs	A4W	S	MSSA	PN	CA	Unknown	Demised
92	**TBH 90**	Male	28 yrs	D9	M	MSSA	PB	[a]	Negative	Demised
93	**TBH 91**	Female	41 yrs	A7	A7	MSSA	CSI	HCA	Negative	Discharged
94	**TBH 92**	Female	6 mnths	G10	Pd	MSSA	Unknown		Negative	Discharged

Patient details		Patient Demographics					Clinical Data			
95	**TBH 93**	Male	9 days	G1	N	MSSA	PB	[a]	Unknown	Discharged
96	**TBH 94**	Female	8 mnths	KHA	KHA	MSSA	SSTI	HA	Unknown	Transferred to another HP
97	**TBH 95**	Female	32 yrs	D10	M	**MRSA**	SA	HA	Negative	Transferred to another HP
98	**TBH 96**	Male	31 yrs	A1B	A1B	**MRSA**	Unknown	HA	Unknown	Demised
99	**TBH 97**	Female	9 yrs	G3	Pd	MSSA	CSI	HA	Negative	Demised
100	**TBH 98**	Male	81 yrs	A1B	A1B	MSSA	SSTI	HA	Unknown	Demised
101	**TBH 99**	Male	73 yrs	A1W	A1W	**MRSA**	SSTI	HA	Negative	Transferred to another HP
102	**TBH 100**	Male	21 yrs	KHA	KHA	MSSA	SSTI	HA	Unknown	Discharged
103	**TBH 101**	Female	8 mnths	G9	Pd	**MSSA**	PN	HA	Negative	Discharged
104	**TBH 102**	Female	6 yrs	G10	Pd	MSSA	PN	CA	Negative	Discharged
105	**TBH 103**	Female	53 yrs	A7	A7	MSSA	CSI	HCA	Negative	Discharged
106	**TBH 104**	Female	21 yrs	A4W	S	MSSA	PN	HA	Unknown	Transferred to another HP
107	**TBH 105**	Male	4 mnths	A9E	A9E	MSSA	PB	[a]	Negative	Discharged
108	**TBH 106**	Female	19 days	G1	N	MSSA	PB	[a]	Negative	Discharged
109	**TBH 107**	Female	44 yrs	A1W	A1W	**MRSA**	PN	HA	Negative	Discharged
110	**TBH 108**	Female	43 yrs	D10	M	MSSA	Unknown		Negative	Discharged
111	**TBH 109**	Female	60 yrs	F1	M	MSSA	PB	[a]	Unknown	Demised
112	**TBH 110.a**	Male	35 yrs	A1B	A1B	MSSA	CSI	HA	Unknown	Discharged
113	**TBH 110.b**					**MRSA**	PB	[a]		

(**CSI**) = Catheter and prosthetic device-related sepsis

(**PN**) = Pneumonia

(**IE**) = Infective endocarditis

(**Unknown**) = clinical significance not clear, possible contaminants

([a]) = PB excluded from analysis for HA/HCA/CA category due to unknown source

(**HA**) = Hospital acquired infection;

(**HCA**) = Health care associated infections; (**CA**) = Community acquired infection

(**SA**) = Septic arthritis (including osteomyelitis)

(**SSTI**) = Skin and soft tissue infections

(**PB**) = Primary bacteraemia

(**Transferred to another HP**) = Transferred to another hospital

Table B2: Phenotypic and Genotypic results obtained from all isolates

| Patient details | | Phenotypic results | Genotypic results | | | | | |
# of strains	Study #	MRSA/MSSA	*mecA*	*spa* band	*pvl* +	*Spa*-type	Repeats	Cluster groups
1	**TBH 1**	MSSA	-	+	-	t127	7	*spa*-CC 174
2	**TBH 2**	MSSA	-	+	-	t030	7	*spa*-CC 012
3	**TBH 3**	MSSA	-	+	+	t317	7	Singleton
4	**TBH 5**	**MRSA**	+	+	-	t1257	10	*spa*-CC 701
5	**TBH 6**	**MRSA**	+	+	-	t037	7	*spa*-CC 012
6	**TBH 7.a**	MSSA	-	+	-	t002	10	*spa*-CC 002
7	**TBH 7.b**	**MRSA**	+	+	-	t037	7	*spa*-CC 012
8	**TBH 8**	MSSA	-	**NT**	+	*	*	*
9	**TBH 9**	**MRSA**	+	+	-	t018	11	*spa*-CC 012
10	**TBH 10**	MSSA	-	+	-	t701	10	*spa*-CC 701
11	**TBH 11**	MSSA	-	+	+	t891	11	Singleton
12	**TBH 12**	MSSA	-	+	-	t2360	11	*spa*-CC 701
13	**TBH 13**	**MRSA**	+	+	-	t1443	11	*spa*-CC 701
14	**TBH 14**	MSSA	-	+	-	t346	10	*spa*-CC 346/085
15	**TBH 15**	MSSA	-	+	+	t433	9	*spa*-CC 012
16	**TBH 16**	**MRSA**	+	+	-	t037	7	*spa*-CC 012
17	**TBH 17**	**MRSA**	+	+	-	t015	10	*spa*-CC 015
18	**TBH 18**	MSSA	-	+	-	t026	3	Excluded
19	**TBH 19**	**MRSA**	+	+	-	t451	9	*spa*-CC 701
20	**TBH 20**	MSSA	-	**NT**	-	*	*	*
21	**TBH 21**	MSSA	-	+	-	t214	11	*spa*-CC 002
22	**TBH 22**	**MRSA**	+	+	-	t287	3	Excluded
23	**TBH 23**	MSSA	-	+	-	t002	10	*spa*-CC 002
24	**TBH 24**	MSSA	-	**NT**	-	*	*	*
25	**TBH 25**	MSSA	-	+	+	t1597	8	Singleton
26	**TBH 26.a**	MSSA	-	+	+	t174	6	*spa*-CC 174
27	**TBH 26.b**	MSSA	-	+	-	t253	12	*spa*-CC 012
28	**TBH 26.c**	MSSA	-	+	-	t253	12	*spa*-CC 012
29	**TBH 27**	MSSA	-	+	-	t1476	8	*spa*-CC 701
30	**TBH 28**	MSSA	-	+	-	t174	6	*spa*-CC 174
31	**TBH 29**	**MRSA**	+	+	-	t1257	10	*spa*-CC 701
32	**TBH 30.a**	MSSA	-	+	-	t015	10	*spa*-CC 015
33	**TBH 30.b**	MSSA	-	+	-	t015	10	*spa*-CC 015
34	**TBH 30.c**	MSSA	-	+	-	t015	10	*spa*-CC 015
35	**TBH 30.d**	MSSA	-	+	+	t891	11	Singleton
36	**TBH 30.e**	MSSA	-	+	-	t012	10	*spa*-CC 012
37	**TBH 31**	MSSA	-	+	-	t015	10	*spa*-CC 015
38	**TBH 32**	MSSA	-	+	-	t002	10	*spa*-CC 002
39	**TBH 33**	**MRSA**	+	**NT**	-	*	*	*
40	**TBH 34**	MSSA	-	+	-	t148	11	Singleton
41	**TBH 35**	MSSA	-	+	-	t002	10	*spa*-CC 002
42	**TBH 36**	MSSA	-	+	-	t045	7	*spa*-CC 002
43	**TBH 37**	MSSA	-	+	-	t1848	9	*spa*-CC 012
44	**TBH 38**	MSSA	-	+	-	t275	8	*spa*-CC 701
45	**TBH 39**	MSSA	-	+	+	t891	11	Singleton
46	**TBH 40.a**	**MRSA**	+	+	-	t021	9	*spa*-CC 012
47	**TBH 40.b**	**MRSA**	+	+	-	t021	9	*spa*-CC 012

# of strains	Study #	MRSA/MSSA	mecA	spa band	pvl +	Spa-type	Repeats	Cluster groups
		Patient details	**Phenotypic results**		**Genotypic results**			
48	**TBH 41**	MSSA	-	+	+	t891	11	Singleton
49	**TBH 42**	**MRSA**	+	+	-	t037	7	spa-CC 012
50	**TBH 43**	**MRSA**	-	+	-	t2360	11	spa-CC 701
51	**TBH 44**	**MRSA**	+	+	-	t1257	10	spa-CC 701
52	**TBH 45**	MSSA	-	+	-	t085	10	spa-CC 346/085
53	**TBH 46**	**MRSA**	+	+	-	t451	9	spa-CC 701
54	**TBH 47**	MSSA	-	+	-	t5474	12	spa-CC 701
55	**TBH 48**	**MRSA**	+	+	-	t1257	10	spa-CC 701
56	**TBH 49**	**MRSA**	+	+	-	t021	9	spa-CC 012
57	**TBH 50**	MSSA	-	+	-	t267	10	No founder
58	**TBH 51**	MSSA	-	+	+	t409	6	Singleton
59	**TBH 52**	MSSA	-	+	-	t002	10	spa-CC 002
60	**TBH 53**	**MRSA**	+	+	-	t021	9	spa-CC 012
61	**TBH 55**	MSSA	-	+	-	t888	6	Singleton
62	**TBH 57**	MSSA	-	+	-	t306	11	spa-CC 002
63	**TBH 58**	MSSA	-	+	-	t318	10	spa-CC 012
64	**TBH 59**	**MRSA**	+	+	-	t1257	10	spa-CC 701
65	**TBH 60**	MSSA	-	+	+	t891	11	Singleton
66	**TBH 61**	**MRSA**	+	+	-	t451	9	spa-CC 701
67	**TBH 62**	MSSA	-	+	-	t1597	8	Singleton
68	**TBH 63**	**MRSA**	+	**NT**	-	*	*	*
69	**TBH 65**	MSSA	-	+	-	t4576	8	spa-CC 015
70	**TBH 66**	**MRSA**	+	+	-	t1443	11	spa-CC 701
71	**TBH 67**	MSSA	-	+	-	t2393	10	Singleton
72	**TBH 68**	MSSA	-	+	+	t891	11	Singleton
73	**TBH 69**	**MRSA**	+	+	-	t037	7	spa-CC 012
74	**TBH 71**	**MRSA**	+	+	-	t064	10	spa-CC 701
75	**TBH 72**	MSSA	-	+	-	t701	10	spa-CC 701
76	**TBH 73**	MSSA	-	+	+	t002	10	spa-CC 002
77	**TBH 75**	MSSA	-	+	+	t311	9	spa-CC 002
78	**TBH 76**	**MRSA**	+	+	-	t064	10	spa-CC 701
79	**TBH 77**	MSSA	-	+	-	t521	11	No founder
80	**TBH 78**	MSSA	-	**NT**	-	*	*	*
81	**TBH 79**	MSSA	-	+	-	t701	10	spa-CC 701
82	**TBH 80**	MSSA	-	**NT**	-	*	*	*
83	**TBH 81**	MSSA	-	+	-	t1597	8	Singleton
84	**TBH 82**	MSSA	-	+	-	t1597	8	Singleton
85	**TBH 83**	**MRSA**	+	+	-	t012	10	spa-CC 012
86	**TBH 84**	MSSA	-	+	-	t015	10	spa-CC 015
87	**TBH 85**	MSSA	-	+	-	t5396	14	spa-CC 346/085
88	**TBH 86**	**MRSA**	+	+	-	t021	9	spa-CC 012
89	**TBH 87**	MSSA	-	+	-	t012	10	spa-CC 012
90	**TBH 88**	MSSA	-	+	-	t375	8	Singleton
91	**TBH 89**	MSSA	-	+	-	t5472	6	spa-CC 701
92	**TBH 90**	MSSA	-	**NT**	-	*	*	*
93	**TBH 91**	MSSA	-	+	-	t5473	11	spa-CC 701
94	**TBH 92**	MSSA	-	+	-	t174	6	spa-CC 174
95	**TBH 93**	MSSA	-	+	-	t465	9	spa-CC 015
96	**TBH 94**	MSSA	-	+	-	t2763	6	Singleton

Patient details		Phenotypic results	Genotypic results					
# of strains	Study #	MRSA/MSSA	*mecA*	spa band	pvl +	Spa-type	Repeats	Cluster groups
97	TBH 95	MRSA	+	+	-	t1257	10	*spa*-CC 701
98	TBH 96	MRSA	+	+	-	t037	7	*spa*-CC 012
99	TBH 97	MSSA	-	+	-	t2623	9	*spa*-CC 015
100	TBH 98	MSSA	-	+	-	t064	10	*spa*-CC 701
101	TBH 99	MRSA	+	+	-	t037	7	*spa*-CC 012
102	TBH 100	MSSA	-	+	-	t148	11	Singleton
103	TBH 101	MSSA	-	NT	+	*	*	*
104	TBH 102	MSSA	-	NT	+	*	*	*
105	TBH 103	MSSA	-	+	-	t292	8	*spa*-CC 701
106	TBH 104	MSSA	-	+	-	t030	6	*spa*-CC 012
107	TBH 105	MSSA	-	+	+	t891	11	
108	TBH 106	MSSA	-	+	-	t1443	11	*spa*-CC 701
109	TBH 107	MRSA	+	+	-	t037	7	*spa*-CC 012
110	TBH 108	MSSA	-	NT	-	*	*	*
111	TBH 109	MSSA	-	+	+	t5471	6	*spa*-CC 174
112	TBH 110.a	MSSA	-	+	-	t1476	8	*spa*-CC 701
113	TBH 110.b	MRSA	+	+	-	t037	7	*spa*-CC 012

(+) = Positive for detection of the *mecA, spa* and *pvl* genes

(-) = No detection (negative) of the *mecA, spa* and *pvl* genes

(NT) = Non-typeable strains with no *spa* band

(*) = Could not be further analysed due to non-typeable strains

## 8.3 APPENDIX C: Multiplex PCR gel images obtained after agarose gel Electrophoresis

Images include a multiplex PCR for the detection of the *mecA, pvl* and *spa* genes. The *pvl* gene is approximately 80 bp, the *mecA* is 162 bp and the *spa* gene varies between 200 and 500 bp. The negative (neg.) control excluded DNA. Positive controls included ATCC 49775 - PVL positive (+ve), MSSA and ATCC 43300 – PVL negative (–ve) MRSA). **(M)** = Molecular size marker (100 bp); **(N/A)** = excluded from all analysis. **(RP: TBH)** = Samples that were re-tested (Consisted of samples with no *spa* band. These isolates were confirmed to be *S. aureus* by phenotypic methods. Genotypic results revealed a lack of the *spa* gene, which could not be typed: Non-typeable strains).
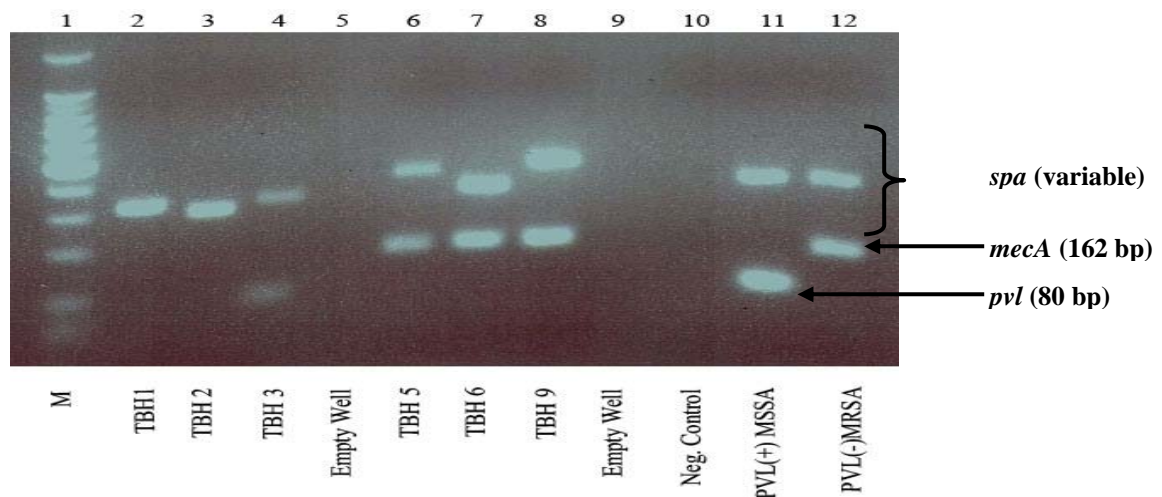
Figure C1: Multiplex PCR agarose gel electrophoresis image # 1
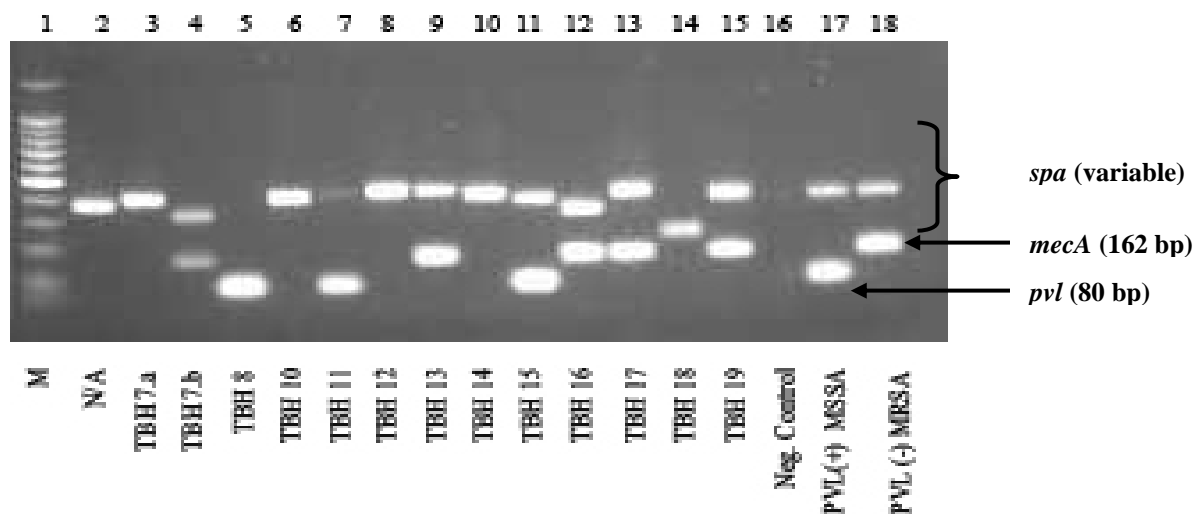
Figure C2: Multiplex PCR agarose gel electrophoresis image # 2
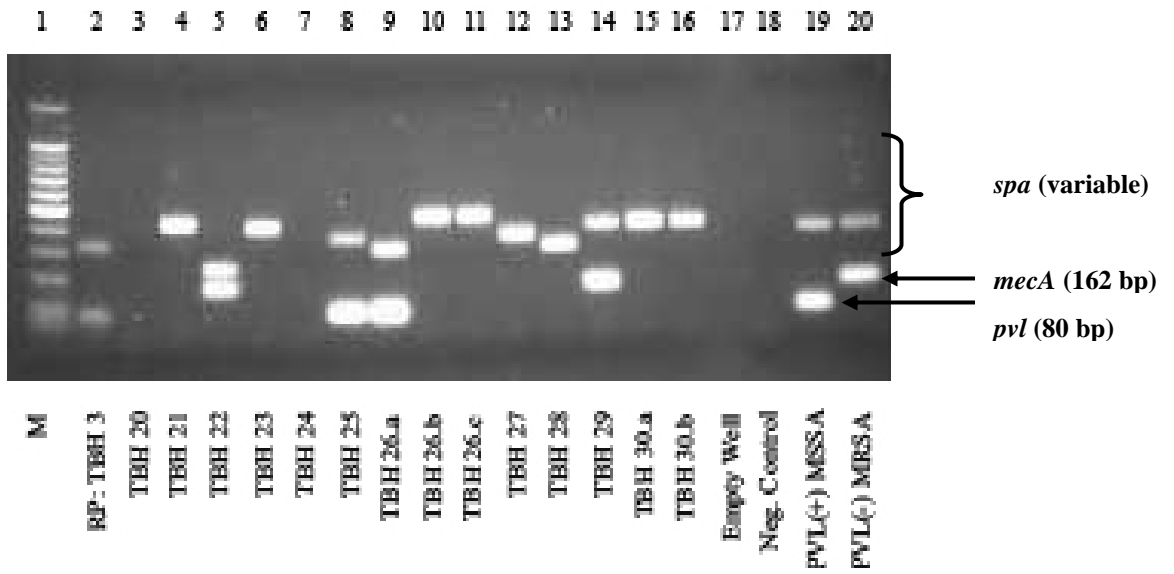
Figure C3: Multiplex PCR agarose gel electrophoresis image # 3

Figure C4: Multiplex PCR agarose gel electrophoresis image # 4

Figure C5: Multiplex PCR agarose gel electrophoresis image # 5

Figure C6: Multiplex PCR agarose gel electrophoresis image # 6
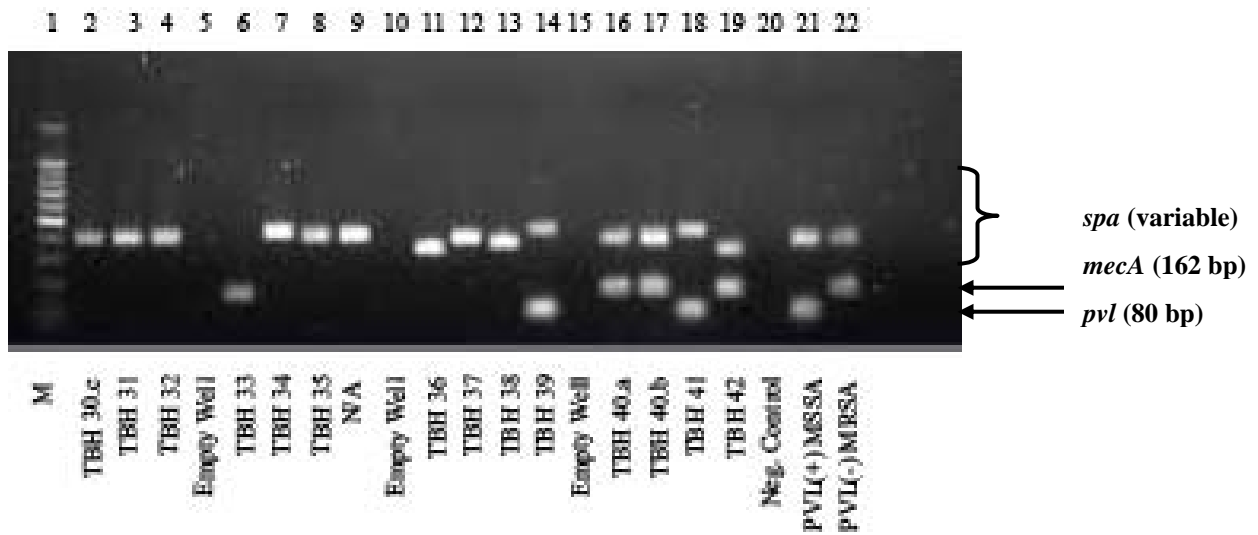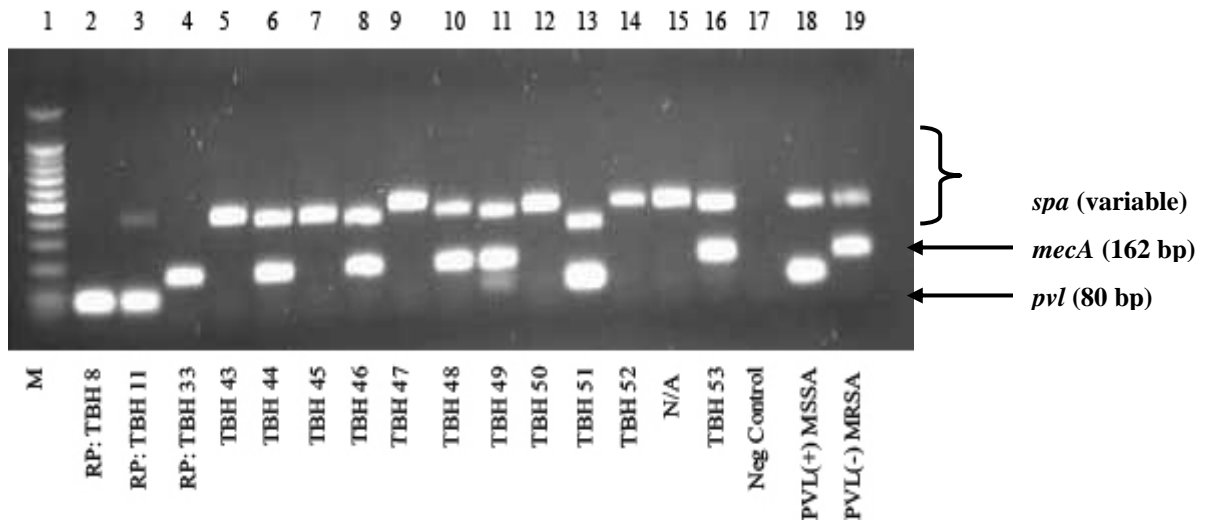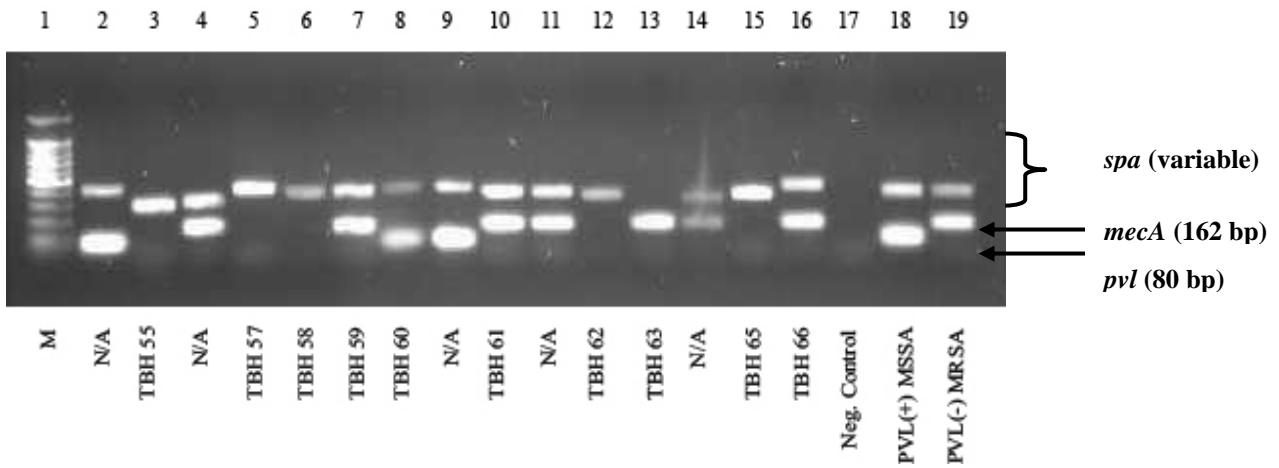
155
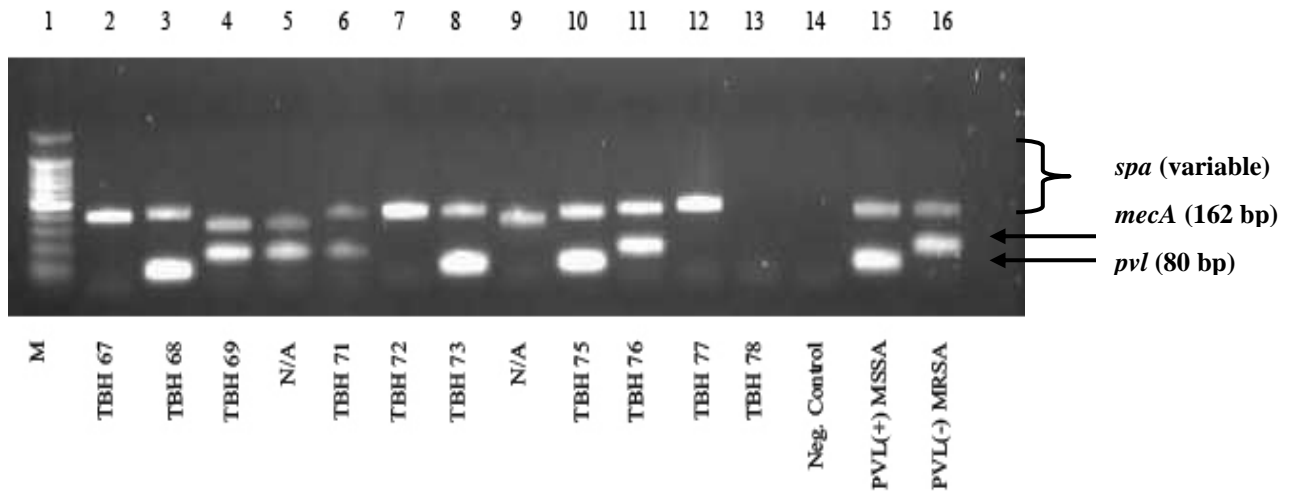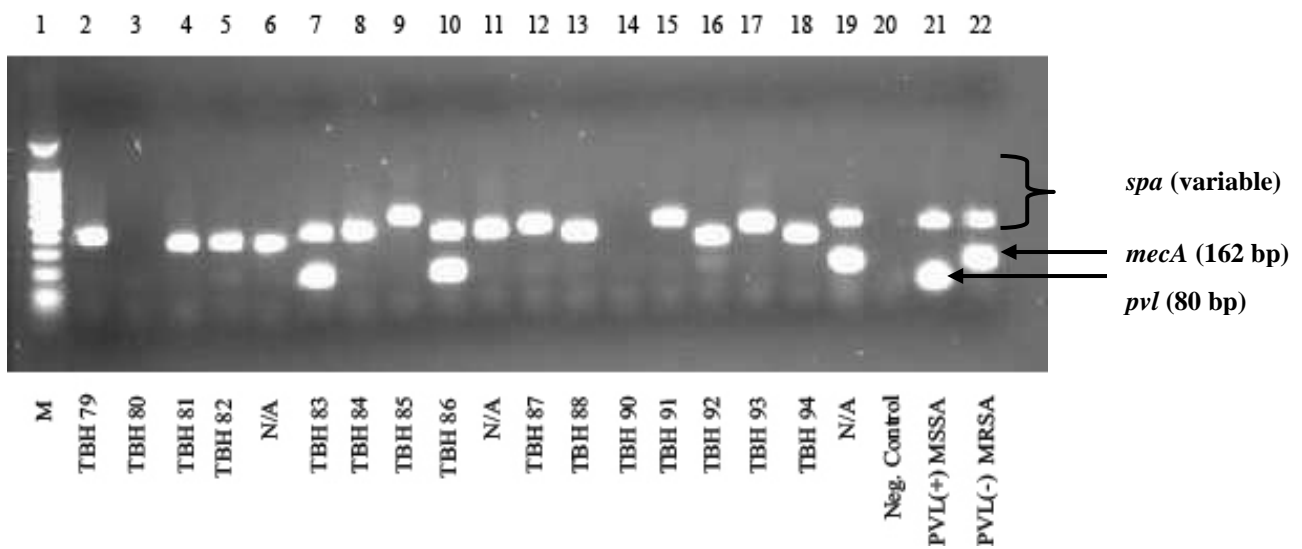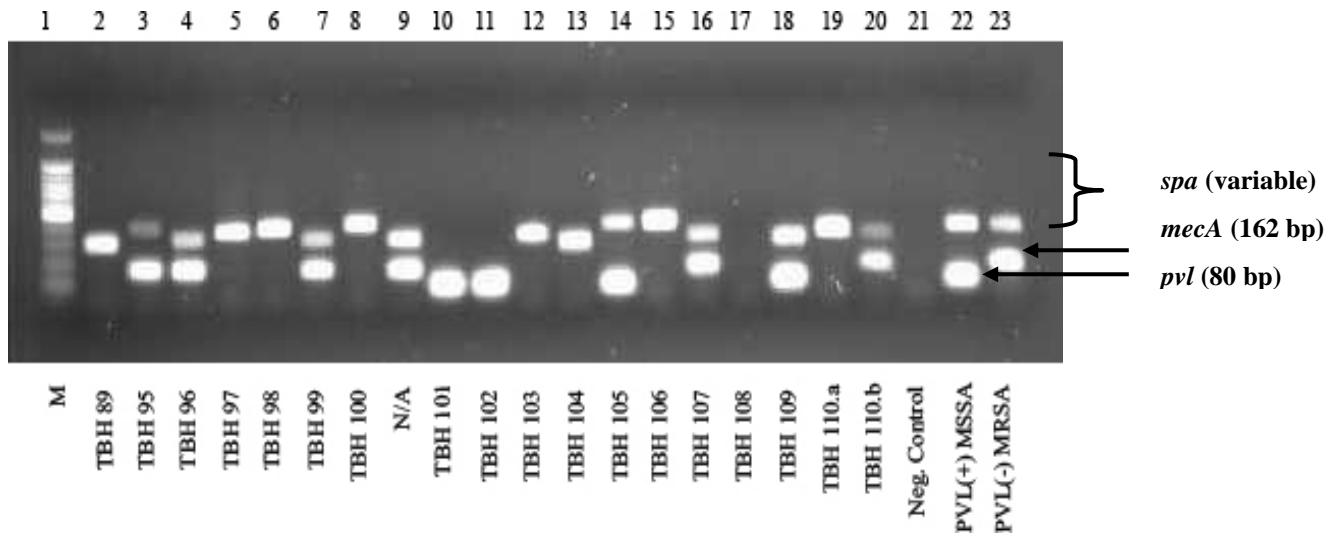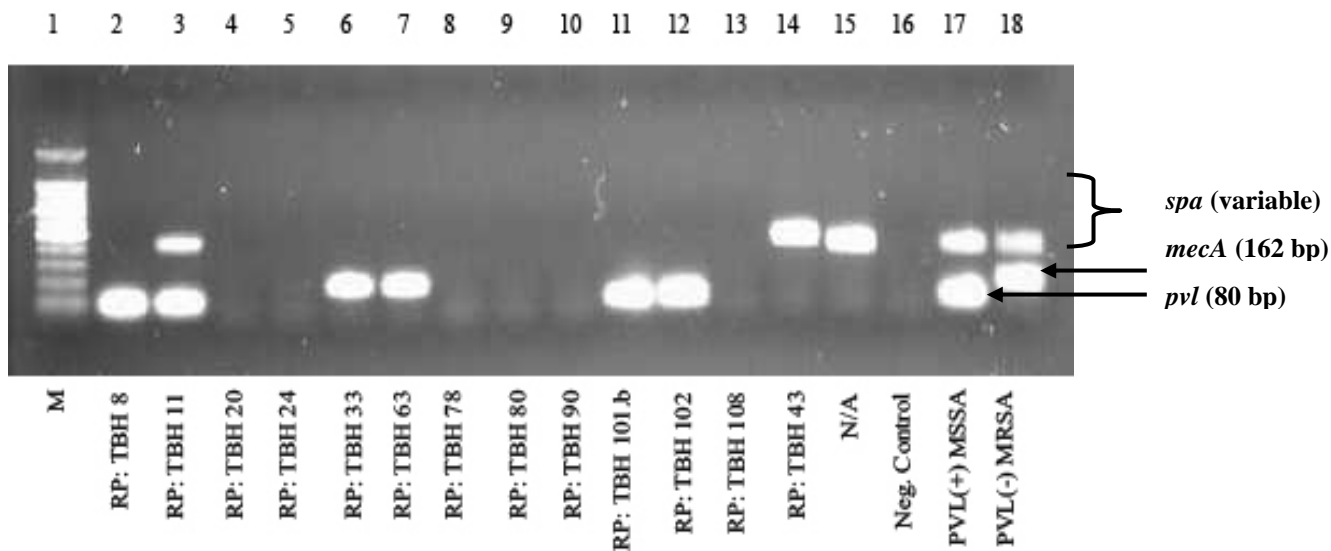
Figure C7: Multiplex PCR agarose gel electrophoresis image # 7

Figure C8: Multiplex PCR agarose gel electrophoresis image # 8

Figure C9: Multiplex PCR agarose gel electrophoresis image # 9

Figure C10: Multiplex PCR agarose gel electrophoresis image # 10

8.4 APPENDIX D: Ridom sequences and Geographical spread of all *spa* types

Spa type	Geographical Distribution	Ridom Sequence of *Spa* types
t002	Aus, Bel, Can, Chi, Cro, Cyp, Czr, Den, Est, Fin, Fra, Ger, Hun, Ice, Isr, Ita, Jap, Jor, Leb, Mar, Net, New, Nor, Pol, Rom, SA, Spa, Swe, Swi, Tai, UK, USA	26-23-17-34-17-20-17-12-17-16
t012	Aus, Bel, Can, Cyp, Czr, Den, Fin, Fra, Ger, Ice, Ita, Jor, Lat, Leb, Net, New, Nor, Pol, SA, Spa, Swed, Swi, UK, USA	15-12-16-02-16-02-25-17-24-24
t015	Aus, Bel, Cro, Czr, Den, Fin, Fra, Ger, Hun, Ice, Ind, Ita, Lat, Net, New, Nor, Pol, Rom, SA, Spa, Swe, Swi, Tai, UK, USA	08-16-02-16-34-13-17-34-16-34
t018	Aus, Bel, Can, Czr, Den, Fra, Ger, Ice, Ita, Jor, Net, New, Nor, Pol, SA, Spa, Swe, Swi, UK, USA	15-12-16-02-16-02-25-17-24-24-24
t021	Aus, Bel, Den, Fin, Fra, Ger, Ice, In, Ita, Jor, Lat, Leb, Net, New, Nor, Pol, SA, Spa, Swe, Swi, UK, USA	15-12-16-02-16-02-25-17-24
t026	Aus, Bel, Cro, Czr, Den, Fin, Fra, Ger, Net, New, Nor, Pol, Slo, SA, Spa, Swe, Swi, Tai	08-16-34
t030	Aus, Bul, Chi, Cro, Cyp, Czr, Den, Fra, Ger, Leb, Net, Nor, Rom, SA, Swe, Swit, Tur	15-12-16-02-24-24
t037	Aus, Bel, Bul, Can, Chi, Cro, Czr, Den, Fra, Ger, Ice, Ita, Jor, Lat, Leb, Mal, Net, New, Nor, Pol, SA, Spa, Swe, Swi, Tai, UK	15-12-16-02-25-17-24
t045	Aus, Bel, Chi, Den, Fra, Ger, Isr, Jap, Net, New, Nor, SA, Spa, Swe, Swi, USA	26-17-20-17-12-17-16
t064	Aus, Bel, Can, Czr, Den, Ger, Ice, Isr, Net, Nor, SA, Swe, Swi, UK, USA	11-19-12-05-17-34-24-34-22-25
t085	Bel, Den, Fin, Fra, Ger, Net, New, Nor, Pol, SA, Spa, Swe, Swi	07-23-12-34-34-12-23-02-12-23
t127	Aus, Bel, Chi, Cro, Cyp, Den, Fin, Fra, Ger, Ice, Ind, Lat, Leb, Net, New, Nor, Pol, Rom, SA, Spa, Swe, Swi, Tai, UK, USA	07-23-21-16-34-33-13
t148	Aus, Bel, Cro, Den, Fra, Ger, Ice, Ind, Net, Nor, Pol, SA, Spa, Swe, Swi, USA	07-23-12-21-12-17-20-17-12-12-17
t174	Aus, Net, SA, Swe, UK	14-21-16-34-33-13
t214	Den, Fra, Ger, Jor, Net, Nor, SA, Spa, Swe, UK	26-23-17-34-17-20-17-12-17-16-16
t253	Bel, Den, Ger, Net, Nor, Pol, SA, UK	15-12-16-02-16-02-25-17-24-24-24-24
t267	Aus, Bel, Cyp, Den, Fra, Ger, Ita, Jor, Leb, Net, New, Nor, SA, Spa, Swe, Tai, UK, USA	07-23-12-21-17-34-34-34-33-34
t275	Aus, Bel, Chi, Ger, Net, New, Nor, SA,	15-12-16-02-25-17-24

	Swe, UK	
t287	Bel, Ger, Net, Nor, SA, Spa, Swe, Tai	04-12-17
t292	Den, Ger, SA	11-12-17-34-24-34-22-25
t306	Den, Fra, Ger, Jap, Net, Nor, SA, Swe, USA	26-23-17-34-17-20-17-12-17-17-16
t311	Bel, Chi, Czr, Den, Fra, Gab, Ger, Leb, Net, New, Nor, SA, Spa, Swe, Swi, UK	26-23-17-34-20-17-12-17-16
t317	SA, Spa	08-17-23-18-23-18-17
t318	Bel, Cap, Chi, Den, Fin, Fra, Ger, Ind, Lat, Leb, Net, Nor, Pol, SA, Spa, Swe, Swi, UK, USA	15-12-16-16-02-16-02-25-17-24
t346	Bel, Den, Fin, Fra, Ger, Jor, Net, Nor, Pol, SA, Swe, Swi, USA	07-23-12-34-12-12-23-02-12-23
t375	Chi, Den, Fin, Ger, Net, Nor, SA, Spa, Swe	49-13-23-05-17-34-33-34
t409	SA, Nor, Ger, Ind	60-61-34-22-34-17
t433	Fra, Ger, Lat, Net, Nor, SA, Swi, USA,	15-12-16-16-02-16-02-25-17
t451	Aus, Bel, Chi, Ger, Net, New, Nor, SA, Swe, UK	11-12-05-17-34-24-34-22-25
t465	Den, Ger, SA	08-23-16-34-13-17-34-16-34
t521	Fra, Ger, Ind, Net, Nor, SA, Zim	07-23-12-21-17-34-34-34-34-33-34
t701	Bel, Den, Fra, Gab, Ger, Ind, Jap, Jor, Net, New, Nor, SA, Spa, Swe, Tai	11-10-21-17-34-24-34-22-25-25
t888	Den, Fin, Ger, Nor, SA, Swe, Swi	07-23-21-24-33-17
t891	Den, Fra, Ger, SA, Swi	26-23-13-23-31-05-17-25-17-25-28
t1257	Den, Ger, Nor, SA	11-19-34-05-17-34-24-34-22-25
t1443	SA	11-19-12-05-17-34-24-24-34-22-25
t1476	Bel, Gab, Ger, Net, Nor, SA	11-10-17-34-24-34-22-25
t1597	Fra, Ger, Ind, Net, Nor, SA, Zim	15-12-17-20-17-12-12-17
t1848	Fra, SA	15-12-17-16-02-16-02-25-17
t2360	SA	11-10-21-17-34-24-34-22-25-25-25
t2393	Fra, Nor, SA, Swe, UK	07-12-21-17-13-13-13-34-33-34
t2623	Aus, Net, SA	08-16-02-16-13-17-13-16-34
t2763	SA	26-13-17-34-16-13
t4576	Ger, SA	08-16-02-16-34-34-16-13
t5471	SA	35-21-16-34-33-13
t5472	SA	11-10-17-34-24-34
t5473	SA	11-19-12-21-21-12-34-24-34-22-25
t5474	SA	11-10-21-17-34-24-34-22-25-25-25-25

Abbreviatians are as follows:

(Aus) Austria; **(Bel)** Belgium; **(Bul)** Bulgaria; **(Can)** Canada; **(Chi)** China; **(Cro)** Croatia; **(Cyp)** Cyprus; **(Czr)** Czech Republic; **(Den)** Denmark; **(Est)** Estonia; **(Fin)** Finland; **(Fra)** France; **(Gab)** Gabon; **(Ger)** Germany; **(Hun)** Hungary; **(Ice)** Iceland; **(In)** India; **(Ind)** Indonesia, **(Isr)** Israel; **(Ita)** Italy; **(Jap)** Japan; **(Jor)** Jordan; **(Lat)** Latvia; **(Leb)** Lebanon; **(Mal)** Malaysia; **(Mar)** Martinique; **(Net)** The Netherlands; **(New)** New Zealand; **(Nor)** Norway; **(Pol)** Poland; **(Rom)** Romania; **(SA)** South Africa; **(Swe)** Sweden; **(Slo)** Slovenia; **(Spa)** Spain; **(Swi),** Switzerland; **(Tai)** Taiwan; **(Tur)** Turkey; **(UK)** United Kingdom; **(USA)** United States of America; **(Zim)** Zimbabwe.

8.5 APPENDIX E: *spa* Type Repeat Motif Alignments

Table E1: The alignment of *spa* type repeat patterns within *spa*-CC 701 (Cluster 1)

spa type	Repeat motif			
t064	11-19-	12-05-	-17-34-24-	-34-22-25
t292	11-12-	- -	-17-34-24-	-34-22-25
t451	11-12-	05-	-17-34-24-	-34-22-25
t701	11-10-	21-	-17-34-24-	-34-22-25-25
t1257	11-19-34-05-		-17-34-24-	-34-22-25
t1443	11-19-	12-05-	-17-34-24-24-34-22-25	
t1476	11-10-		-17-34-24-	-34-22-25
t2360	11-10-	21-	-17-34-24-	-34-22-25-25-25
t5472	11-10-	- -	-17-34-24-	-34
t5473	11-19-	12-21-21-12-34-24-		-34-22-25
t5474	11-10-	21-	-17-34-24-	-34-22-25-25-25-25

Table E2: The alignment of *spa* type repeat patterns within *spa*-CC 012 (Cluster 2)

spa type	Repeat motif		
t012	15-12-	-16-	-02-16-02-25-17-24-24
t018	15-12-	-16-	-02-16-02-25-17-24-24-24
t021	15-12-	-16-	-02-16-02-25-17-24
t030	15-12-	-16-	-02- -24-24
t037	15-12-	-16-	-02- -25-17-24
t253	15-12-	-16-	-02-16-02-25-17-24-24-24-24
t275	15-12-	-16-	-02- -25-17-24-24
t318	15-12-	-16-16-02-16-02-25-17-24	
t433	15-12-	-16-16-02-16-02-25-17	
t1848	15-12-17-16-	-02-16-02-25-17	

Table E3: The alignment of *spa* type repeat patterns within *spa*-CC 002 (Cluster 3)

spa type	Repeat motif	
t002	26-23-17-34-17-20-17-12-17-	-16
t045	26- -17-20-17-12-17-	-16
t214	26-23-17-34-17-20-17-12-17-	-16-16
t306	26-23-17-34-17-20-17-12-17-17-16	
t311	26-23-17-34- -20-17-12-17-	-16

Table E4: The alignment of *spa* type repeat patterns within *spa*-CC 015 (Cluster 4)

spa type	Repeat motif
t015	08-16-02-16-34-13-17-34-16-34
t465	08-23- -16-34-13-17-34-16-34
t2623	08-16-02-16- -13-17-13-16-34
t4576	08-16-02-16-34- -34-16-13

Table E5: The alignment of *spa* type repeat patterns within *spa*-CC 174 (Cluster 5)

spa type	Repeat motif
t127	07-23-21-16-34-33-13
t174	14- -21-16-34-33-13
t5471	35- -21-16-34-33-13

Table E6: The alignment of *spa* type repeat patterns within *spa*-CC 346/085 (Cluster 6)

spa type	Repeat motif
t346	07-23-12- -34- -12-12-23-02-12-23
t085	07-23-12- -34-34-12- -23-02-12-23
t5396	07-23-12-13-23-12-34-34-12-12-23-02-12-23

Table E7: The alignment of *spa* type repeat patterns within Cluster 7, No founder

spa type	Repeat motif
t521	07-23-12-21-17-34-34-34-34-33-34
t267	07-23-12-21-17-34-34-34- -33-34

8.6 APPENDIX F: *spa* **Repeat Unit Alignments**

Table F1: Alignment of the nucleotide sequences of repeats r19 and r12

Repeat	Nucleotide sequence
r19	AAAGAAGACAA**T**AACAAGCCTGG**C**
r12	AAAGAAGACAA**C**AACAAGCCTGG**T**

Table F2: Alignment of the nucleotide sequences of repeats r19 and r10

Repeat	Nucleotide sequence
r19	AAAGAAGACAATAACAAGCCTGG**C**
r10	AAAGAAGACAATAACAAGCCTGG**T**

Table F3: Alignment of the nucleotide sequences of repeats r21 and r05

Repeat	Nucleotide sequence
r21	AAAGAAGACAACAA**C**AAGCCTGGC
r05	AAAGAAGACAACAA**A**AAGCCTGGC

Table F4: Alignment of the nucleotide sequences of repeats r21 and r12

Repeat	Nucleotide sequence
r21	AAAGAAGACAACAACAAGCCTGG**C**
r12	AAAGAAGACAACAACAAGCCTGG**T**

Table F5: Alignment of the nucleotide sequences of repeats r17 and r12

Repeat	Nucleotide sequence
r17	AAAGAAGAC**GG**CAACAAGCCTGGT
r12	AAAGAAGAC**AA**CAACAAGCCTGGT

Table F6: Alignment of the nucleotide sequences of repeats r16 and r23

Repeat	Nucleotide sequence
r16	AAAGAAGACGGCAACAAACCTGG**T**
r23	AAAGAAGACGGCAACAAACCTGG**C**

Table F7: Alignment of the nucleotide sequences of repeats r34 and r13

Repeat	Nucleotide sequence
r34	AAAGAAGACAACAA**A**AAACCTGGT
r13	AAAGAAGACAACAA**C**AAACCTGGT

Table F8: Alignment of the nucleotide sequences of repeats r14 and r07

Repeat	Nucleotide sequence
r14	GAGGAAGACAACAACAAACCTGG**C**
r07	GAGGAAGACAACAACAAACCTGG**T**

Table F9: Alignment of the nucleotide sequences of repeats r14 and r35

Repeat	Nucleotide sequence
r14	GAGGAAGACAACAA**C**AAACCTGGC
r35	GAGGAAGACAACAA**A**AAACCTGGC