The epidemiology and virulence characteristics of *Staphylococcus aureus* isolates from bacteraemic patients at Tygerberg Hospital, South Africa

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ABSTRACT

Introduction: *Staphylococcus aureus* is a versatile pathogen that produces multiple virulence factors which work together to establish and maintain infections. The accessory gene regulator (agr) locus is a quorum sensing two-component system which regulates at least 23 virulence factors. There are four different agr types, I-IV, and mutations within the agr locus may result in a dysfunctional agr. These can result in altered gene expression which may affect disease presentation and outcome. Data on the molecular epidemiology of *S. aureus* and its association with clinical outcome in South Africa is limited. This study aimed to determine the effect of epidemiology and agr-associated virulence characteristics on the clinical outcome of bacteraemic patients at Tygerberg Hospital.

Methods: *S. aureus* isolates were collected from blood cultures from February 2015 to March 2017. Genotyping was performed using staphylococcal protein A (*spa*) typing and multi-locus sequence typing (MLST); and staphylococcal cassette chromosome *mec* (SCC*mec*) typing was performed on all methicillin resistant *S. aureus* (MRSA) isolates. Agr typing was performed by PCR and agr functionality was assessed using a phenotypic δ-haemolysin assay and matrix assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS). Associations between patient- and strain- characteristics, and the final outcomes mortality, methicillin resistance and length of stay were investigated by means of regression models.

Results and discussion: Of the 199 *S. aureus* isolates collected, 27% were MRSA. Seventy three *spa* types were identified; reflecting a diverse population. MRSA isolates were more clonal than methicillin susceptible *S. aureus* (MSSA) isolates. A previously described novel variant SCC*mec* type (NV) and SCC*mec* type IV were most common among MRSA isolates. Agr type I was the dominant agr type, while agr type IV was least prevalent; consistent with the literature. The dominant clone in this study was an MRSA outbreak strain, t045-ST5-MRSA-NV, agr type II (*spa*-CC 002, CC5), which appears to be circulating in multiple hospital settings in South Africa. The most prevalent MSSA strain/clone was t318-ST1865, agr type III. Pandemic clones such as t037-ST239-MRSA-III, t032-ST22-MRSA-IV and t012-ST36-MRSA-II were also identified. A previously described association between MRSA and *spa*-CC 002 (CC5) was confirmed in this study; however this association may have been driven by the MRSA outbreak. Agr dysfunctionality was low at 12.6% and 6% using the phenotypic assay and MALDI-TOF MS respectively. Agr dysfunctionality was not clone specific and there was no difference in agr dysfunctionality between MRSA and MSSA isolates. A borderline association between agr dysfunction and shorter length of stay was identified, but needs further investigation. The overall mortality rate was 29% and older age was associated with increased mortality. Hospital acquired (HA) infections were also associated with a higher mortality, which could be explained by the complicated nature of these infections, leading to death. An association between HA infections and MRSA was identified, which is consistent with previous studies and not surprising considering antibiotic selective pressure is higher in hospitals.

Conclusion: This study provides insights into the associations between *S. aureus* epidemiology and agr related virulence characteristics and clinical outcome, despite the limited clinical data available.

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OPSOMMING

Inleiding: *Staphylococcus aureus* is 'n veelsydige patogeen wat verskeie virulensie-faktore produseer wat saamwerk om infeksies te veroorsaak en onderhou. Die geassosieerde geen reguleerder (agr) lokus is 'n dubbele-komponent kworumwaarnemingstelsel wat minstens 23 virulensie-faktore reguleer. Daar is vier verskillende agr tipes, I-IV. Mutasies in die agr lokus lei dikwels tot 'n disfunksionele agr wat daaropvolgend lei tot wisselende geen uitdrukking, wat die voorkoms en uitkoms van siektes kan beïnvloed. Data oor die molekulêre epidemiologie van *S. aureus* en die assosiasie daarvan met kliniese uitkoms in Suid-Afrika is beperk. Hierdie studie het gepoog om die effek van epidemiologiese- en agr-verwante virulensie eienskappe op die kliniese uitkoms van pasiënte met bakteremie by Tygerberg Hospitaal te bepaal.

Metodes: *S. aureus* isolate van bloedkulture was vanaf Februarie 2015 tot Maart 2017 versamel. Genotipering was uitgevoer met behulp van stafilokokkale proteïn A (*spa*) tipering en multi-lokus volgordebepaling (MLST). "*Staphylococcal chromosome complex mec"* (SCC*mec*) tipering was op alle metisillienweerstandige *Staphylococcus aureus* (MRSA) isolate uitgevoer. Agr tipering was deur polimerasekettingreaksie (PKR) uitgevoer en agr funksionaliteit was met behulp van 'n fenotipiese δ-hemolisien toets en "*matrix assisted laser desorption ionisation-time of flight mass spectrometry*" (MALDI-TOF MS) ondersoek. Assosiasies tussen pasiënt- en stam-eienskappe met die finale uitkomstes, mortaliteit, metisillienweerstandigheid en lengte van hospitaalverblyf, was ondersoek.

Resultate en bespreking: Sewe-en-twintig persent van die 199 *S. aureus*-isolate wat ingesamel is, was metisillienweerstandig. Drie-en-sewentig *spa*-tipes is geïdentifiseer; wat 'n diverse bevolking weerspieël. MRSA-isolate was meer klonaal as metisillien-vatbare *S. aureus* (MSSA) isolate. 'n Nuwe variant SCC*mec*-tipe (NV), voorheen beskryf, en SCC*mec*-tipe IV was die algemeenste tipe in die MRSA groep. Agr tipe I was die dominante agr tipe, terwyl agr tipe IV die minste voorgekom het; dit stem ooreen met wat in literatuur beskryf word. Die dominante kloon in hierdie studie was 'n MRSA-uitbraakstam, t045-ST5-MRSA-NV, agr tipe II (*spa*-CC 002, CC5), wat in verskeie hospitale in Suid-Afrika voorkom. Die algemeenste MSSA kloon was t318-ST1865, agr tipe III. Pandemiese klone soos t037-ST239-MRSA-III, t032-ST22-MRSA-IV en t012-ST36-MRSA-II is ook geïdentifiseer. 'n Voorheen beskryfde assosiasie tussen MRSA en *spa*-CC 002 (CC5) is in hierdie studie bevestig; dit is egter moontlik dat dit deur die MRSA-uitbraak gedryf kon word. Agr disfunksie was laag, 12.6% en 6%, soos bepaal deur die fenotipiese δ-hemolisien toets en MALDI-TOF MS onderskeidelik. Agr disfunksionaliteit was nie kloon spesifiek nie en daar was geen verskil in agr disfunksionaliteit tussen MRSA en MSSA isolate nie. Die assosiasie tussen agr disfunksie en 'n korter hospitaalverblyf het gegrens aan statistiese betekenisvolheid, maar dit benodig verdere ondersoek. Die totale sterftesyfer was 29% en ouer ouderdom is geassosieer met verhoogde sterftes. Hospitaalverworwe (HA) infeksies is ook geassosieer met 'n hoër sterftesyfer, wat verklaar kan word deur die ingewikkelde aard van hierdie infeksies wat tot die dood lei. 'n Assosiasie tussen HA-infeksies en MRSA is geïdentifiseer; dit stem ooreen met vorige studies en is nie verbasend nie, aangesien antibiotika-selektiewe-druk in hospitale hoër is.

Gevolgtrekking: Hierdie studie bied insig oor die assosiasies tussen *S. aureus* epidemiologie en agr-verwante virulensie eienskappe en kliniese uitkoms, ten spyte van die beperkte kliniese data wat beskikbaar was.

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If I have seen further it is by standing on the shoulders of giants. - Isaac Newton

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LIST OF ABBREVIATIONS

agr	accessory gene regulator
AIP	autoinducing peptide
ATCC	American Type Culture Collection
bp	base pair
CA	community acquired
CC	clonal complex
ccr	cassette chromosome recombinase
CHCA	α-cyano-4-hydroxycinnamic acid
Clf	clumping factor
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ETA, ETB	exfoliative toxins A and B
FnbpA, FnbpB	fibronectin-binding proteins A and B
GERMS-SA	Group for Enteric, Respiratory and Meningeal Disease Surveillance in South Africa
HA	hospital acquired
HCA	health-care associated
hla, hlb, hld	α-, β-, δ-haemolysin
HR	hazards ratio
HREC	Health Research Ethics Committee
IG	immunoglobulin
IQR	interquartile range
IS	insertion sequence
J region	joining region
MEGA	Molecular Evolutionary Genetic Analysis
MALDI-TOF MS	Matrix Assisted Laser Desorption Ionisation Time of Flight Mass Spectrometry

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MGE	mobile genetic element
MLST	multilocus sequence typing
MLVA	multilocus variable tandem repeat analysis
MRSA	methicillin resistant *Staphylococcus aureus*
CA-MRSA	community acquired methicillin resistant *Staphylococcus aureus*
HA-MRSA	hospital acquired methicillin resistant *Staphylococcus aureus*
MSCRAMM	microbial surface component recognizing adhesive matrix molecules
MSSA	methicillin susceptible *Staphylococcus aureus*
NHLS	National Health Laboratory Services
NT	non-typeable
NV	novel variant
OR	odds ratio
ORF	open reading frame
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
PMF	peptide mass fingerprint
PSM	phenol soluble modulins
PTSAgs	pyrogenic toxin superantigens
PVL	Panton-Valentine leukocidin
rep-PCR	repetitive element palindromic PCR
S. aureus	*Staphylococcus aureus*
SAASP	South African Antibiotic Stewardship Program
SCC*mec*	staphylococcal cassette chromosome *mec*
SNP	Single nucleotide polymorphism
spa	staphylococcal protein A
SSSS	staphylococcal scalded skin syndrome
ST	sequence type
TH	Thomson
TSST-1	toxic shock syndrome toxin-1

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VNTR	variable number tandem repeat
WGS	whole genome sequencing
WHO	World Health Organisation
WT	wild type

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CHAPTER 1: Literature review

Staphylococcus aureus

Staphylococcus aureus is a Gram positive, non-motile, coccus shaped bacterium, which often forms clusters. *S. aureus* is an extremely versatile pathogen, responsible for a wide range of superficial infections (e.g. wound infections), deep-seated infections (e.g. pneumonia) and toxaemic syndromes such as staphylococcal scarlet fever and toxic shock syndrome (Ben Ayed *et al.*, 2008).

Asymptomatic colonization of the nasopharynx, perineum or skin of human hosts, by *S. aureus,* is far more common than infection and often occurs shortly after birth and may re-occur at any time thereafter (Chambers, 2001). *S. aureus* is transferred by direct contact and therefore family members and contacts of colonised individuals may become colonised. Colonization may be transient or persistent and may last for years (Chambers, 2001). *S. aureus* carriage rates are between 25% and 50%, with higher rates observed in injection drug users, individuals with insulin-independent diabetes, health care workers, patients with dermatologic conditions, as well as patients with long-term indwelling intravascular catheters. Children tend to have higher colonization rates than adults because of their repeated person-to-person interaction and their recurrent contact with respiratory secretions (Adcock *et al.*, 1998).

S. aureus is a frequent hospital-acquired (HA) pathogen that is commonly isolated from blood cultures. Before antibiotics were available for the treatment of *S. aureus* bacteraemia the mortality rate was as high as 82%-98% and the introduction of penicillin in the early 1940's had a great impact on treatment outcome (Stefani *et al.*, 2012). In 1959, methicillin was introduced to overcome problems caused by strains resistant to penicillin G and penicillin V (Enright *et al.*, 2000). However, methicillin resistant *S. aureus* (MRSA) strains quickly developed, causing the fatality rate to remain high (15-50%) and resulting in major problems for hospitals world-wide (Speller *et al.*, 1997; Perovic *et al.*, 2015). The first methicillin resistant strain was reported in 1961 in the United Kingdom and MRSA strains were soon reported in European countries followed by Japan, Australia and the United States. MRSA is now a worldwide problem and is increasingly isolated from hospitals and the community (Voss and Doebbeling, 1995; Green *et al.*, 2012; Earls *et al.*, 2017). Methicillin resistance is acquired by the insertion of the staphylococcal cassette chromosome *mec* (SCC*mec*) element into the chromosome of antibiotic susceptible strains (Hiramatsu *et al.*, 2001). The SCC*mec* element carries the gene responsible for resistance, namely *mecA,* encoding for a methicillin-resistant penicillin-binding protein that is absent in methicillin susceptible strains (Enright *et al.*, 2002). Although the SCC*mec* element is widely dispersed and exclusively found in staphylococci, the exact origin of the element is unknown (Hiramatsu *et al.*, 2001).

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Infections can be classified as hospital acquired (HA), health care associated (HCA) or community acquired (CA), depending on the source of the infection. HA-MRSA and HCA-MRSA isolates are often isolated from immunocompromised patients and are resistant to multiple antimicrobials, while CA-MRSA isolates used to be associated with colonization and were usually susceptible to other antimicrobials (Earls *et al.*, 2017). These distinctions between the groups have however become blurred (Earls *et al.*, 2017). Recent studies have shown that MRSA strains from multiple different clinical sources are often resistant to many other classes of antibiotics and show decreased susceptibility to glycopeptides, which threatens the ability to treat infections caused by MRSA strains (Enright *et al.*, 2000; Earls *et al.*, 2017).

Molecular epidemiology of *Staphylococcus aureus*

Strain typing methods

Many epidemiological studies have used various strain typing methods to describe the distribution of different *S. aureus* strains within and between settings and to investigate the cross-border differences in molecular epidemiology at a global level (Stefani *et al.*, 2012; Abdulgader *et al.*, 2015). There are multiple different bacterial strain typing methods available, including phenotypic methods, such as serotyping, phage typing and antibiotic resistance profiles, and genotypic based methods. Appropriate strain typing technique should include the following (Stefani *et al.*, 2012):

- portable data, highly reproducible and unambiguous;
- inter- and intra-laboratory compatibility;
- low-cost methodology;
- rapid throughput;
- easy processing, storage and exchange/distribution of data;
- standardised nomenclature recognised internationally;
- quality control of ‘raw’ typing data (external quality control);
- flexibility of use for a variety of microorganisms

The methods which are frequently used for *S. aureus* strain typing include *spa* typing, pulsed field gel electrophoresis (PFGE), multilocus sequence typing (MLST), repetitive element palindromic PCR (rep-PCR), multilocus variable tandem repeat analysis (MLVA) and whole genome sequencing (WGS); as well as staphylococcal chromosomal cassette *mec* (SCC*mec*) typing for MRSA strains (Struelens *et al.*, 2009). Although none of these strain typing methods meet all the requirements listed above; *spa* typing, MLST and SCC*mec* typing are generally preferred over PFGE and MLVA (Stefani *et al.*, 2012). The reason for this may be that PFGE and MLVA have limited portability and no standard nomenclature. Table 1.1 describes the strengths and limitations of strain typing methods used for *S. aureus*.

Table 1.1: Features of frequently used *S. aureus* strain typing methods. (Adapted from Ross *et al*., 2005; Struelens *et al.,* 2009; Stefani *et al.,* 2012).

Method	Principle	Strengths	Limitations
***spa* typing**	Amplification and sequencing of the hypervariable X region of the *spa* gene for *S. aureus* surface protein A	Rapid, standard nomenclature, portable, high throughput	Misclassification of a small number of lineages because of homoplasy
PFGE	Restriction polymorphisms of the whole genome	High discriminatory index	Laborious, slow, limited portability, misclassification of some lineages, multiple nomenclatures
MLST	Core genetic population (amplification and sequencing of seven housekeeping genes)	Defines core genetic population, standard nomenclature, portable	Low throughput, high cost
rep-PCR	Amplification of the regions between the non-coding repetitive sequences in the genome	High discriminatory index, rapid	poor inter-laboratory reproducibility, misclassification of some lineages, no standard nomenclature
MLVA	Polymorphisms in chromosomal VNTR elements	High throughput, rapid	No standard protocol or nomenclature, misclassification of some lineages
WGS	Sequencing of the entire genome	Reproducible, high discriminatory index	Expensive, complex sample preparation, interpretation of results is difficult
SCC*mec* typing	MGEs	Standard nomenclature	Low throughput, high cost, evolving nomenclature

*variable number tandem repeat (VNTR), mobile genetic elements (MGEs)

spa typing

spa typing involves PCR amplification and determination of the sequence polymorphisms in the hypervariable region of the *spa* gene, specifically the polymorphic X region; and is currently one of the most popular *S. aureus* typing methods (Hallin *et al.,* 2009; Struelens *et al.,* 2009). The *spa* gene encodes protein A, which is a cell-wall component that is bound by its carboxy-terminal to the peptidoglycan of *S. aureus* (Hallin *et al.*, 2009). The N-terminal of the *spa* gene encodes four to five binding units for immunoglobulin G (IgG), while the X region is on the C-terminal and displays a variable number of short repeat units (24bp) flanked by well-conserved regions (Hallin *et al.*, 2009). Figure 1.1 features the C-terminal, which is divided into two regions; the constant Xc region that encodes for cell-wall attachment and the hypervariable Xr region that displays the variable number tandem repeats (VNTR). The Xr region is amplified during *spa* typing and the strain type is characterised based on the VNTR succession (Hallin *et al.*, 2009). The nomenclature for this method is as follows: for each new base composition within the polymorphic region the strain is assigned a unique repeat code and each n-long repeat code corresponds to a repeat succession. A repeat succession is allocated to each strain, which determines the *spa* type of the strain. A “type” number preceded by a “t” is assigned to every repeat profile. (Source: Harmsen *et al.,* 2003).

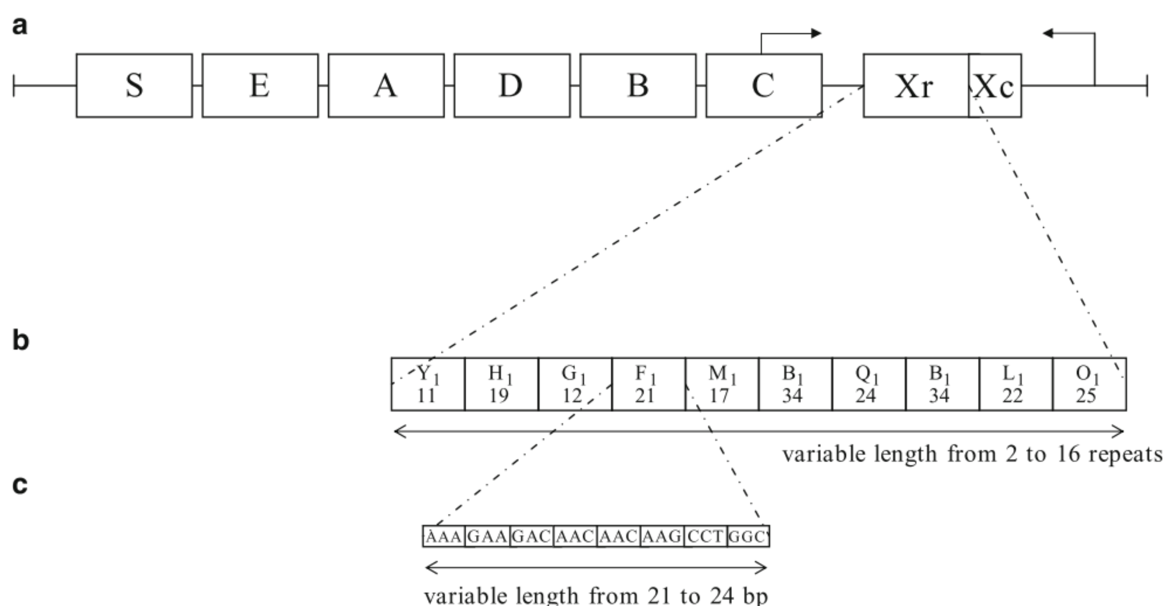

Figure 1.1: The *spa* typing principle. a) Map of the *spa* gene. X represents the C-terminal and is divided into two regions; the constant Xc region encoding for cell-wall attachment and the hypervariable (VNTR) Xr region, which is amplified and used for *spa* typing. b) Xr region repeat structure. c) The DNA sequence of *spa* repeat 21 (Ridom). (Source: Hallin *et al.,* 2009).

spa typing has many practical advantages which may be the reason for its popularity. These advantages include high throughput, reproducibility and portability of data; and isolates are assigned *spa* types through the internet, which enables access to an international database and allows comparison on a worldwide scale (Struelens *et al.*, 2009). The Ridom *spa* typing server (https://www.spaserver.ridom.de) provides a standard universally recognized nomenclature as well

as integral quality control for *spa* typing. The Ridom StaphType software (Ridom GmbH, Germany) also allows related *spa* types to be clustered into clonal complexes (CCs) using the based upon repeat patterns (BURP) algorithm; these *spa*-CCs show good congruency with MLST-CCs.

The combination of *spa* typing with the based upon repeat pattern (BURP) analysis provides a strong epidemiological typing tool that is highly reproducible, and may be useful for both national and international surveillance of *S. aureus* strains. However, this single-locus-based method should be accompanied by other typing methods as well as detection of other markers in parallel to *spa* typing. This may increase the cost and time of the analysis, therefore, these markers should be selected based on the questions in need of answering and results from the *spa* typing-BURP analysis (Strommenger *et al.*, 2008).

Pulsed field gel electrophoresis (PFGE)

Pulsed field gel electrophoresis (PFGE) involves the genomic macrorestriction of fragments separated by pulsed-field gel electrophoresis and has been used for outbreak investigation as well as surveillance at national and regional levels (Struelens *et al.*, 2009). PFGE is highly discriminatory and many studies have been published with validated interpretation criteria and harmonised protocols for the investigation of regional transmission and outbreak investigation (Blanc *et al.*, 2001; Murchan *et al.*, 2003). Yet, it is a laborious and low-throughput method and multiple technical difficulties in achieving inter-laboratory comparability and standardization have been identified, resulting in limited portability (Struelens *et al.*, 2009). Furthermore, some strains (such as ST398) are un-typeable because of restriction site methylation (Struelens *et al.*, 2009).

Multi-locus sequence typing (MLST)

Multi-locus sequence typing (MLST) characterizes strains based on the sequences of approximately 450 base pairs (bp) of internal fragments of seven housekeeping genes (*arc, aroE, glpF, gmk, pta, tpi, yqiL)*. An allele number is assigned to the sequences of each of the seven gene fragments, and each combination of allele numbers represents a unique sequence type (ST) (Enright *et al.*, 2000). STs are then grouped into clonal complexes (CCs) based on the similarity of their allelic profiles. MLST is highly discriminatory, the typing results are easily comparable between laboratories, and the ability to compare results on the internet is a major advantage (Enright *et al.*, 2002). However, for epidemiological typing, MLST remains too labour intensive and expensive to use as the primary strain typing tool (Struelens *et al.*, 2009).

Other strain typing methods

Other high throughput typing methods such as rep-PCR and multilocus variable tandem repeat analysis (MLVA) also exist. Rep-PCR varies in terms of discriminatory power, but it has been shown to be poorly reproducible when using protocols that are not kit-based (Struelens *et al.*, 2009). A study

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performed by Ross *et al.* in 2005 compared rep-PCR to PFGE for outbreak investigation. A kit-based rep-PCR assay (by Diversilab, Houston, TX, USA) showed outstanding reproducibility but moderate discriminatory power in comparison to PFGE. An advantage to rep-PCR is it's low running cost (assuming the lab has the necessary equipment), ease of use and real time results, however interpretation guidelines and standard nomenclature are still lacking (Ross *et al.*, 2005). MLVA, like rep-PCR, has a rapid turnaround time, high throughput, and variable discriminatory power however, to our knowledge, no international nomenclature or standard protocol is available (Stefani *et al.*, 2012).

Lastly, whole genome sequencing (WGS), including single nucleotide polymorphism (SNP) analysis, is an alternative to the typing techniques mentioned above. WGS is becoming the gold standard because of its high discrimination compared to the above mentioned molecular typing methods, but it is too expensive to use as a primary typing tool (Earls *et al.*, 2017). It is however over time becoming more available and affordable. The typing techniques discussed allow the identification of multiple *S. aureus* strains and CCs and as additional sequence variants are identified in these *S. aureus* strains a standard hierarchical SNP catalogue can be developed and used for high throughput SNP typing (Struelens *et al.*, 2009).

High concordance between *spa* typing and PFGE has been reported (Hallin *et al.* 2007). The majority of the inconsistencies were found in MLST CC8 and CC5, in which a number of MRSA isolates appeared to be sporadic by PFGE, but were shown to be more clonal by *spa* typing (t008/CC8 and t002/CC5). This may be due to the nature of the typing methods; *spa* typing being a single locus and PFGE being a whole genome typing method and has a higher discriminatory power than *spa* typing. Genetic events such as insertions and/or deletions of mobile genetic elements which occur across the genome may result in diverse PFGE patterns, while the targets for *spa* typing and MLST remain unaffected. It has been concluded that *spa* typing can be used for the same purposes as PFGE, however, additional typing schemes should be used for resistant strains such as SCC*mec* typing or further investigating markers such as resistance or virulence genes; especially in rapidly evolving strains (such as strains from *spa*-CC5 and *spa*-CC8) (Hallin *et al.,* 2007). It is now common to define clones by the combination of their chromosomal background by different typing methods, for example *spa* type in combination with MLST ST (such as t00x-STy) and in the case of MRSA isolates, the SCC*mec* type is added (such as t00x-STy-MRSA-z).

Staphylococcal chromosomal cassette mec typing (SCCmec typing)

The SCC*mec* element is a mobile genetic element composed of the *mec* gene complex encoding methicillin resistance and the *ccr* gene complex encoding recombinases responsible for the mobility of the element (Hiramatsu *et al.*, 2001). SCC*mec* elements have been grouped into thirteen types defined by the combination of *mec* and *ccr* gene complex types (Table 1.2) (Ito *et al.*, 2009; Martínez-Meléndez *et al.*, 2015; Baig *et al.*, 2018). SCC*mec* typing can assist with surveillance of transmission

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and evolution of MRSA strains between hospitals and even internationally (Struelens *et al*., 2009). There are several methods available for typing and subtyping of MRSA, either by PCR mapping of the cassette elements or by sequence determination of *ccrB* (Struelens *et al*., 2009). A combination of these two methods is recommended for reliable MRSA typing.

Table 1.2: Currently identified SCC*mec* types. (Adapted from Ito *et al.,* 2009; Martínez-Meléndez *et al.,* 2015; Baig *et al.,* 2018)**.**

SCC*mec* type	*ccr* gene complex	*mec* gene complex
I	1 (A1B1)	B
II	2 (A2B2)	A
III	3 (A3B3)	A
IV	2 (A2B2)	B
V	5 (C1)	C2
VI	4 (A4B4)	B
VII	5 (C1)	C1
VIII	4 (A4B4)	A
IX	1 (A1B1)	C2
X	7 (A1B6)	C1
XI	8(A1B3)	E
XII	9 (C2)	C2
XIII	9 (C2)	A

The *mec* gene complex consists of the *mecA* gene which encodes a methicillin-resistant penicillin-binding protein, *mecA* regulatory genes (*mecR1* and *mecI*), as well as associated insertion sequences (IS) (Figure 1.2). There are five different *mec* classes, classes A-E (Table 1.3). Class A *mec* is the prototype complex and consists of *mecA*, the regulatory genes (complete *mecR1* and *mecI*) upstream of *mecA*, the hypervariable region (HVR) and the insertion sequence IS431 downstream of *mecA* (Katayama *et al*., 2001). The class B *mec* gene complex contains the *mecA* gene and a truncated *mecR1* (*ΔmecR1*) (as a result of IS1272) upstream of the *mecA* while the HVR and IS431 insertion sequence are downstream of *mecA* (Ito *et al*., 2009). The class C *mec* gene complex contains *mecA* and *ΔmecR1* (as a result of IS431) upstream of *mecA*; another IS431 insertion sequence and HVR are present downstream of *mecA* (Ito *et al*., 2009). There are two different class C *mec* gene complexes; in the first (class C1 *mec* gene complex), the two IS431 insertion sequences upstream and downstream of *mecA* have the same orientation, while in the

second (class C2 *mec* gene complex), the orientation of IS431 upstream of *mecA* is reversed (Katayama *et al*., 2001). The two class C2 *mec* complexes are likely to have evolved independently and are therefore regarded as two different *mec* complexes. The class D *mec* gene complex contains *mecA*, *ΔmecR1* and no insertion sequence downstream of *ΔmecR1* (Ito *et al.,* 2009). Class E is similar to class D, but contains a greater degree of deletion in *ΔmecR1* (Martínez-Meléndez *et al*., 2015). Variants within the major classes have been identified and described; these include insertions of IS431 and/or IS1182 upstream of the *mec* gene in the class A *mec* gene complex as well as insertion of Tn4001 upstream of the *mecA* gene in the class B *mec* gene complex. These variants have been designated a number following the class for example the class B *mec* gene complex variant is called the class B_2 *mec* gene complex (Ito *et al*., 2009).

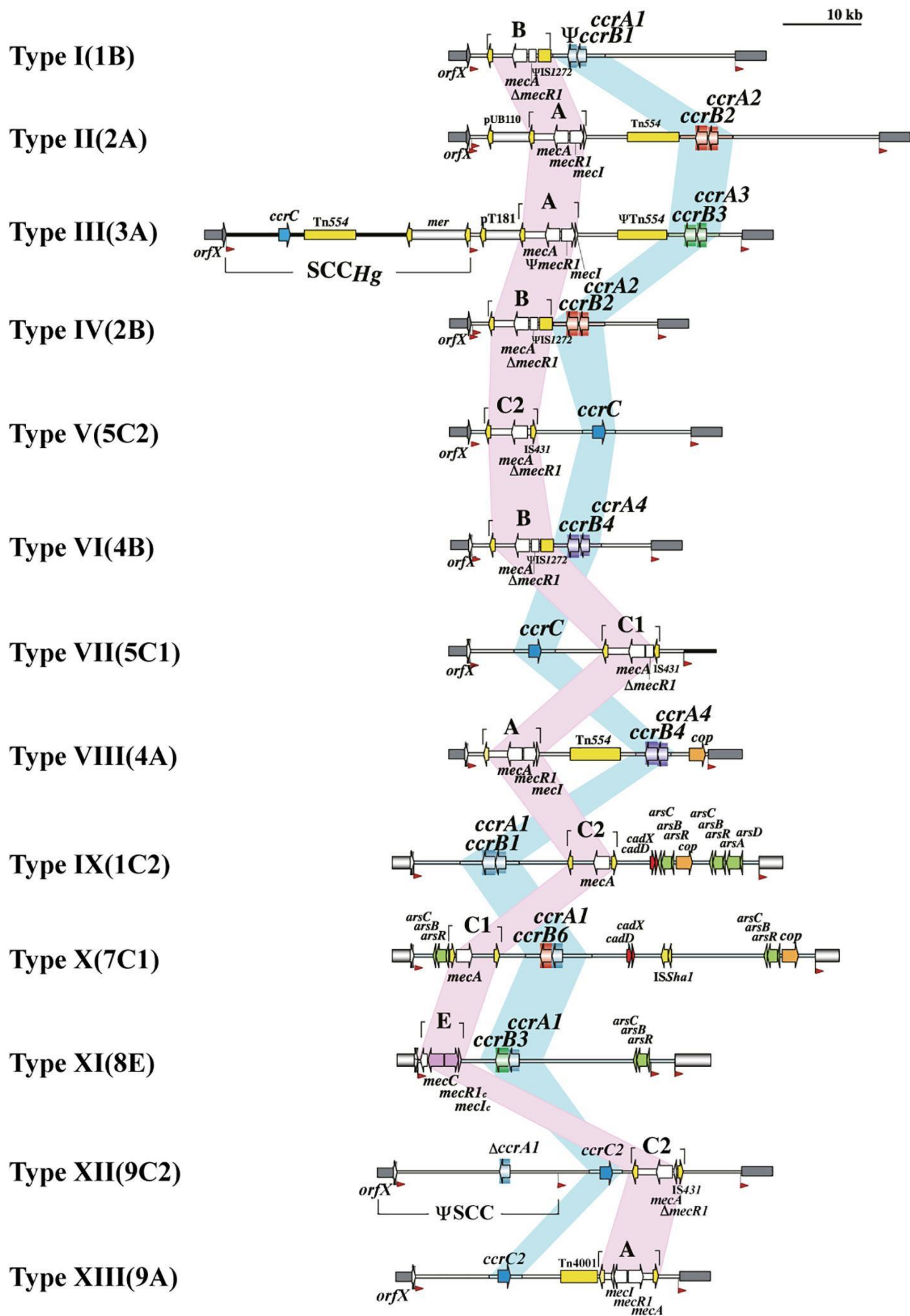

Figure 1.2: The basic structure of the SCC*mec* elements of SCC*mec* types I-XIII. (Source: Baig *et al.,* 2018).

Table 1.3: Structure of *mec* classes A-E. (Adapted from Martines-Malendez *et al.,* 2015).

***mec* class**	***mec* structure**
A	*mecI–mecR1–mecA*–IS*431*
B	IS1272–Δ*mecR1–mecA*–IS*431*
C	IS431–Δ*mecR1–mecA*–IS*431*
D	Δ*mecR1–mecA*–IS*431*
E	Δ*mecR1–mecA*–IS*431*

The *ccr* gene complex consists of a combination of three *ccr* gene types identified in *S. aureus*, namely *ccrA*, *ccrB* and *ccrC,* and the surrounding open reading frames (ORFs). The three different *ccr* genes have DNA sequence similarities of less than 50% and four allotypes have been identified for the *ccrA* and *ccrB* genes (Ito *et al.*, 2009). There is only one *ccrC* allotype since all of the *ccrC* genes share greater than 87% similarity (Figure 1.3) (Ito *et al.*, 2009).

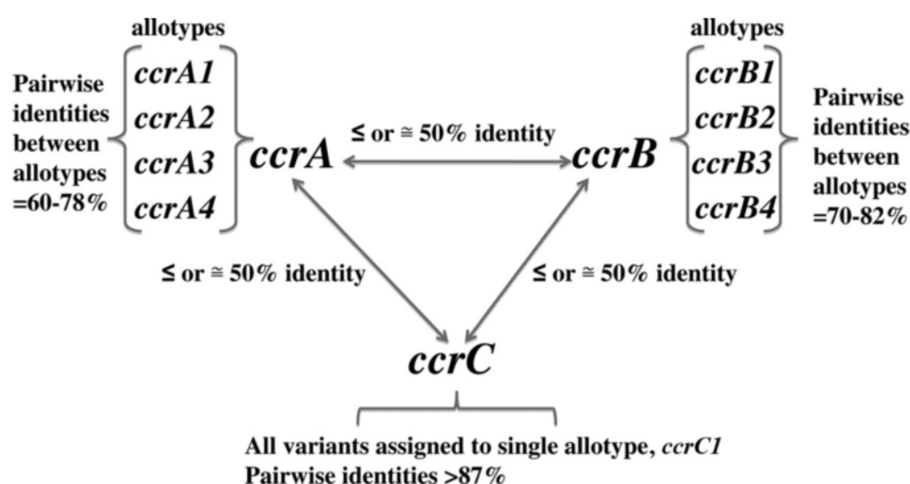

Figure 1.3: Schematic representation of the *ccr* genes, the DNA sequence similarity between them and the different allotypes present within each *ccr* gene type. (Source: Ito *et al.*, 2009).

Epidemiology of *Staphylococcus aureus*

The majority (88%) of MSSA isolates have been shown to cluster into eleven MLST CCs, namely CC1, CC5, CC8, CC9, CC12, CC15, CC22, CC25, CC30, CC45, and CC51/121, with CC30 being the dominant MSSA CC (Enright *et al.*, 2000, 2002; Hallin *et al.*, 2007; Chambers and DeLeo, 2009; Breurec *et al.*, 2011). MRSA, however, has been shown to be more clonal. Five major CCs, namely CC5, CC8, CC22, CC30 and CC45, have been defined as the dominant clones among HA-MRSA isolates from all continents (Stefani *et al.*, 2012). CC8 and CC30 are pandemic lineages and are, together with CC1 and CC80, also associated with CA-MRSA (Abdulgader *et al.*, 2015).

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Regional clones have also been described in many countries or regions, and include ST93 in Australia (Chua *et al.*, 2011), ST612 in South Africa and Australia (Moodley *et al.*, 2010), ST72 in South Korea (Kim *et al.*, 2014), ST88 in Africa and Asia (Otto and Chatterjee, 2013) and ST772 in India (Shambat *et al.*, 2012).

In Africa STs from CC5 have been shown to be most prevalent among MRSA isolates and ST239 (Brazillian/Hungarian clone) has been described in nine African countries (Abdulgader *et al.*, 2015). ST22-MRSA-IV, ST612-IV and ST36-MRSA-II were limited to South Africa (Moodley *et al.*, 2010; Jansen van Rensburg *et al.*, 2011; Oosthuysen *et al.*, 2013), while the European clone ST80-MRSA-IV was only reported in North African countries; Algeria, Egypt and Tunisia (Ramdani-bouguessa *et al.*, 2006; Enany *et al.*, 2010; Abdulgader *et al.*, 2015). CA-MRSA clones ST8 and ST88 have also frequently been reported in Africa (Abdulgader *et al.*, 2015).

Epidemiology of *Staphylococcus aureus* in South Africa

The epidemiology of *S. aureus* in South Africa has been investigated in six studies, in four provinces, between 2001 and 2012 (Table 1.4).

Two studies described the epidemiology of *S. aureus* from multiple clinical sources in KwaZulu-Natal between January 2001 and August 2003. ST239 and ST5 were among the MRSA clones detected in both studies. Specifically, the MRSA clones circulating in 16 hospitals during January 2001 and December 2002 were ST8-MRSA-IV, ST239-MRSA-III, ST8-MRSA-II, ST5-MRSA-IV and ST45-MRSA-IV (Essa *et al.*, 2009). While, MRSA strain types t064-ST1173-MRSA-IV, t064-ST1338-MRSA-IV, t037-ST239-MRSA-III and t045-ST5-MRSA-III were dominant in 13 health-care institutions during March 2001 to August 2003 (Shittu *et al.*, 2009). The difference in strain types between the two studies may be attributed to the geographical origin of the samples and the fact that the samples were obtained from different clinical specimens, however this was not elaborated on in these studies. Only one of the two studies investigated both MSSA and MRSA isolates and reported MSSA strains to be more diverse compared to the MRSA strains, with ST1, ST5, ST8, ST9 and ST88 being the dominant STs among the MSSA isolates (Essa *et al.*, 2009). Despite the differences in clones detected in the two studies, SCC*mec* type IV was the most prevalent *SCCmec* type among MRSA isolates in both studies. This may be due to the fact that SCC*mec* type IV is a smaller and potentially more mobile SCC*mec* type compared to other SCC*mec* types.

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Table 1.4: Distribution of *S. aureus* strain types in South Africa.

Province	Collection period	Clinical source	*spa* type	*spa*-CC	MLST ST	MLST CC	SCC*mec* type	agr type	Reference
KwaZulu-Natal	2001-2002	Skin & soft tissue, otitis media, surgical site, septicaemia, other	-	-	ST8, ST239, ST5, ST45, ST1, ST9, ST22, ST30, ST88	-	IV, III, II	-	Essa *et al.*, 2009
KwaZulu-Natal	2001-2003	Wound, sputum, otitis media, blood, urine, eye, endotracheal aspirate	t064, t037, t045	-	ST1173, ST1338, ST239, ST5	-	IV, III, IIIa, II, IIIb, I	-	Shittu *et al.*, 2009
Western Cape	2007-2008	Pus, pus swabs, respiratory tract specimens, urine, central venous catheter tips, blood	t045, t037, t1443, t2196, t1143, t012, t064	-	ST612, ST5, ST239	CC8, CC5	IV, I, II, III	-	Jansen van Rensburg *et al.*, 2011
Western Cape	2008-2009	Blood cultures	t037, t891, t1257, t002, t015, t021	CC012, CC701, CC002, CC015	ST239, ST612	CC8, CC30, CC45, CC5	IV, III, II, V	-	Orth *et al.*, 2013

Province	**Collection period**	**Clinical source**	***spa* type**	***spa*-CC**	**MLST ST**	**MLST CC**	**SCC*mec* type**	**agr type**	**Reference**
Western Cape	2009-2010	Skin & soft tissue, bone & joint, respiratory tract, prosthetic device, eye, urinary tract, unknown, other	t891	CC891, CC021, CC015, CC064	ST22, ST1865, ST45, ST36, ST612, ST8, ST1862, ST239	CC22, CC30, CC8, CC45, CC5, CC15	IV, I, II, III, V	I, III, II, IV	Oosthuysen *et al.,* 2013
Gauteng, Western Cape, Free State, KwaZulu-Natal	2010-2012	Blood cultures	t037, t1257, t045, t064, t012	CC064, CC037/012, CC045	ST239,ST612, ST5, ST36	CC8, CC5, CC30	III, IV	-	Perovic *et al.,* 2015

**spa* (staphylococcal protein A), CC (clonal complex), MLST (multi-locus sequence type), ST (sequence types), SCC*mec* (staphylococcal cassette chromosome *mec*), agr (accessory gene regulator).

Three studies described the epidemiology of *S. aureus* in the Western Cape between 2007 and 2010; two studies were performed on isolates from Tygerberg Hospital while the other investigated isolates from five hospitals in Cape Town. ST239 and ST612 were present in all three these studies. Among isolates collected from January 2007 to December 2008 from five hospitals in the Cape Town (Western Cape), the dominant SCC*mec* type among the isolates was SCC*mec* type IV followed by I, II, III. Four clones were identified and accounted for 92% of the isolates, namely, ST612-MRSA-IV (multiple *spa* types including t064, t1443, t1257 and t2196), t045-ST5-MRSA-I, t012/t021-ST34-MRSA-II and t037-ST239-MRSA-III. ST612 has infrequently been reported in South Africa and Australia, which suggests that it is an old local clone that has undergone clonal expansion over time. t002-ST650-MRSA-IV, t032-ST22-MRSA-IV and t3092-ST72-MRSA-NT were among the PFGE sporadic isolates (Jansen van Rensburg *et al.*, 2011). Furthermore, at Tygerberg Hospital, Cape Town (Western Cape), 113 isolates were collected from blood cultures from April 2008 to May 2009 and 30% were MRSA isolates (Orth *et al.*, 2013). The dominant *spa* types were t037 and t891, and all the *spa* types clustered into four major *spa*-CCs (*spa*-CC701, *spa*-CC012, *spa*-CC002, *spa*-CC015). Majority of the MRSA isolates clustered into *spa*-CC012 (53%) and *spa*-CC701 (43%), while MSSA strains showed more diversity, spanning all *spa*-CCs. SCC*mec* typing showed that SCC*mec* IV is the dominant type followed by SCC*mec* III, II and V. ST239 (CC5) and ST612 (CC5) were the dominant STs. CC8, CC30, CC45, CC5 were amongst the CCs observed and form part of the major CCs found worldwide (Stefani *et al.*, 2012). Similar results were reported by another study performed at Tygerberg Hospital (Oosthuysen *et al.*, 2013). Altogether 367 *S. aureus* were collected over a one year period in 2009-2010 and 15,3% were MRSA. The dominant *spa* type was t891 (*spa*-CC891) and SCC*mec* IV was, once again, the most prevalent SCC*mec* type. The MRSA and MSSA isolates clustered into different clones with the exception of ST612 which contained an MSSA isolate. In addition to the strain typing methods, agr typing was also performed and the most prevalent agr type among the isolates was agr type I, accounting for more than half of the isolates. In both the studies performed at Tygerberg Hospital the dominant clone among the MRSA isolates was ST612-MRSA-IV (in concordance with Jansen van Rensburg *et al.,* 2011) and common worldwide epidemic MRSA clones, namely ST239-MRSA-III and ST36-MRSA-II, were also identified (Oosthuysen *et al.*, 2013; Orth *et al.*, 2013).

A multicentre study investigated 2709 isolates from bacteraemic patients in four different provinces (Western Cape, Free State, Gauteng and KwaZulu-Natal), collected between June 2010 and July 2012. The prevalence of MRSA was 46%. The five dominant *spa* types and their corresponding ST were as follow; t037-ST239 (CC8), t1257-ST612 (CC8), t045-ST5 (CC5), t064-ST612 (CC8) and t012-ST36 (CC30). The strain t037-ST238 was related to HA infections while t1257-ST612 was related to CA infections. The dominant SCC*mec* type was type III followed by type IV and are associated with HA and CA infections, respectively (Perovic *et al.,* 2015).

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To conclude, in South Africa a diverse *S. aureus* population has been described, with t037-ST239 (CC8), t1257-ST612 (CC8) and t045-ST5 (CC5) being the most prevalent strains. The MRSA rate in South Africa is high and majority of the MRSA isolates belong to SCC*mec* types IV and III. The high prevalence of SCC*mec* IV is alarming since it has been associated with CA infections.

Staphylococcus aureus virulence

S. aureus has the ability to adhere to epithelial surfaces, invade, evade immune responses and secrete harmful proteins that contribute to its ability to cause a wide range of infections. *S. aureus* produces multiple virulence factors that work together to establish and maintain an infection. The pathogenicity of *S. aureus* is extremely complex and involves the expression of a diverse range of cell wall associated virulence factors as well as extracellular proteins at different stages of infection (Bien *et al.*, 2011). During the exponential growth phase, *S. aureus* expresses a number of surface molecules or adhesins. This allows the bacteria, at low density, to adhere to and colonise the host cells as well as implanted medical devices (Korem *et al.*, 2003). At a higher cell density the bacteria produce exotoxins, which aid in the survival and dissemination of the bacteria as well in establishing infection (Korem *et al.*, 2003). This density dependant regulation is under the control of complex quorum sensing mechanisms and genetic loci such as accessory gene regulator (agr), *sar*, *sae* and *traP* (Korem *et al.*, 2003).

Adhesins

S. aureus initiates the colonization process by attaching to surfaces. Multiple adhesins are responsible for regulating the attachment to surfaces. Microbial surface component recognizing adhesive matrix molecules (MSCRAMMs) are a major class of *S. aureus* adhesion proteins, which are anchored to the peptidoglycan of the cell wall. MSCRAMMs recognise components of the extracellular matrix (ECM) or blood plasma. Members of the MSCRAMM family include staphylococcal protein A (SpA), clumping factor (Clf) A and B and collagen-binding fibronectin-binding proteins A and B (FnbpA and FnbpB) (Bien *et al.*, 2011). SpA is one of the main membrane bound virulence factors, produced by almost all clinical *S. aureus* isolates (Huntzinger *et al.*, 2005). The protein binds to the von Willebrand factor, which is a large glycoprotein that mediates platelet adhesion at damaged endothelial sites (Huntzinger *et al.*, 2005). SpA also interferes with Immunoglobulin (Ig)-mediated opsonisation (Patel *et al.*, 1987) and has B-cell super antigenic properties - it acts as a natural B-cell toxin by inducing programmed cell death of the targeted B-cell (Huntzinger *et al.*, 2005).

Exoproteins

A second type of virulence factor is exoproteins, which include exotoxins that promote disease, and degradative enzymes that help the organism invade the host. Exoproteins are expressed during the

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late exponential phase of growth when expression of adhesins is downregulated. This allows the bacteria to detach from the original colonization site and establish an invasive infection (George and Muir, 2007; Bien *et al.*, 2011).

There are different types of exotoxins produced by *S. aureus*. One group has cytolytic activity, and includes α-haemolysin, β-haemolysin, γ-haemolysin, δ-haemolysin and Panton-Valentine leukocidin (PVL). Cytolytic exotoxins form pores in the plasma membrane resulting in lysis of the host cell due to content leakage (Bien *et al.*, 2011).

S. aureus produces another group of exotoxins known as pyrogenic toxin superantigens (PTSAgs). These include the staphylococcal enterotoxins (SEA, SEB, SECn, SED, SEE, SEG, SEH and SEI) and the toxic shock syndrome toxin-1 (TSST-1). The enteroexotoxins have the ability to stimulate proliferation of T-lymphocytes (superantingenicity), and to cause diseases such as food poisoning, toxic shock syndrome and staphylococcal scalded skin syndrome (SSSS). *S. aureus* also produces exfoliative toxins (ETA and ETB) which have been recognised to possess mitogenic activity toward T-lymphocytes, but whether these exfoliative toxins are superantigens remains controversial (Bien *et al.*, 2011).

The expression of virulence genes in bacteria is tightly regulated; virulence gene expression is turned on under appropriate conditions allowing the bacteria to survive within the host and initiate infection by evading the host defence system (Huntzinger *et al.*, 2005). *S. aureus* virulence is regulated by multiple global regulatory loci that form part of a complex signalling pathway, which, together with environmental and intracellular signals, regulates the expression of virulence factors (George and Muir, 2007). *S. aureus* has developed quorum-sensing systems that enable cell-cell communication as well as the regulation of virulence factors (Yarwood and Schlievert, 2003). These quorum-sensing systems facilitate its ability to cause disease and occupy niches within the host.

The accessory gene regulator (agr)

The accessory gene regulator (agr) locus is one of the quorum-sensing systems that plays a critical role in virulence regulation in *S. aureus*. Previous proteomic and microarray studies have shown that the agr locus regulates the expression of more than 70 genes, of which, 23 are known virulence factors (George and Muir, 2007). The expression of the agr locus down-regulates the production of cell-wall associated virulence factors, such as SpA, and up-regulates the expression of secreted virulence factors (exotoxins) such as α-, β-, δ-haemolysin (encoded by *hla, hlb, hld*) and Panton-Valentine leukocidin (PVL) (Sakoulas *et al.*, 2002). Bacteria can cause an infection by detaching from their original colonization site during the late exponential and stationary growth phases (George and Muir, 2007). During these stages of growth, the exotoxins are secreted to assist in detachment which can also be regarded as a mechanism for spreading (George and Muir, 2007). The expression of the agr locus also appears to play a role in invasion and apoptosis of epithelial cells (Yarwood and Schlievert, 2003).

Peng *et al.,* first described the agr two-component quorum sensing gene cluster in 1988. It contains five genes (*agrB, agrD, agrC, agrA* and *hld*) which form part of a multifaceted network (Peng *et al.*, 1988). The *agrD* transcript is a propeptide that is processed by AgrB, an integral membrane protein, to produce and secrete a mature autoinducing peptide (AIP) to the outside of the cell (Dufour *et al.*, 2002; George and Muir, 2007). The AIPs activate this two-component signalling pathway by activating AgrC, a transmembrane receptor histidine kinase, which undergoes autophosphorylation resulting in the activation of AgrA, the response regulator. This activates transcription of the agr locus at P2 and P3 (Figure 1.4), resulting in the expression of two RNA transcripts, RNAII and RNAIII (Lina *et al.*, 2003; George and Muir, 2007). Expression of RNAIII (the effector molecule) is highly dependent on the expression of *agrB, agrD, agrC* and *agrA,* which are co-transcribed on RNAII. The RNAIII transcript also encodes the exoprotein δ-haemolysin (*hld*), which serves as a marker for agr activity (Figure 1.4) (Cheung *et al.*, 1997).

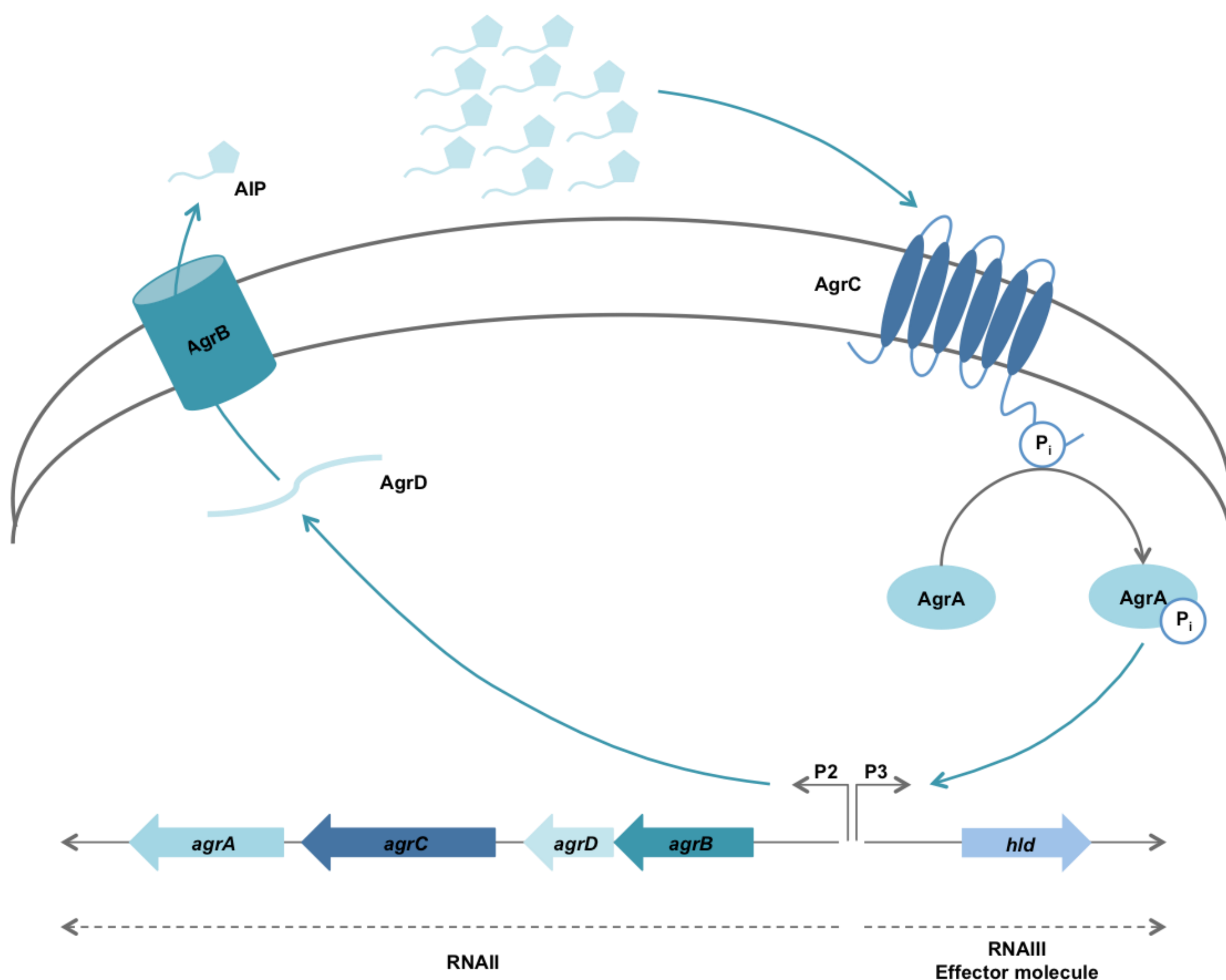

Figure 1.4: Schematic representation of the agr locus. The expression of RNAII and RNAIII is initiated by promoters P2 and P3 respectively. Five genes are expressed, *agrB, agrD, agrC, agrA* and *hld* (δ-haemolysin). Autoinducing peptides (AIPs) accumulate outside the cell and when a certain threshold is reached, AgrC is activated. This results in the expression of RNAIII (the effector molecule).

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RNAIII: The effector molecule

The agr locus regulates the expression of virulence factors, firstly, by the direct AgrA-induced expression of virulence genes such as phenol soluble modulins (PSM), and secondly, by AgrA-induced expression of RNAIII which affects the transcription and translation of other virulence factors. RNAIII is a multifunctional regulatory molecule that acts as both an antisense and a messenger RNA. It consists of 514 nucleotides, encodes δ-haemolysin and forms a stable 14 stem-loop motif structure (Figure 1.5), which is responsible for the up-regulation of expression of exotoxins and the reduced production of surface proteins during the post-exponential and exponential growth phases, respectively (Sterba *et al.*, 2003; Huntzinger *et al.*, 2005; George and Muir, 2007).

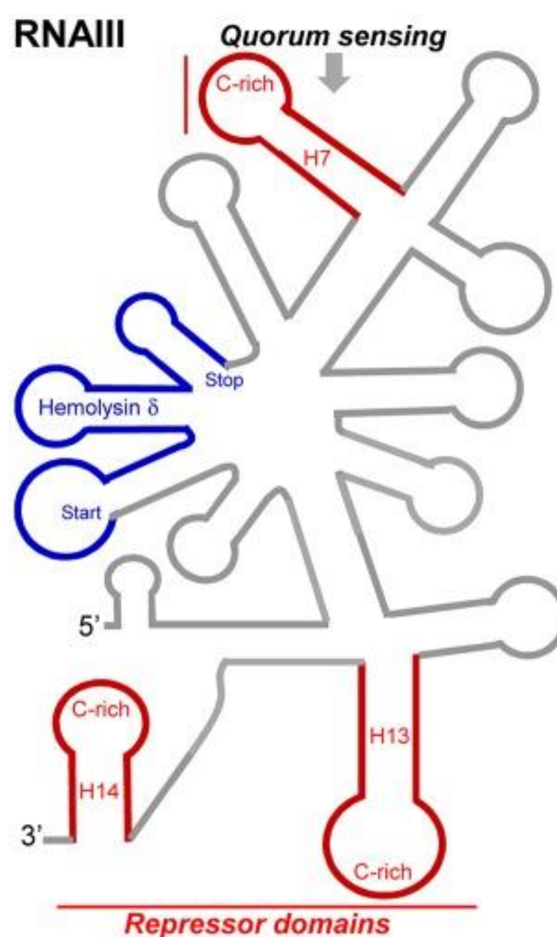

Figure 1.5: Schematic representation of RNAIII. The blue region indicates the location of δ-haemolysin, while the red areas indicate three known repressor domains. (Source: Guillet *et al.,* 2013).

One way in which RNAIII is involved in the regulation of other genes is by positive regulation of translation. An example of this is when the 5'-end of RNAIII competes with an intramolecular RNA secondary structure in *hla* mRNA which sequesters of the *hla* ribosomal binding site, thereby increasing α-haemolysin expression (Huntzinger *et al.*, 2005). Another way is the translational repression of expression, for example repression of staphylococcal protein A (*spa*) synthesis.

Studies by Novic (2003) and Huntzinger *et al.* (2005) suggested that sequence complementarity between *spa* mRNA and the 3'-end of RNAIII represses the translation of *spa* mRNA to inhibit the production of SpA (Novick, 2003; Huntzinger *et al.*, 2005).

agr types

There are four different agr types, I-IV, defined by sequence variation within the hypervariable region of the agr locus (*agrB, agrC, agrD*) (George and Muir, 2007). Each agr type encodes a separate AIP made up of seven to nine amino acids and a pentapeptide thiolactone macrocycle (Figure 1.6). All of the different AIPs can bind to the AgrC receptor of all of the agr types, but only a related AIP-AgrC (intratype) interaction leads to the activation of AgrA. Intertype AIP-AgrC interactions inhibit the phosphorylation of AgrC and can block activation of AgrA. Type I and IV AIPs differ by a single amino acid and are the only exception to the intertype interference (George and Muir, 2007).

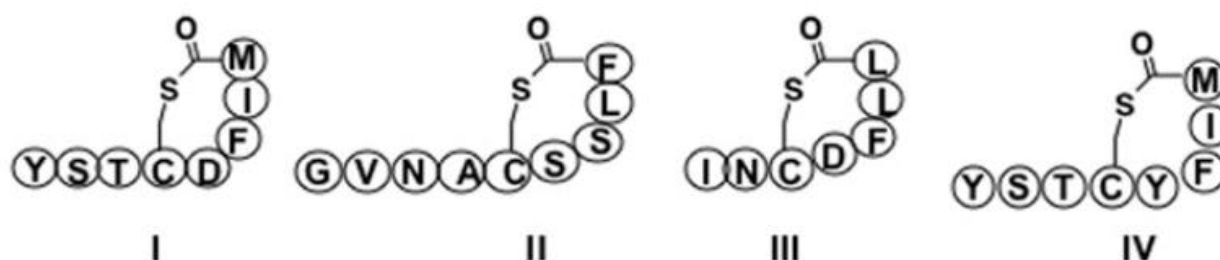

Figure 1.6: Four different AIPs (amino acid sequences) generated by the different agr types. (Source: George & Muir 2007).

Different agr types have been associated with certain diseases, however the exact reason for the association between the disease and agr type is not yet clear (Yarwood and Schlievert, 2003). Previous studies have reported that agr type I strains are most commonly isolated from clinical cultures, while agr type IV is found in the minority of *S. aureus* isolates (Robinson *et al.*, 2005; Traber *et al.*, 2008; Schweizer *et al.*, 2011). Agr types I and II have been associated with resistance to glycopeptides, while agr type III strains were isolated from patients with community acquired MRSA (CA-MRSA) as well as toxic shock syndrome. Agr type IV is associated with staphylococcal scalded skin syndrome (Robinson *et al.*, 2005).

A study done by Jarraud *et al.* (2002) investigated the relationship between agr types and disease presentation and reported a strong correlation between agr type and genetic background and, ultimately, certain disease presentations. A link between agr type IV and exfoliative toxin-mediated disease as well as agr type I and II with endocarditis was also observed. Although they could define causation, the authors did speculate that the preferential association between certain agr types, strain types and toxin genes may result in a more effective activation of virulence factors (Jarraud *et al.*, 2002).

Agr functionality

Mutations in *agrA* or any of the other genes encoded by RNAII can prevent activation of transcription at both promoters (Cheung *et al.*, 1997; Traber *et al.*, 2008). This results in failure to produce the effector molecule RNAIII, and therefore, altered expression of virulence genes, which can affect disease manifestation. *S. aureus* strains which don't produce RNAIII are known as agr dysfunctional strains, while wild type strains are referred to as agr functional strains (Paulander *et al.*, 2012). The prevalence of agr dysfunctional strains can range from 15-60% in HA infections and is much lower among CA infections, at approximately 4%.

The functionality or dysfunctionality of the agr locus can be determined by investigating the production of δ-haemolysin (Sakoulas *et al.*, 2002). When the agr locus is expressed, the *hld* gene is also transcribed (as part of RNAIII) resulting in the production of δ-haemolysin; therefore the presence of δ-haemolysin suggests agr functionality. There are several methods to determine the functionality of the agr locus (Sakoulas *et al.*, 2002; Gagnaire *et al.*, 2012; Paulander *et al.*, 2012). These include the observation of haemolysis using a phenotypic synergistic haemolysis assay, detecting the presence of δ-haemolysin by whole cell Matrix Assisted Laser Desorption Ionisation Time of Flight Mass Spectrometry (MALDI-TOF MS) and the quantitation of RNAIII by qPCR.

A study done by Schwan *et al.* (2003) investigated the effects of agr dysfunction on disease in murine models and reported that *S. aureus* agr dysfunctional strains are selected for in wounds and abscesses, while haemolytic strains are associated with systemic infections (Schwan *et al.*, 2003). Data also suggest that dysfunctionality of the agr locus in *S. aureus* encourages disease development in device related infections (Kong *et al.*, 2006).

A prominent correlation between dysfunctionality of the agr locus and enhanced biofilm formation has been reported, possibly due to the up-regulation of attachment molecules and down-regulation of the detachment molecules (Vuong *et al.*, 2000). This can be advantageous for the bacterium, because it reduces pro-inflammatory responses within the host (Kong *et al.*, 2006). Biofilm formation includes distinct stages: initial attachment to the matrix, cell-to-cell adhesion and proliferation, maturation and detachment. In *S. aureus,* quorum sensing plays a role in biofilm formation at many of these stages (Kong *et al.*, 2006). Transition of the cells from free-floating planktonic to a biofilm phase is a crucial step. This involves the expression and interaction of several adhesion molecules that permits the physiochemical interactions between the planktonic cells and the surface. As biofilm matures, small clusters of cells detach from the established structure, which is a fundamental step for the spread of infections that are associated with biofilm formation (Kong *et al.*, 2006). Studies by Yarwood *et al.* (2004) suggest that the agr locus plays a role in the detachment of cells from biofilms. They performed biofilm time course experiments and reported that the agr locus was, at most times, not expressed in most areas of the of the biofilm. However, cells that did express the agr locus were present, but were released from the biofilm. The release of virulent *S. aureus* isolates enables it to

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spread to new sites (Yarwood *et al.*, 2004). The formation of thicker biofilms in dysfunctional agr strains compared to the wild type is due to the inability of the cells to detach from the mature biofilm (Vuong *et al.*, 2000).

The observation that the agr locus is involved in the regulation of many virulence factors leads to the assumption that it is vital in *S. aureus* pathogenesis and that a loss of agr function leads to a decrease in virulence (Kong *et al.*, 2006). However, some studies have called into question the importance of the agr locus since agr dysfunctional strains are still able to cause disease (Kielian and Cheung, 2001; Kong *et al.*, 2006). Previous studies reported no difference in virulence between agr dysfunctional and functional strains in mouse brain abscess model and in a rabbit endocarditis model (Cheung *et al.*, 1994; Kielian and Cheung, 2001); therefore further investigation is required.

Problem Statement

S. aureus is a highly virulent bacterium that is capable of causing a variety of infections when exposed to mucous membranes or damaged skin. *S. aureus* has mastered evading the host immune system as well as becoming resistant to antibiotics; strains resistant to the last resort antibiotics, such as vancomycin, have already been identified (George and Muir, 2007). Data regarding *S. aureus* epidemiology and agr related virulence characteristics in South Africa is limited. Of the six South African epidemiology studies described in Table 1.4, only one investigated the association between agr type and strain type. One other study performed in our setting investigated agr type as well as agr functionality, but the data is unpublished. Furthermore, data describing the link between strain type and agr related virulence characteristics and clinical outcome is also extremely limited. Different agr types have been associated with different disease types and agr dysfunction has been associated with prolonged bacteraemia, increased resistance to antibiotics and an increased mortality (Gagnaire *et al.*, 2012; Paulander *et al.*, 2012). It is clear that the agr locus plays a significant role in the regulation of *S. aureus* virulence factors, but the exact role of agr locus and agr functionality is controversial and complex. Investigating the role of strain type and the agr locus in virulence and its effects on the clinical outcome may ultimately provide a foundation for progress in the treatment and prevention of Staphylococcal disease.

Aim

The aim of this study was to determine the effect of genetic background and agr-associated virulence characteristics on the clinical outcome of bacteraemic patients at Tygerberg Hospital.

Objectives

- To investigate the molecular epidemiology of the *S. aureus* isolates.
- To determine the agr type and agr functionality or dysfunctionality of the isolates.
- To correlate the strain type and agr-associated virulence characteristics with clinical data.

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CHAPTER 2: The population structure of patients with *Staphylococcus aureus* bacteraemia at Tygerberg Hospital

Introduction

Staphylococcus aureus is one of the most common human pathogens and a leading cause of hospital-acquired (HA), healthcare-associated (HCA) and community-acquired (CA) infections (Kaech *et al.*, 2006; Perovic *et al.*, 2015). Although invasive *S. aureus* infections can manifest in many different ways, in the majority of these cases the organism can be detected in the patient's blood, resulting in bacteraemia (Turnidge *et al.*, 2009). More than 80% of *S. aureus* bacteraemia is endogenous in origin, which could be a result of the high carriage rates (25-40%) among individuals is (von Eiff *et al.*, 2001; Kaech *et al.*, 2006).

Despite the availability of antibiotics for the treatment of *S. aureus* bacteraemia, the mortality rate remains high and ranges from 29-63% (Perovic *et al.*, 2015). In the last two decades methicillin resistant *S. aureus* (MRSA) isolates, which were previously only found in nosocomial infections, have increasingly been isolated from patients with CA infections (Perovic *et al.*, 2015). The emergence of CA-MRSA has caused a shift in epidemiology and an increase in the number of MRSA infections (Larsen *et al.*, 2008). CA-MRSA isolates mainly cause skin and soft tissue infections, but invasive infections such as necrotizing pneumonia and bacteraemia have been described (Larsen *et al.*, 2008). Although CA-MRSA isolates are infrequently isolated from blood, they are found in 45-85% of cases of bacteraemia without focus (Kaech *et al.*, 2006).

About 40-80% of *S. aureus* bacteraemia is caused by HA isolates and, in contrast to CA infections, most of these cases have an obvious portal of entry, e.g. surgical sites or intravenous catheters. The high rate of bacteraemia in health care settings may be due to increasing use of central venous lines, as intravenous catheter-related *S. aureus* bacteraemia has been shown to be a major problem (Kaech *et al.*, 2006).

S. aureus bacteraemia is a serious disease with a high mortality rate. Difficult to treat MRSA isolates are frequently isolated from blood cultures, and isolates resistant to last resort antibiotics have been identified. This is threatening our ability to treat infections caused by this organism. In this section we describe the study population, clinical data and *S. aureus* isolates collected from patients who presented with *S. aureus* bacteraemia at Tygerberg Hospital between January 2015 and March 2017.

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Methodology

Study population

This study included patients of all ages with *S. aureus* bacteraemia at Tygerberg Hospital between January 2015 and March 2017. Tygerberg Hospital is a 1384 bed tertiary academic hospital in the Western Cape of South Africa, which serves a population of approximately 1.9 million. *S. aureus* bacteraemia was defined based on the isolation of *S. aureus* from at least one blood culture.

Sample collection

Blood cultures collected by clinicians as part of routine care were submitted to the National Health Laboratory Service (NHLS) microbiology laboratory at Tygerberg Hospital for culture and identification; as per routine diagnostic procedures. Blood culture bottles were incubated in the BACTEC Blood Culture System (Becton Dickinson, USA) for up to 5 days, or until they flagged positive. Standard morphological methods, such as Gram morphology, catalase, mannitol fermentation and DNAse activity, were used to identify *S. aureus* isolates and if necessary the Pastorex Staph-Plus test (Bio-Rad Laboratories, USA) was performed. In the case of a negative or weakly positive mannitol fermentation or DNAse test, the Vitek®2 system (bioMérieux, France) was used to confirm *S. aureus* identification. Isolates were classified as MRSA using the Vitek®2 system (bioMérieux, France). *S. aureus* isolates were collected from the NHLS microbiology laboratory by convenience collection and stored for further analysis in this study. Only non-duplicate isolates were included in this study; where duplicate isolates were defined as isolates collected from the same patient within a two-week period.

Ethical approval for this study has been obtained from the Health Research Ethics Committee (HREC) of Stellenbosch University (Ethics Reference #: N14/06/065).

Patient characteristics

Clinical data of all patients with *S. aureus* blood infections at Tygerberg Hospital was collected by the Group for Enteric, Respiratory and Meningeal Disease Surveillance in South Africa (GERMS-SA) as part of routine surveillance. This data includes age, gender, ethnicity, diagnosis, final outcome (crude mortality), source of the infection, risk factors as well as the admission date and dates of blood culture collection and final outcome.

The patients were divided into four groups based on age; neonates were defined as babies younger than 28 days, children as 28 days to 13 years, adults as older than 14 years but younger than 50 years and elderly as 50 years and older. Diagnosis was documented as bacteraemia with focus or bacteraemia without a known focus, and the final outcome as death or discharge. The length of stay (in days) was calculated using the admission and final outcome dates.

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The infection was classified as hospital acquired (HA), community acquired (CA), or health-care associated (HCA). HA infections were defined as a positive blood culture taken more than three days after the admission date. HCA infections were defined as positive blood cultures taken at hospital admission or within three days of admission, with one or more of the following risk factors:

- hospitalisation within the year prior to the culture date,
- prior dialysis in the year before the culture date,
- prior surgery in the year before the culture date,
- residence in a long term care facility in the year before the current culture date.

CA infections were defined, as modified from Perovic *et al.* (2017), as a positive blood culture taken at hospital admission or within three days of admission without any of the above mentioned risk factors (Perovic *et al.*, 2017). The time of admission of the patients was often unavailable, therefore three days was used as the cut-off instead of 48 hours to avoid potentially misclassifying some patients with HAI as CA.

Results

Study population and sample collection

During the study period, 473 *S. aureus* bacteraemia cases were documented by GERMS-SA and 199 non-duplicate *S. aureus* isolates were randomly collected from the NHLS microbiology laboratory at Tygerberg Hospital from January 2015 to March 2017. Of the 199 isolates collected, clinical profiles were missing for six patients. To determine whether the study sample set is representative of the complete population, a comparison was done between the profiles of the 193 patients for whom *S. aureus* isolates and clinical data was available and the full data set of 473 cases recorded by GERMS-SA. No significant difference was observed between the two groups, therefore, the study population was concluded to be representative of the entire population of patients with *S. aureus* bacteraemia during the study period (Table 2.1).

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Table 2.1: Comparison of the patient profiles in the complete clinical data set and the study population.

	Complete data set (n=473)	Study data set (n=193)
Age in days – median (IQR)	11285 (15450)	11181.5 (15490)
Adult wards	334 (71%)	138 (71.5%)
Paediatric wards	139 (29%)	55 (28.5%)
Male patients	253 (53%)	113 (58.5%)
Female patients	220 (47%)	80 (42.5%)
Race		
Black	193 (41%)	83 (43%)
Coloured	234 (49%)	96 (49.7%)
White	35 (8%)	10 (5.2%)
Unknown	11 (2%)	4 (2.1%)

Patient characteristics

Amongst the study population, the median age of the patients was 30.8 years (11181.5 days; IQR=42.4 years), ranging from 2 days to 87 years (Table 2.1). Forty eight percent of the patients (n=96) were classified as adults, and 56.8% (n=113) were males (Table 2.2). HA infections represented 52.8% (n=105) of the cases, while HCA and CA were 23.1% (n=46) and 19.6% (n=39), respectively. The source of the organism for nine (4.5%) of the isolates was unknown (Table 2.2). The median length of stay was 24 days (IQR=34.5 days); ranging from 0 to 212 days.

Table 2.2: Summary of clinical data for the total study population and classified as MSSA and MRSA infections.

	Total (n=199)	MSSA (n=145)	MRSA (n=54)
Length of stay in days – Median (IQR)	24 (34.5)	20 (24)	46 (49)
Final outcome			
Deceased	58 (29.1%)	39 (26.9%)	19 (35.2%)
Discharged	141 (70.9%)	106 (73.1%)	35 (64.8%)
Age groups			
Neonates (<28 days)	21 (10.6%)	9 (6.2%)	12 (22.2%)
Children (≥28 days – <14 years)	32 (16.1%)	24 (16.6%)	8 (14.8%)
Adults (≥14 year – <50 years)	96 (48.2%)	71 (49%)	25 (51.9%)
Elderly (≥50 year)	50 (25.1%)	41 (28.3%)	9 (16.7%)
Gender			
Male	113 (56.8%)	54 (37.2%)	26 (51%)
Female	80 (42%)	87 (60%)	26 (48%)
Unknown	6 (3%)	4 (27.6%)	2 (11%)
Diagnosis			
Bacteraemia with focus	57 (28.6%)	46 (31.7%)	11 (20.4%)
Bacteraemia without focus	129 (64.8%)	90 (63.4%)	39 (72.2%)
Unknown	13 (6.5%)	9 (62%)	4 (74.1%)
Source of organism			
HA	105 (52.8%)	62 (42.8%)	43 (79.6%)
HCA	46 (23.1%)	39 (26.9%)	7 (13.7%)
CA	39 (19.6%)	37 (25.5%)	2 (3.7%)
Unknown	9 (4.5%)	7 (4.8%)	2 (3.7%)

Twenty seven percent (n=54) of the patients were infected with MRSA isolates. The mortality rate amongst patients with MRSA isolates was 35.2% (n=19), while 26,9% (n=39) of patients with MSSA infections passed away. Of all the patients 21.6% were infected by HA-MRSA infections, while only 1% (n=2) of the patients had CA-MRSA infections. HA-MSSA infections accounted for the largest group with 31.2% (n=62), followed by HCA-MSSA and CA-MSSA infections accounting for 19.6%

(n=39) and 18.6% (n=37) of the cases respectively. Altogether, 19.6% (n=39) of the patients were diagnosed with bacteraemia without focus due to MRSA, while 45.2% (n=90) of the patients had bacteraemia without focus caused by MSSA infections (Table 2.2). Patients who had bacteraemia with a known focus caused by MRSA infections accounted for the smallest number of isolates (5,5%; n=11), while 23.1% (n=46) of the patients had bacteraemia with a known focus caused by MSSA infections.Figure 2.1 features the diagnoses reported in the study; 64.8% (n=129) of the patients had bacteraemia without focus and 28.6% (n=57) had bacteraemia with a known focus (LRTI, bone infection, joint infection, skin and soft tissue infection etc.); 13 (6.5%) patients had an unknown diagnosis. The crude mortality rate was 29.1% (n=58) during the study period (Table 2.2).

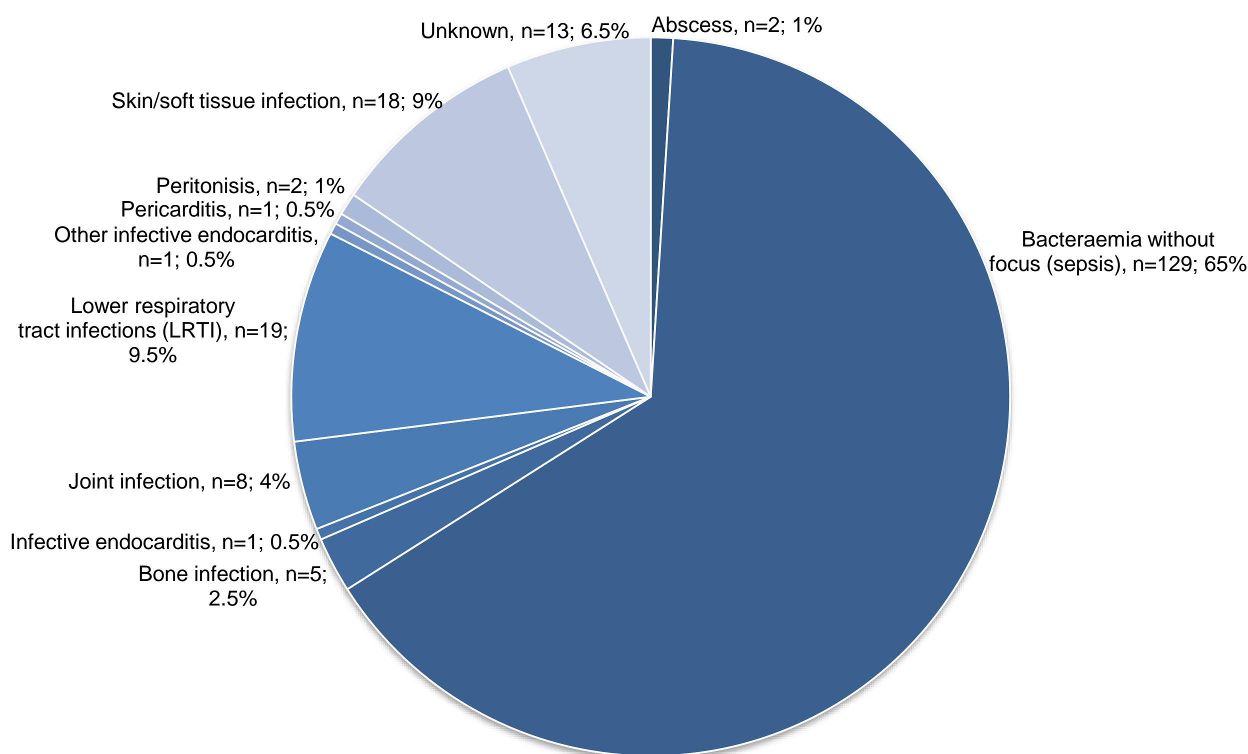

Figure 2.1: Patient diagnoses.

Discussion

During the period between January 2015 and March 2017, 473 cases of *S. aureus* bacteraemia were identified at Tygerberg hospital as part of the GERMS-SA surveillance. 199 *S. aureus* isolates were collected from the NHLS microbiology laboratory, of which clinical data was collected for 193 of these. This subset of cases were concluded to be representative of the larger population. In many cases the data of the patients were incomplete and six of the collected isolates had no patient profile

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match which is a limitation to this study and may have impacted the results. The missing profiles could be because the patients died or were discharged before the clinical data could be collected.

During this study period the prevalence of MRSA in *S. aureus* bacteraemia at Tygerberg hospital was 27.1%, which was lower than that reported by other studies. Perovic *et al.* (2015) reported an MRSA prevalence of 37% in the Western Cape in 2012 (Perovic *et al.,* 2015), while previous studies at Tygerberg hospital have reported that the MRSA prevalence amongst routinely collected *S. aureus* isolates from adult and paediatric patients with *S. aureus* infections from multiple clinical sources from 2008-2012 varied from 30% to 44% (Oosthuysen *et al*., 2013; Karayem, 2014). According to the GERMS-SA annual reports the MRSA prevalence in Gauteng and the Western Cape has decreased from 33% in 2015 to 24% in 2017 (Crowther-Gibson & Quan 2016; Crowther-Gibson & Quan 2017).

The neonate age group was the only group that showed a higher prevalence of MRSA (57.1%) than MSSA. This may be attributed to an MRSA outbreak which occurred in the paediatric and neonatal wards during this period.

In Africa, the prevalence of MRSA varies between countries, however a systematic review on MRSA in Africa reported a prevalence of lower than 50% for the majority of the African countries with Egypt (52–82%) and Algeria (35-75%) being the exceptions (Falagas *et al*., 2013).

In this study we observed a high proportion of HA-MRSA (79.6% of MRSA infections) which is consistent with a previous study performed in our setting (Orth *et al.,* 2013); which reported that 88% of MRSA infections were HA (Orth *et al*., 2013). This is not unexpected since antibiotic selective pressure is higher in hospitals than in the community and favours the selection of resistant strains (Chambers, 2001).

In our study there was no major difference in distribution of bacteraemia without focus compared to bacteraemia with focus, 49% compared to 44% respectively, among CA infections. Furthermore, 79% of the HA infections were bacteraemia without focus, while 19% were bacteraemia with focus. Which states that majority of the HA infections did not have a clearly identifiable portal of entry. A previous study performed on blood culture isolates from patients from a Swiss tertiary-care centre reported a high prevalence of bacteraemia without focus, ranging between 45 and 85%, among CA *S. aureus* bacteraemia (Kaech *et al*., 2006). This result is consistent with our study since 49% of the CA infections were bacteraemia without focus. The Swiss study did, however, also report that in most cases, nosocomial infections have a portal of entry such as surgical sites or intravenous catheters (Kaech *et al*., 2006). Our results are contradictory to this since only 19% of the HA infections were as a results of bacteraemia with a known focus. It is possible that, when the clinical data was collected, the focus of the bacteraemia may not have been available or communicated to the GERMS-SA surveillance officer as the time of data collection. The way in which the diagnosis of patients was reported may be a limitation to this study.

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Conclusion

In conclusion, 199 *S. aureus* isolates were collected from patients with bacteraemia at Tygerberg hospital between 2015 and 2017. Clinical data was collected from 193 of the patients. We report a 29% mortality rate, and a lower MRSA prevalence (27,1%) than previously described in our setting. HA-MRSA accounted for almost/approximately 80% of the MRSA infections and almost half of the cases of bacteraemia without focus were CA infections. The following chapters will describe the genetic diversity of bacteraemic *S. aureus* isolates from Tygerberg hospital and their accessory gene regulator (agr)-associated virulence characteristics (Chapters 3 and 4); and further investigate the impact of strain type and agr-associated virulence characteristics on the clinical data (Chapter 5).

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CHAPTER 3: The epidemiology of *Staphylococcus aureus* bacteraemia isolates from patients at Tygerberg Hospital

Introduction

The World Health Organisation (WHO) has defined epidemiology as “the study of the distribution and determinants of health-related states or events (including disease), and the application of this study to the control of diseases and other health problems” (WHO, n.d.). Strain typing is fundamental for effective characterisation and discrimination of isolates in order to determine clonal dominance and strain relatedness (Oosthuysen *et al.*, 2013). Bacterial isolates are geographically diverse and strain typing may be used to determine common and dominant strain types in certain regions (Perovic *et al.*, 2015). This may provide insight into evolutionary relationships between isolates and permit the study of routes of transmission and to assess the source of the infection (Perovic *et al.*, 2015).

There are multiple techniques available for *Staphylococcus aureus* strain typing amongst which staphylococcal protein A (*spa*) typing and multi-locus sequence typing (MLST) are two of the most common methods used. Pulsed field gel electrophoresis (PFGE), single nucleotide polymorphisms (SNPs) and whole genome sequencing (WGS) is also available for epidemiological studies (Stefani *et al.*, 2012). PFGE is considered to be the gold standard for *S. aureus* strain typing because of its high discriminatory index. However, it is a laborious technique, has limited portability and does not have a standardised nomenclature. Based on this, *spa* typing is often considered an acceptable alternative to PFGE, especially when additional methods such as SCC*me*c typing are included for MRSA isolates (Hallin *et al.*, 2007).

Data regarding the molecular epidemiology of *S. aureus* in South Africa is limited (Oosthuysen *et al.*, 2013). Of the four previous studies in Cape Town and our setting specifically, only two focused on blood cultures alone while the other two studied isolates from multiple sources. It is important to continuously describe the genetic diversity of isolates in our setting to determine whether clonal expansion has taken place. Investigating and understanding molecular characteristics of *S. aureus* isolates is important as this may have an impact on patient outcome and treatment (Perovic *et al.*, 2015). Information on the genetic relatedness of the isolates may provide a basis for further studies comparing virulence-related characteristics of different strains and the impact of these strains on patients.

In this section we aimed to describe the molecular epidemiology of *S. aureus* isolates collected from bacteraemic patients at Tygerberg Hospital. This was done by *spa* typing of all of the isolates, MLST on a subset of isolates and SCC*mec* typing of MRSA isolates.

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Methodology

Sample collection

S. aureus isolates were collected from blood cultures from patients with bacteraemia at Tygerberg Hospital from February 2015 to March 2017, as described in Chapter 2. Isolates were collected from the National Health Laboratory Services (NHLS) microbiology diagnostic laboratory at Tygerberg Hospital, following routine diagnostic procedures. All isolates were stored on Microbank beads (Pro-lab diagnostics, Canada) at -80°C. Only non-duplicate isolates were included in this study; where duplicate isolates were defined as isolates collected from the same patient within a two-week period.

Bacterial culture

S. aureus isolates were retrieved from the Microbank beads by aseptically removing one bead from the tube and streaking it out on 5% horse blood agar plate (Green Point Media Lab, South Africa). Cultures were aerobically incubated overnight at 37°C. Following overnight incubation, a single colony was re-streaked onto a blood agar plate and incubated for a further 24 hours at 37°C to allow growth of pure cultures.

Extraction of genomic DNA

Genomic DNA was extracted using an in house crude heat-lysis DNA extraction method. A few colonies of the pure culture were re-suspended in 400 µl nuclease free water in a micro-centrifuge tube and placed in a heating block at 95°C for 30 minutes. The heating step was followed by freezing for 30 minutes at -20°C after which the micro-centrifuge tubes were thawed and centrifuged at maximum rpm for 10 minutes. The supernatant (containing the DNA) was transferred to a new micro-centrifuge tube and the extracted DNA was stored at -20°C for use in downstream analyses. A negative DNA extraction control was included in every batch of extractions to indicate possible contamination.

spa typing

The extracted genomic DNA was used in the PCR amplification of the hypervariable region of the *spa* gene. All PCR reactions were set up and run on a Proflex PCR thermal cycler (Applied Biosystems, United States). PCR amplification was performed using the KAPA Taq Readymix PCR kit (KAPA Biosystems, Switzerland). For each PCR reaction, 12.5 µl of KAPA Taq, 1 µl of each primer (10 pmol/µl), 8.5 µl nuclease free water and 2 µl template DNA (extracted genomic DNA) was added. A PCR negative control containing all the reagents except genomic DNA was included in each PCR run to detect possible contamination. The first primer set, 1095F and 1517R, was used to amplify the X region of the *spa* gene (Table 3.1) (Harmsen *et al.*, 2003). When PCR amplification

using these primers failed, samples were amplified using the second and third primer sets as described by Hallin *et al.* (2009). (Table 3.1).

Table 3.1: PCR primers for *spa* typing. (Adapted from Harmsen *et al.*, 2003; Hallin *et al.,* 2009).

#	Primer	Sequence (5' – 3')	Product size (bp)
1	1095F 1517R	AGACGATCCTTCGGTGAGC GCTTTTGCAATGTCATTTACTG	200-500
2	239F 1717R	ACTAGGTGTAGGTATTGCATCTGT TCCAGCTAATAACGCTGCACCTAA	± 1200
3	1084F 1618R	ACAACGTAACGGCTTCATCC TTAGCATCTGCATGGTTTGC	± 550

Amplification consisted of an initial denaturation step at 95°C for 3 minutes; 30 cycles of denaturation at 95°C for 30 seconds, primer annealing at 62°C for 30 seconds and elongation at 72°C for 1 minute; the reaction was concluded with a final extension at 72°C for 1 minute. The PCR reaction was kept at 4°C until removal of the PCR tubes from the PCR machine.

The PCR products were separated using agarose gel electrophoresis on a 2% agarose gel in 1 x TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) for 30 minutes at 120 V. Five microliters of PCR product was added to 1 µl Novel Juice (GeneDirex, United States) on parafilm and mixed by pipetting up and down; 5 µl of the mixture was used for electrophoresis. A 5 µl of KAPA Universal Ladder (KAPA Biosystems) was included in each gel for determination of the sizes of the PCR products. Amplicon bands were visualised on agarose gels with the UVItec Alliance 2.7 gel documentation system (UVItec, UK). PCR products were sequenced at Inqaba Biotec (Pretoria) using both the forward and reverse PCR primers used during amplification (Table 3.1).

The Ridom StaphType v.2.1.1 (Ridom GmbH, Germany) software package was used for *spa* type elucidation (Harmsen *et al.*, 2003). A built-in Based Upon Repeat Pattern (BURP) algorithm was used to cluster the *spa* types into *spa* Clonal Complexes (*spa*-CCs). *spa* types that were too short to presume ancestry (<5 repeats within the hypervariable Xr region of the *spa* gene) were excluded from the BURP analysis. A phylogenetic tree of the identified *spa* type data was constructed using the Molecular Evolutionary Genetic Analysis (MEGA) software using the neighbour-joining method.

spa typing was performed on 72 of the 199 *S. aureus* isolates as part of my BSc Honours project in 2016.

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Multi-locus sequence typing (MLST)

MLST was performed as previously described by Enright *et al.* (2000). Seven housekeeping genes (*arcC, aroE, glpF, gmk, pta, tpi, yqiL*) were amplified and sequenced using the primers in Table 3.2. Singleplex PCRs were performed for each gene using extracted genomic DNA. Each reaction contained 12.5 µl KAPA Taq Readymix (KAPA Biosystems), 1 µl of each primer (10 pmol/µl), 9.5 µl nuclease free water and 1 µl template DNA to make up a final volume of 25 µl. A PCR negative control containing all the reagents except genomic DNA was included in each PCR run to detect possible contamination.

The PCR conditions were as follows: an initial denaturation step of 5 minutes at 95°C, followed by 30 cycles of 95°C for 45 seconds, 56°C for 45 seconds and 72°C for 1 minute. A final elongation step was carried out at 72°C for 10 minutes. The PCR reaction was kept at 4°C until removal of the PCR tubes from the PCR machine. The PCR products were separated using agarose gel electrophoresis as described in the *spa* typing section.

PCR products were sequenced at Inqaba Biotec using the forward and reverse primers used during the PCR reaction. Sequences for each of the seven housekeeping genes were inspected and trimmed in BioEdit Sequence Alignment Editor (Ionis Pharmaceuticals, United States) using allele 1 of each of the housekeeping genes as reference genes (http://saureus.beta.mlst.net/#). The forward and reverse sequences were used to generate a consensus sequence which was then copied into the *S. aureus* MLST database (http://saureus.beta.mlst.net/#) to confer MLST STs.

Table 3.2: Primers for the seven housekeeping genes used in MLST. (Source: Enright *et al.*, 2000)**.**

MLST Group	Gene	Primer	Primer Sequence (5'-3')	Product size	Alleles	Polymorphic sites
A	*arcC* carbamate kinase	arcC-Up	TTGATTCACCAGCGCGTATTGTC	456 bp	17	19
		arcC-Dn	AGGTATCTGCTTCAATCAGCG			
B	*aroE* shikimate dehydrogenase	aroE-Up	ATCGGAAATCCTATTTCACATTC	456 bp	17	23
		aroE-Dn	GGTGTTGTATTAATAACGATATC			
C	*glpF* glycerol kinase	glpF-Up	CTAGGAACTGCAATCTTAATCC	465 bp	11	14
		glpF-Dn	TGGTAAAATCGCATGTCCAATTC			
D	*gmk* guanylate kinase	gmk-Up	ATCGTTTTATCGGGACCATC	417 bp*	11	13
		gmk-Dn	TCATTAACTACAACGTAATCGTA			
E	*pta* phosphate acetyltransferase	pta-Up	GTTAAAATCGTATTACCTGAAGG	474 bp	15	18
		pta-Dn	GACCCTTTTGTTGAAAAGCTTAA			
F	*tpi* triosephosphate isomerase	tpi-Up	TCGTTCATTCTGAACGTCGTGAA	402 bp	14	18
		tpi-Dn	TTTGCACCTTCTAACAATTGTAC			
G	*yqiL* acetyl coenzyme A acetyltransferase	yqiL-Up	CAGCATACAGGACACCTATTGGC	516 bp	16	19
		yqiL-Dn	CGTTGAGGAATCGATACTGGAAC			

SCC*mec* typing

SCC*mec* typing was performed on all MRSA isolates, as classified using the Vitek®2 system (bioMérieux, France), using a multiplex PCR that was previously described by Milheirico *et al.* (2007). The 20 primers described in Table 3.3 were used to amplify different regions of the SCC*mec* element, including genes from the *mec* and *ccr* gene complexes, joining regions J1 and J3. The *mec*A primers (*mec*A P4 and *mec*A P7) were used as an internal control. SCC*mec* types were determined by the combination of the different amplified targets.

Table 3.3: Primers used in SCC*mec* typing by multiplex PCR. (Source: Milheirico *et al.,* 2007)**.**

Primer Name	Primer Sequence (5'-3')	Target: Type; region	Size (bp)
kdpF1 **kdpR1**	AATCATCTGCCATTGGTGATGC CGAATGAAGTGAAAGAAAGTGG	**II**; J1	284
CIF2 F2 **CIF2 R2**	TTCGAGTTGCTGATGAAGAAGG ATTTACCACAAGGACTACCAGC	**I**; J1	495
RIF5 F10 **RIF5 R13**	TTCTTAAGTACACGCTGAATCG GTCACAGTAATTCCATCAATGC	**III**; J3	414
SCC*mec* III J1 F **SCC*mec* III J1 R**	CATTTGTGAAACACAGTACG GTTATTGAGACTCCTAAAGC	**III**; J1	243
SCC*mec* V J1 F **SCC*mec* V J1 R**	TTCTCCATTCTTGTTCATCC AGAGACTACTGACTTAAGTGG	**V**; J1	377
***mecI* P2** ***mecI* P3**	ATCAAGACTTGCATTCAGGC GCGGTTTCAATTCACTTGTC	**II, III**; *mec* complex	209
dcs F2 **dcs R1**	CATCCTATGATAGCTTGGTC CTAAATCATAGCCATGACCG	**I, II, IV, VI**; J3	342
***mecA* P4** ***mecA* P7**	TCCAGATTACAACTTCACCAGG CCACTTCATATCTTGTAACG	Internal control	162
***ccrB2* F2** ***ccrB2* R2**	AGTTTCTCAGAATTCGAACG CCGATATAGAAWGGGTTAGC	**II, IV**; *ccr*	311
***ccrC* F2** ***ccrC* R2**	GTACTCGTTACAATGTTTGG ATAATGGCTTCATGCTTACC	**V**; *ccr*	449

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PCR amplification was performed using the KAPA 2G Fast Multiplex Mix Kit (2X) (KAPA Biosystems) and each reaction had a final volume of 25 µl. For each PCR reaction 12.5 µl of KAPA 2G Fast Multiplex Mix Kit (2X), 0.1 µl of each primer in Table 3.3 (50 pmol/µl), 9.5 µl nuclease free water and 1 µl template DNA (extracted genomic DNA) was added. Seven control strains Table 3.4, each representing a different SCC*mec* type, were included in every multiplex PCR run. A PCR negative control containing all the reagents except genomic DNA was included in each PCR run to detect possible contamination.

Table 3.4: SCC*mec* typing control strains. (Adapted from Milheirico *et al.,* 2007; Nel van Zyl 2016).

SCC*mec* type	Control strain	Origin
I	BAA-38	ATCC (American Type Culture Collection)
II	BAA-1681	ATCC
III	BAA-39	ATCC
IV	BAA-1680	ATCC
V	WIS	Division of Medical Microbiology, University of Cape Town
V	BAA-1688	ATCC
VI	BAA-42	ATCC

The PCR cycle conditions were as follow: 95°C initial denaturation for three minutes; 30 cycles of denaturation at 95°C for 15 seconds, primer annealing at 60°C for 30 seconds and elongation at 72°C for 45 seconds; the reaction was concluded with a final extension at 72°C for three minutes. The PCR reaction was kept at 4°C until removal of the PCR tubes from the PCR machine.

The PCR products were separated using agarose gel electrophoresis, essentially as previously described, on a 3% agarose gel in 1 x TAE buffer for 80 minutes at 100 V (Nel van Zyl, 2016). The band patterns of the isolates were compared to those of the control strains in order to assign SCC*mec* types.

Results

The molecular epidemiology was determined for the 199 *S. aureus* isolates collected from blood cultures from bacteraemic patients at Tygerberg Hospital from January 2015 to March 2017, as described in Chapter 2.

spa typing

spa typing provided information on the genetic diversity of the identified *S. aureus* isolates. Figure 3.1 is a representative *spa* typing gel, showing the presence of the expected bands of between 200 bp and 500 bp. The different product sizes are as a result of the different numbers of repeats within the hypervariable Xr region of the *spa* gene. The band size is however not an indication of the *spa* type and cannot be used to discriminate between strain types; sequence analysis is essential for *spa* type elucidation. A total of 189 (95%) of the 199 isolates were successfully typed. PCR amplification failed for 10 isolates even after repeated DNA extraction and attempted PCR amplification with all three *spa* typing primer sets, and were classified as non-typeable.

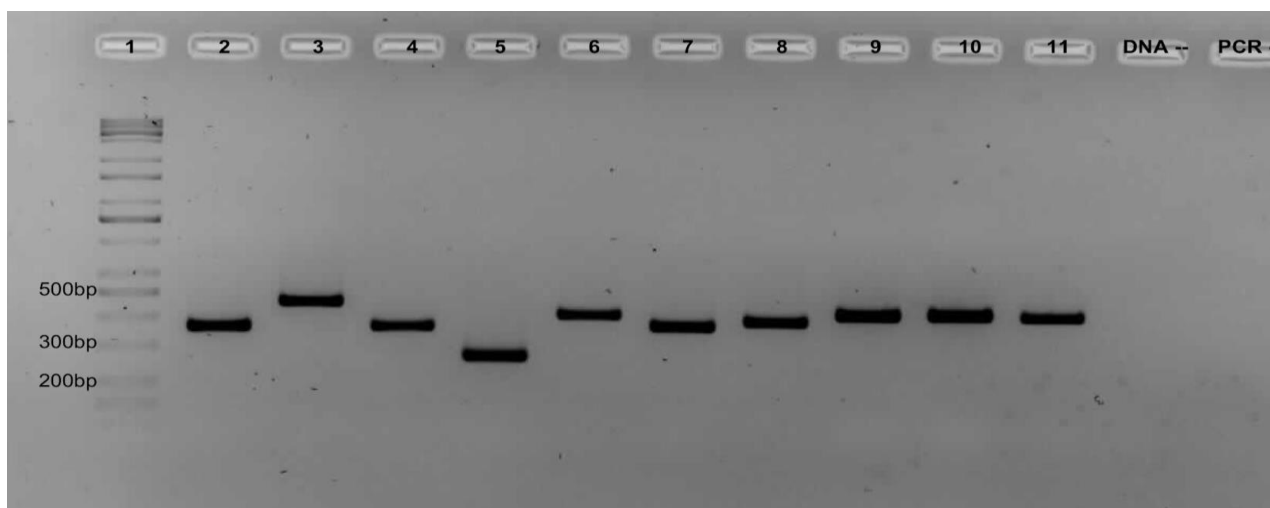

Figure 3.1: Representative agarose gel of the *spa* typing PCR products. Lane 1 contains the DNA ladder, lanes 3-11 contain *spa* PCR products of ten *S. aureus* isolates and lanes 12 and 13 contain the DNA extraction and PCR negative controls respectively.

Interpretable sequencing results were obtained for all *spa* PCR products. *spa* types with globally recognised patterns through the Ridom Spa server are labelled with a "t" before the succession number (Shutt *et al.,* 2005). Seventy-three different *spa* types were identified, of which seven (9.6%) were identified as new *spa* types namely t18222 – t18228. t045 (n=22; 11.1%) was the most abundant *spa* type followed by t318 (n=15; 7.5%) and t002 (n=13; 6.5%). The BURP algorithm clustered the 73 *spa* types into twelve *spa*-CCs (Table 3.5, Figure 3.2), which accounted for 76.9% (n=153) of the isolates. *spa*-CC 002 is the largest group of isolates (n=42; 21.1%), followed by *spa*-CC 012 (n=34; 17%), *spa*-CC 701/2360 (n=23; 11.6%) and *spa*-CC 032/578 (n=13; 6.5%). No founders were identified for four of the *spa*-CCs, and 18 (n=32; 16.1%) of the *spa* types were classified as singletons (Table 3.5). Three *spa* types (n=4; 2%) were excluded from the analysis since they contained less than five repeats within the hypervariable region of the *spa* gene and ancestry could not be assumed

Table 3.5: *spa*-CCs identified using the BURP algorithm of Ridom StaphType.

Clonal Complex (CC)	*spa* types	*spa* types (n)	Isolates (n)	MRSA/MSSA/Mixed
spa-CC 002	**t002**, t045, t071, t242, t509, t1154, t5213, t15306	8	42	Mixed
spa-CC 012	**t012**, t018, t021, t037, t318, t399, t1848	7	34	Mixed
spa-CC 701/2360	t190, **t701**, t1257, t1476, t1971, **t2360**, t4315	7	23	Mixed
spa-CC 015/073	**t015, t073**, t116, t331, t1078, t2171	6	10	MSSA
spa-CC 084	**t084**, t085, t346, t14791, t18222	5	10	MSSA
spa-CC 032/578	**t032, t578**, t891, t1036	4	13	Mixed
spa-CC NF 174	t127, **t174**, t18225, t18227	4	7	MSSA
spa-CC NF 5916	t1490, **t5916,** t18226	3	3	Mixed
spa-CC NF 9	t148, t2409	2	4	Mixed
spa-CC NF 10	t317, t6712	2	3	MSSA
spa-CC NF 11	t1597, t11970	2	2	MSSA
spa-CC NF 12	t258, t349	2	2	MSSA
Singletons	t008, t091, t189, t223, t267, t269, t272, t355, t888, t1467, t2442, t2526, t2763, t6267, t10509	18	32	Mixed
spa types excluded	t026, t2304, t9909,	3	4	MSSA
Non-typeable	N/A	N/A	10	MSSA

*The founder *spa* types for each *spa*-CC are highlighted in bold.

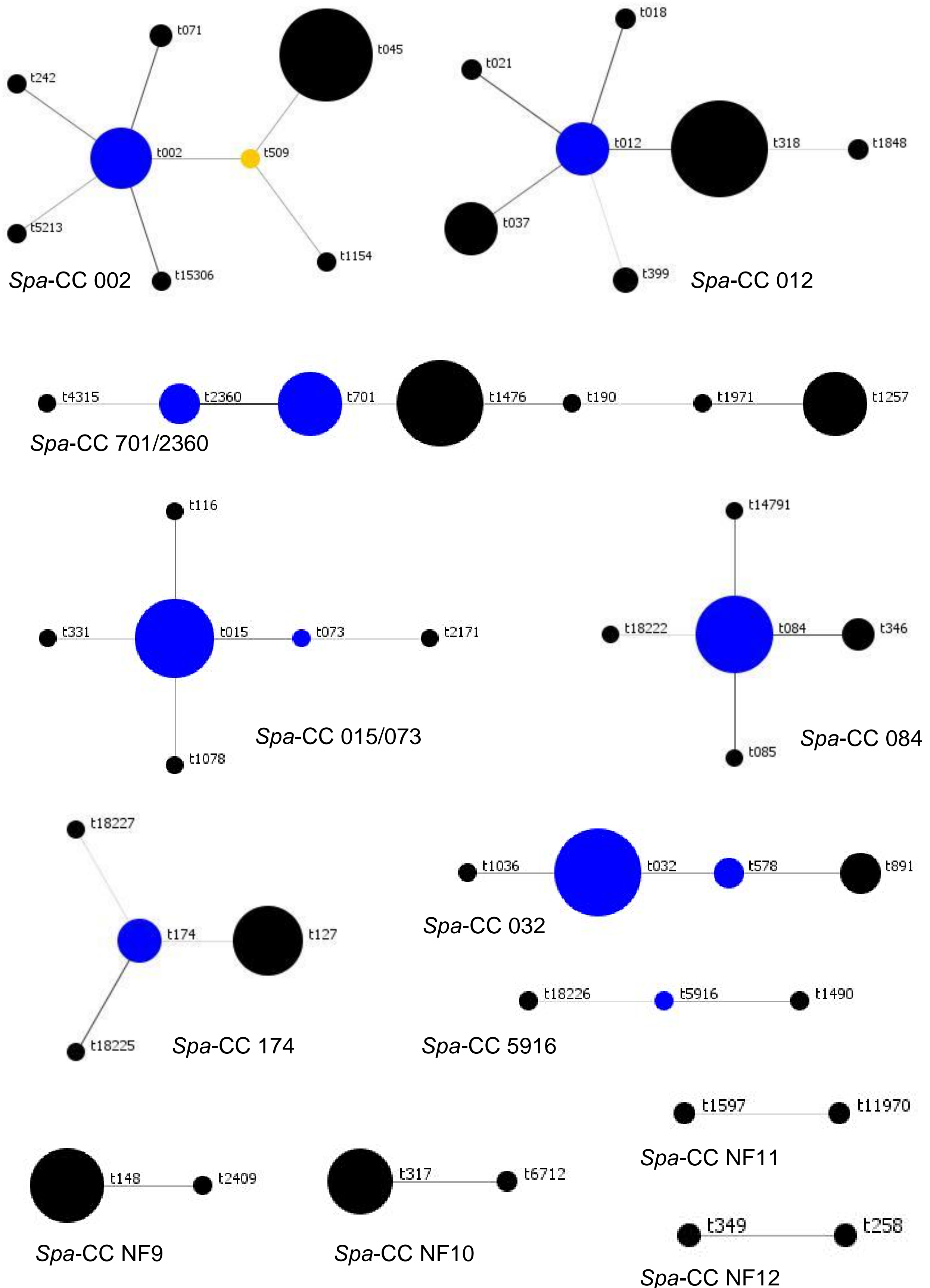

Figure 3.2: Graphical representation of the *spa*-CCs identified using the BURP algorithm of Ridom StaphType. The blue circles represent the founders of the clusters, while the yellow circles represent the co-founders and the black circles represent the other *spa* types present in the cluster. The size of the circle is proportional to the number of isolates belonging to the *spa* type, but is not comparable between cluster diagrams.

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Multi-locus sequence typing (MLST)

MLST was performed on a subset of isolates based on the *spa* typing results. At least one representative isolate from each of the major *spa*-CCs was selected for MLST. Altogether 14 isolates were typed and 11 different STs were identified (Table 3.6). Two representative isolates from the dominant *spa*-CC 002 were ST5, while one isolate was typed as an unknown ST that is most closely related to ST5. The second largest cluster, *spa*-CC 012, contained ST30, ST1865 and ST239. *spa*-CC 701/2360, contained ST8. Amplification of the genes *aroE* and *yqiL* failed for two isolates from *spa*-CC NF10, therefore they were designated non-typeable by MLST (Table 3.6).

Table 3.6: MLST STs from selected representative isolates based of *spa* typing results.

***spa*-CC**	***spa* type**	**MLST ST**	**MLST CC**
002	t002	5	5
	t045	NT (closest related ST is ST5)	5
	t045	5	5
012	t012	30	30
	t318	1865	30
	t037	239	5
701/2360	t1476	8	5
015/073	t015	508	45
084	t084	15	15
032/578	t032	22	22
NF7	t127	1	5
NF9	t148	72	5
NF10	t317	NT	NT
	t6712	NT	NT

*NT: Non-typeable by MLST

SCC*mec* typing

Fifty-four (27.1%) of the *S. aureus* isolates were reported to be methicillin resistant by the Vitek II Advanced Expert System (Biomerieux). SCC*mec* typing was performed on all MRSA isolates as described by Milheirico *et al.,* (2009). Figure 3.3 is a representative SCC*mec* typing gel, showing the typing of the controls as reference and 10 isolates. The SCC*mec* types of ninety-four percent (n=51) of the MRSA could be determined. The remaining three isolates had banding patterns that were non-typeable by the protocol from Milheirico *et al.* (2009) (Figure 3.4).

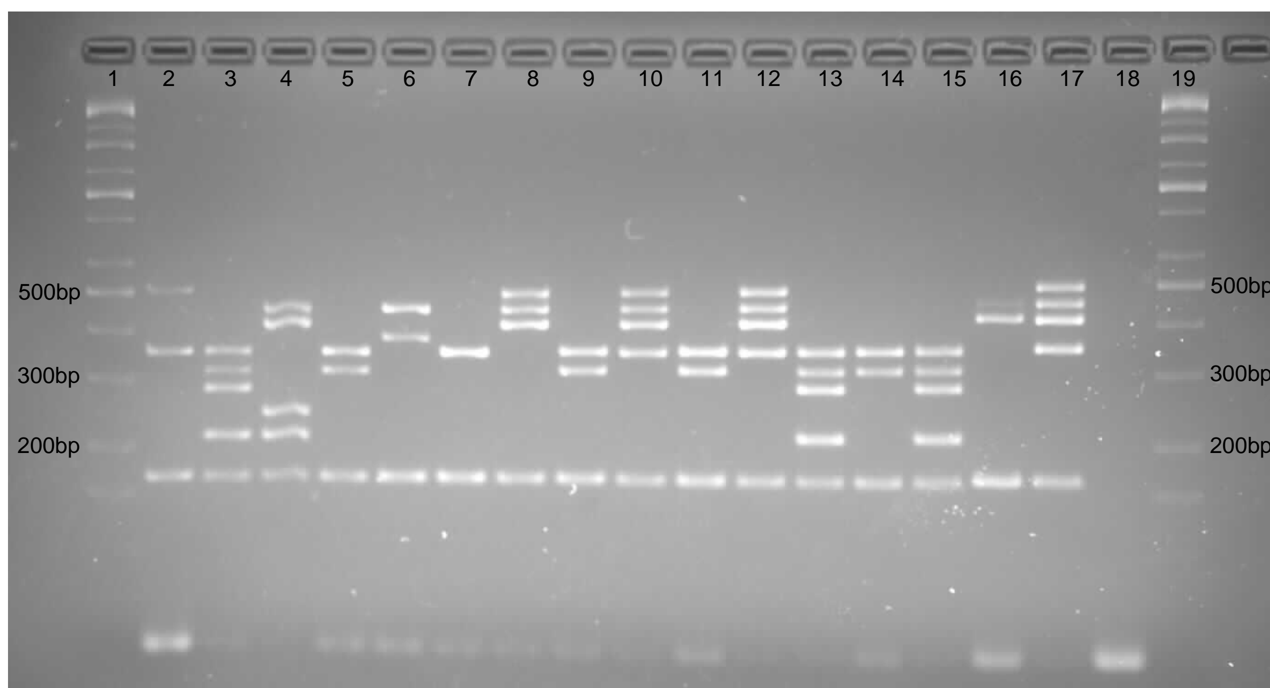

Figure 3.3: Representative SCC*mec* typing gel. Three percent agarose gel for the assignment of SCC*mec* types to isolates. Lane 1 and 19 contain the molecular weight ladder; lanes 2-7 contain the controls for SCC*mec* type I-VI respectively; lanes 8-17 contain ten isolates and lane 18 contains a negative PCR control. Isolates in lanes 9, 11, and 14 were identified as SCC*mec* type IV; lanes 10, 12 and 17 as a novel variant; lanes 13 and 15 as SCC*mec* type II and lane 8 and 16 were non-typeable isolates.

The dominant SCC*mec* types were SCC*mec* type IV and a novel variant (NV) that has previously been described in our setting (Karayem, 2014); each of these types were present in 19 (35.2%) isolates (Figure 3.4). SCC*mec* type III (n=7; 13%) was the third most prevalent type followed by SCC*mec* type II (n=6; 11.1%) (Figure 3.4).

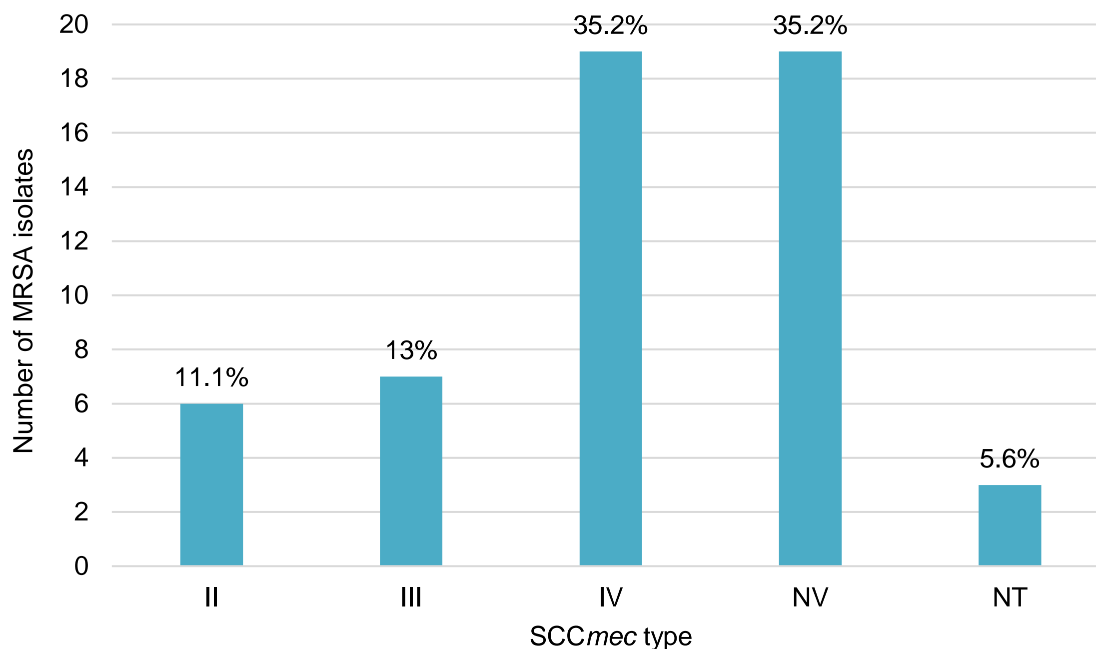

Figure 3.4: The SCC*mec* type distribution amongst the MRSA isolates.

Phylogeny of *S. aureus* isolates based on *spa* typing

The MLST, SCC*mec* typing and methicillin resistance results were correlated on a neighbour-joining phylogenetic tree constructed in MEGA using the *spa* typing data (Figure 3.5). Of the twelve *spa*-CCs identified, six contained only MSSA isolates (*spa*-CC 015/037, *spa*-CC 084, *spa*-CC 174, *spa*-CC NF10, *spa*-CC NF11, *spa*-CC NF12) and six (*spa*-CC 002, *spa*-CC 012, *spa*-CC 701/2360, *spa*-CC 032/578, *spa*-CC 5916, *spa*-CC NF9) consisted of both MRSA and MSSA isolates (Figure 3.5).The dominant MSSA clone was t318-ST1865 (n=14/154; 9.7%), and has previously been described at Tygerberg Hospital followed by t002-ST5 (n=13/154; 9%) and t355 (n=9/154; 6.2%). Amongst MRSA strains, t045-ST5-MRSA-NV accounted for 19 (35.2%) of the strains. This was followed by t037-ST239-MRSA-III and t032-ST22-MRSA-IV, each accounting for seven (13%) MRSA isolates. Strain type t1257-MRSA-IV was assigned to five (9.3%) of the MRSA isolates, and four (7.4%) of the isolates were t012-ST30-MRSA-II. Ten MRSA strain types were limited to one or two isolates only.

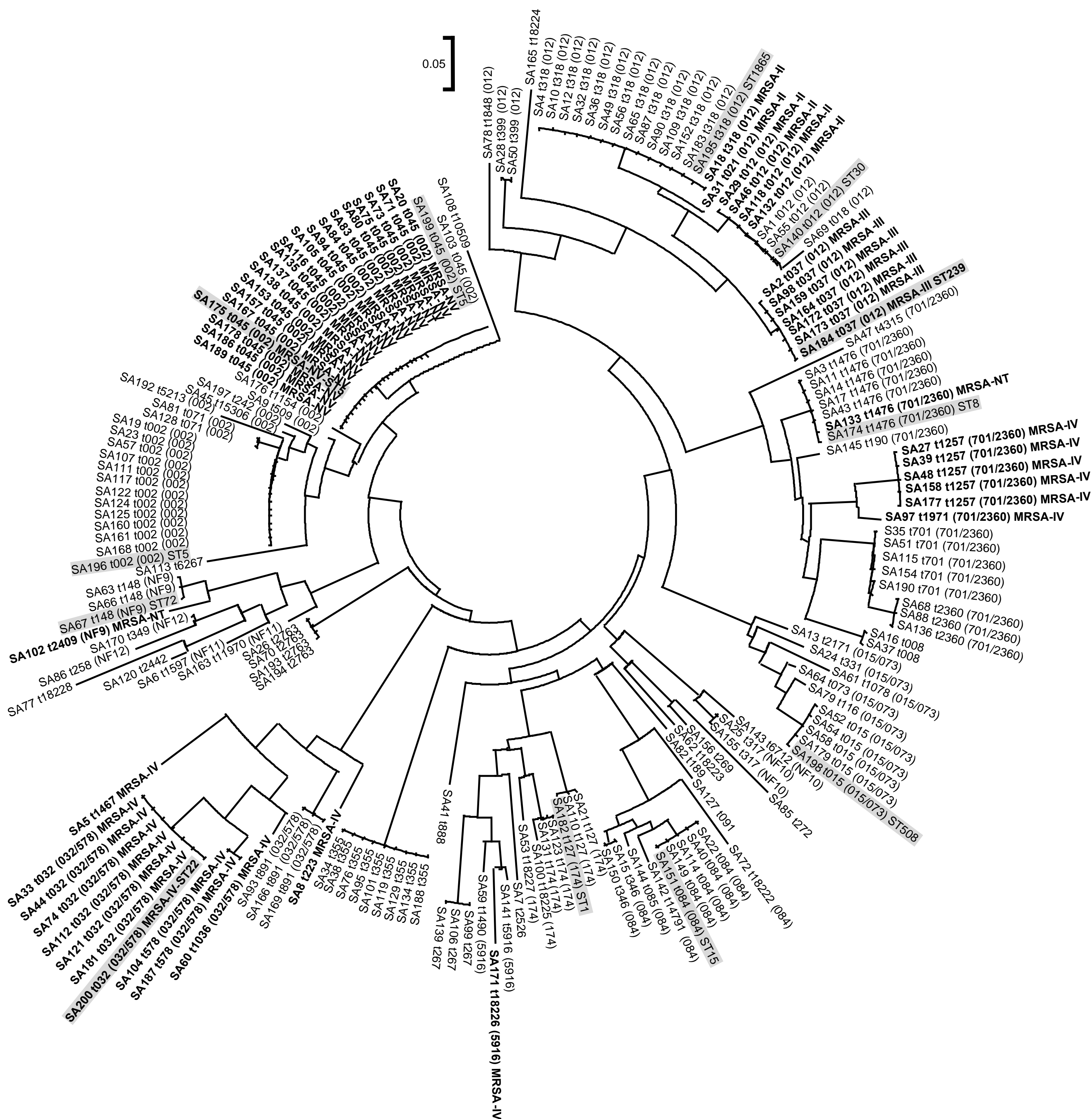

Figure 3.5: Phylogeny of the *S. aureus* isolates based on *spa* type. The phylogeny was constructed with the *spa* typing results, based on the neighbour-joining algorithm in MEGA. The MLST and SCC*mec* typing results were superimposed on the *spa* typing phylogeny. The numbers on the end on each branch are the *spa* type, ST (where analysed), SCC*mec* type and *spa*-CC. The strains in bold are MRSA isolates and the highlighted strains indicate the isolates selected for MLST.

Discussion

This chapter describes the genetic structure of *S. aureus* isolates collected from bacteraemic patients at Tygerberg Hospital between January 2015 and March 2017. The MRSA isolates were shown to be more clonal than the MSSA isolates. The dominant MSSA clone was t318-ST1865 (CC30) and, although not one of the most common clones, has previously been described amongst blood culture isolates from Tygerberg Hospital during 2008 and 2009 (Orth *et al.*, 2013). t318-ST1865 has also been described among isolates from Egypt, Indonesia and multiple countries in Europe (Deurenberg *et al.*, 2010; Enany *et al.*, 2010).

The dominant strain type among the MRSA isolates was t045-ST5-MRSA-NV. ST5-MRSA has previously been described in South-Africa, however, it was linked with various SCC*mec* types. For example, in Kwa-Zulu Natal it was identified with SCC*mec* III (Shittu *et al.,* 2009) and IV (Essa *et al.,* 2009), while in Cape Town it was identified with SCC*mec* type I (Jansen van Rensburg *et al.,* 2011). This indicates that SCC*mec* elements are easily transferred between and acquired by different strains through horizontal gene transfer due to fact that it is a mobile genetic element. ST5-MRSA-IV (also known as the paediatric clone) has also been found in France, Portugal and USA (Essa *et al.*, 2009). A multicentre study performed in South Africa reported the presence of t045-ST5-MRSA with a non-typeable SCC*mec* element (Perovic *et al.,* 2015). In this study 19 t045-ST5-MRSA-NV isolates were collected from paediatric patients and t045 was identified as an paediatric and neonatal outbreak strain which could have resulted in an elevated MRSA rate, especially among the neonate and children age groups.

The SCC*mec* type NV identified in this study has previously been identified among 16.7% of MRSA isolates in our setting by Karayem (2014) and has been described by Nel van Zyl (2016). Following WGS of a SCC*mec* type NV *S. aureus* isolate the NV was found to be similar to SCC*mec* type I (Nel van Zyl 2016). The NV carries the same *mec* type (Class B) and *ccr* type (*ccrA1ccrB1*) as SCC*mec* type I as well as the two insertion sequences, namely IS341 and IS1272. However, a SCC_{Hg}-like element was identified between the J3 and *orfX* regions of the SCC*mec* type NV (Nel van Zyl 2016).

The clone t037-ST239-MRSA-III (CC5) (n=7; 3.5%) described in our study is a pandemic clone also known as the Brazilian/Hungarian clone (Friedrich *et al.*, 2008; Shittu *et al.*, 2009). It has been widespread in hospitals in multiple countries in South America, Asia and Europe (Friedrich *et al.*, 2008). In South Africa, it was first reported in 2009 in 16.4% of isolates from health-care institutions in Kwa-Zulu-Natal (Durban, Pietermaritzburg and Epangeni) (Shittu *et al.,* 2009). The clone t032-ST22-MRSA-IV (CC32) has infrequently been previously described in South Africa in isolates from multiple infection sources from 2005-2008 and to our knowledge has not been described elsewhere in Africa (Jansen van Rensburg *et al.*, 2011; Perovic *et al.*, 2015). This clone is however one of the most frequently (10.4%) described *spa* types worldwide (Friedrich *et al.,* 2008; https://www.spaserver.ridom.de).

Although not selected for MLST, the strain type t1257-MRSA-IV (n=5; 2.5%), identified in this study, has been reported to be associated with ST612, a local dominant ST in South Africa between 2010-2012 (Perovic *et al.,* 2015). ST612 has only been described in hospitals in South Africa and Australia suggesting that clonal expansion of an old local clone has taken place over time (Jansen van Rensburg *et al.*, 2011). Strain type t012 has frequently been described among HA infections in South Africa belonging to ST22, ST36, ST239 and ST612 (Orth *et al.*, 2013; Perovic *et al.*, 2015, 2017), however in our study it was described as t012-ST30-MRSA-II (CC30). ST30 has previously also been described in Egypt and Madagascar (Abdulgader *et al.*, 2015).

Limitations of this study include that the gold standard for *S. aureus* strain typing, PFGE, was not performed and that due to the high expense of MLST only a subset of isolates were subjected to MLST. A single colony was selected for the analysis, which may have caused biasing of the genotype present in patients infected by multiple clones. Future studies may include PFGE typing of the sample set as well as further SCC*mec* typing for the three isolates that were untypeable by the Milheirico *et al.* (2007) protocol.

Conclusion

Our results suggest that the *S. aureus* population amongst bacteremia patients at Tygerberg hospital between 2015 and 2017 was highly diverse. Seventy-three different *spa* types were identified and clustered into 12 *spa*-CCs with *spa*-CC 002 being the dominant *spa* type. The most common *spa* type among the MSSA isolates was t318-ST1865 and MRSA strains were shown to be more clonal than MSSA strains. Pandemic MRSA clones are circulating in our setting, namely t037-ST239-MRSA-III, t032-ST22-MRSA-IV and t012-ST36-MRSA-II. The novel variant SCC*mec* type was predominant in this population and it was linked with t045-ST5-MRSA. Genotyping data from this chapter was used to investigate associations between strain type and agr-associated virulence (Chapter 4) as well as strain type and clinical data (Chapter 5).

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CHAPTER 4: The agr-related virulence characteristics of *Staphylococcus aureus* bacteraemia isolates from patients at Tygerberg Hospital

Introduction

Staphylococcus aureus is an opportunistic pathogen responsible for a wide range of superficial as well as toxin-associated infections. There are several virulence factors involved in establishing and maintaining *S. aureus* infections and many of these are regulated by the accessory gene regulator (agr) locus. Four different agr types, I-IV, have been described; defined by sequence variation within the hypervariable region of the agr locus (*agrB, agrC, agrD*) (George and Muir, 2007). These agr types have been associated with certain types of infections (Yarwood and Schlievert, 2003). Agr type I strains are prevalent in hospital acquired (HA) infections (Sakoulas *et al.*, 2002), agr types I and II have been associated with resistance to glycopeptides (Robinson *et al.*, 2005), agr type III is common amongst community acquired methicillin resistant *S. aureus* (CA-MRSA) infections and has been associated with toxic shock syndrome, while agr type IV is associated with staphylococcal scalded skin syndrome (Robinson *et al.*, 2005).

AgrA is responsible for the regulation of many phenol soluble modulins (PSM) as well as genes involved in metabolism, but the main effector of the agr locus is RNAIII (Vuong *et al.,* 2004, Gagnaire *et al.,* 2012). RNAIII regulates the switch between expression of surface proteins and exotoxins and also includes the mRNA for δ-haemolysin (26 amino acids); production of which is an indicator of agr functionality (Sakoulas *et al.,* 2002). Because of its involvement in the expression of virulence factors, it may be assumed that the agr locus plays an important role in pathogenesis. The agr locus is considered important for transmission between hosts and essential in strains with high virulence (Paulander, 2012) and agr dysfunctional strains have been shown to be less virulent than wild type strains (Abdelnour *et al.*, 1993; Gillaspy *et al.*, 1995; Blevins *et al.*, 2003; George and Muir, 2007).

The vast majority of clinical isolates have a functional agr locus and therefore produce RNAIII. However, agr dysfunctional strains- lacking RNAIII and therefore δ-haemolysin, have been isolated from patients with a variety of nosocomial infections (Shopsin *et al.*, 2008). Previous studies have reported that essentially all *S. aureus* strains from CA infections have a functional agr locus; and that carriage of *S. aureus* with a dysfunctional agr amongst healthy individuals is approximately 4%. This carriage was found to be associated with previous hospitalisation or hospital exposure (Shopsin *et al.*, 2008; Butterfield *et al.*, 2011; Tsuji *et al.*, 2011). In contrast, agr dysfunctionality ranges from 15 to 60% in HA and health care associated (HCA) *S. aureus* infections (Paulander, 2012).

In our setting, agr typing has only been performed in two previous studies, while only a single study performed agr functionality using the synergistic functionality assay. This highlights the lack of agr typing and functionality data in this setting. In this chapter we describe the agr related virulence

characteristics of the *S. aureus* isolates from bacteraemic patients at Tygerberg hospital with regards to agr type and agr functionality.

Methodology

Agr typing and functionality assays were performed on the 199 *S. aureus* isolates from blood cultures collected from bacteraemic patients at Tygerberg Hospital between January 2015 to March 2017, as described in Chapter 2.

Agr typing

Agr typing was performed by multiplex PCR targeting the hypervariable regions of the agr locus (Lina *et al.*, 2003). The extracted genomic DNA (Chapter 3) was used for the PCR amplification. All PCR reactions were set up and run in the PCR laboratory in the Medical Microbiology Division in Tygerberg Hospital. Agr typing was performed in a Proflex PCR thermal cycler (Applied Biosystems, United States) using the KAPA Taq Readymix PCR kit (KAPA Biosystems, United States). For each PCR reaction 12.5 µl of KAPA Taq master mix (2X), 1 µl of each primer (10 pmol/µl), 8.5 µl nuclease free water and 2 µl template DNA was added. A no-template control containing all the reagents except genomic DNA was included in each PCR run to detect contamination. A universal forward primer, for all four agr types, and four reverse primers, specific to each agr type, were used (Table 4.1).

Table 4.1: PCR primers used for agr typing. (Lina *et al.*, 2003)**.**

Primer	Sequence (5' – 3')	Product sizes (bp)
pan agr F	ATGCACATGGTGCACATGC	N/A
agrI R	GTCACAAGTACTATAAGCTGCGAT	439
agrII R	TATTACTAATTGAAAAGTGCCATAGC	572
agrIII R	GTAATGTAATAGCTTGTATAATAATACCCAG	321
agrIV R	CGATAATGCCGTAATACCCG	657

The PCR cycle conditions were as follows: 95°C initial denaturation for 3 minutes; 35-40 cycles of denaturation at 95°C for 30 seconds, primer annealing at 55°C for 30 seconds and elongation at 72°C for 1 minute; with a final extension at 72°C for 1 minute. The PCR reactions were kept at 4°C until removal of the PCR tubes from the PCR machine.

The PCR products were separated using agarose gel electrophoresis on a 2.5% agarose gel in 1 x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) for 80 minutes at 100 V. To allow visualisation of the DNA, 5 µl of each PCR product was added to 1 µl of Novel Juice (GeneDireX,

United States) on parafilm and mixed by pipetting up and down; 5 µl of the mixture was used for electrophoresis. The first lane of each agarose gel contained 5 µl of KAPA Universal Ladder (KAPA Biosystems) for the size determination of the PCR products. Agarose gels were visualised under UV light using the UVItec Alliance 2.7 gel documentation system (UVItec, UK) and agr type was assigned based on the PCR product size (Table 4.1). Previously typed isolates TKN91 (agrI), TKB72 (agrII), TKB83 (agrIII) and THW 187 (agrIV) were added to each set of reactions as positive controls (Karayem, 2014).

Isolates that could not be typed using the KAPA 2G Taq Readymix were typed using KAPA Taq 2G Robust Readymix (Kapa Biosystems) and/or singleplex PCR, using the conditions described above with an annealing temperature of 58°C and the addition of 2.5 µl $MgCl_2$ (25 mM).

Agr typing was performed on 72 of the 199 *S. aureus* isolates as part of my BSc Honours project in 2016.

Agr functionality

Phenotypic synergistic agr functionality assay

Functionality of the agr operon was determined by detecting δ-haemolysin activity using the phenotypic synergistic haemolysis assay (Sakoulas *et al.*, 2002). This assay is based on the synergistic activity of β-haemolysin and δ-haemolysin in the haemolysis of sheep red blood cells. *S. aureus* RN4220 is a β-haemolytic control strain which causes partial haemolysis of red blood cells. When a δ-haemolysin producing (agr functional) isolate is cross-streaked with RN4220, the synergistic activity of δ- and β-haemolysin results in enhanced or complete haemolysis within the β-haemolysis zone of *S. aureus* RN4220 on sheep blood agar plates, allowing its classification as an agr functional isolate (Sakoulas *et al.*, 2002).

S. aureus strain RN4420 was streaked out vertically down the middle of a sheep blood agar plate (Green Point Media Lab, South Africa) and the test isolates were streaked out perpendicular to RN4220 with the haemolysis zones overlapping. The plates were incubated aerobically at 37°C overnight. δ-haemolysin activity was indicated by enhanced or complete haemolysis within the β-haemolysis zone of *S. aureus* RN4220. The experiment was performed in triplicate for each isolate and read by at least two individuals to ensure that the results were properly documented. Positive and negative controls, NRS 149 and NRS 155 respectively, were included for each batch.

Agr functionality using the phenotypic synergistic assay was determined for 72 of the 199 *S. aureus* isolates as part of my BSc Honours project in 2016.

δ-haemolysin toxin screening by MALDI-TOF MS

Agr functionality was also determined using whole cell matrix assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS) to detect the δ-haemolysin toxin, as described by Gagnaire *et al.* (2012). This assay was included to confirm results from the phenotypic synergistic assay, since results from the phenotypic synergistic assay are subjective. Wild-type δ-haemolysin toxin from *S. aureus* is detected as a peak at 3005±5 Thomson (Th), while a 3035±5 Th peak is detected in the case of a functional allelic variant of δ-haemolysin (a substitution causing an amino acid change from glycine to serine at amino acid 10 in δ-haemolysin). Agr functional strains are therefore identified based on the presence of either the WT (3005±5 Th) or variant (3035±5 Th) toxin peak; while dysfunctional strains lack both peaks (Figure 4.1).

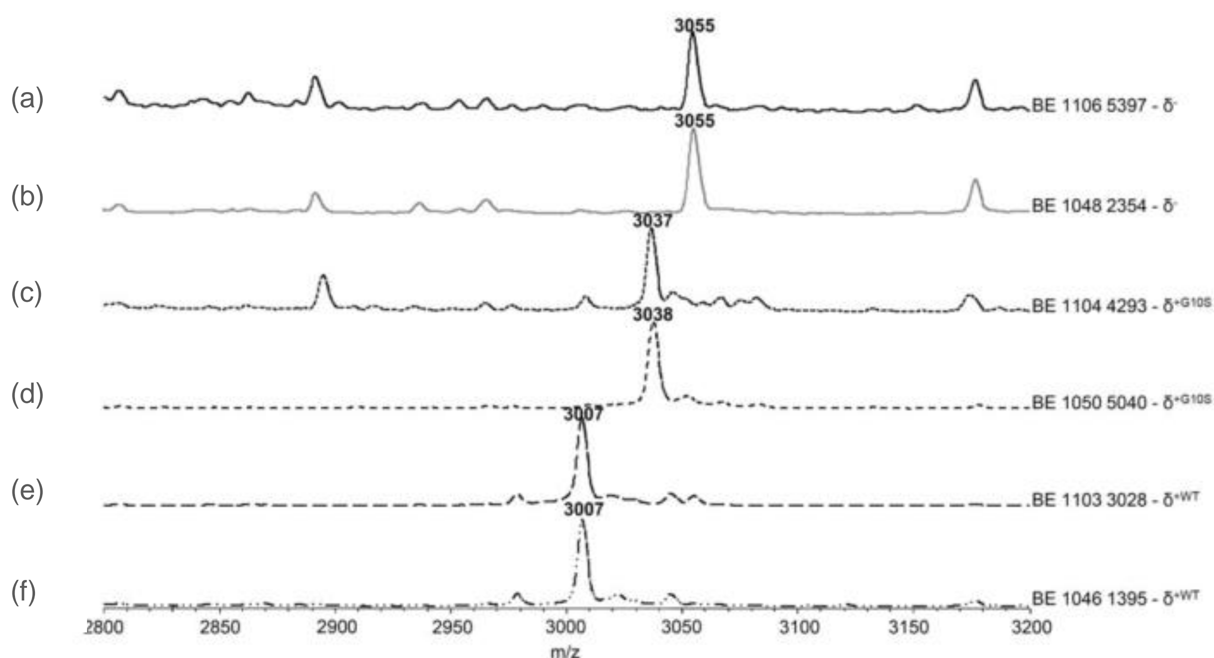

Figure 4.1: Detection of δ-haemolysin toxin peaks by MALDI-TOF MS. Graphs (a) and (b) are both δ-haemolysin negative strains showing no peak at 3005±^{WT} Th or 3035±5^{G10S} Th, while (c) and (d) are two isolates expressing the variant δ-haemolysin toxin; they lack the 3005±5^{WT} Th peak but have a 3035±5^{G10S} Th peak, (e) and (f) are isolates positive for δ-haemolysin showing the 3005±5^{WT} Th peak. *(Source: Gagnaire et al.,* 2012).

For the whole cell MALDI-TOF MS analysis, isolates were grown on blood agar plates (Green Point Media Lab) incubated aerobically at 37°C overnight. One colony was deposited on a stainless steel target slide (Biomerieux, France) and coated with α-cyano-4-hydroxycinnamic acid (CHCA) as matrix. *Escherichia coli* ATCC8739 was used as a control strain for calibration. Mass spectrometry was performed on the VITEK® MS (Biomerieux) at AMPATH at N1 City Hospital in Goodwood, Cape Town, using conventional settings determined for bacterial identification. The VITEK® MS Prep Station Software V2.3.2 (Biomerieux) was used to prepare the target slide and the VITEK® MS Acquisition Station Software was used to visualise the peptide mass fingerprint (PMF). Peaks of

interest (3005±5[WT] Th and 3035±5[G10S] Th) were identified manually by analysing the PMFs generated for all the analytes in the sample. Agr functionality and dysfunctionality were defined by the presence or absence of the peaks respectively. All isolates were run in duplicate and peaks were interpreted as the average of the two replicates.

Statistical analysis

The association between infections with MRSA and the covariates agr type and agr functionality was investigated using univariable and multivariable logistic regression for categorical variables. The two-sample Wilcoxon rank-sum (Mann-Whitney) test was used for univariable analysis, and survival analysis was conducted for the multivariate model. Statistical analysis was done using Stata v.15 (StataCorp LLC, USA) and *P* values of <0.05 were interpreted as significant.

Results

Agr type

Agr typing was successful for all 199 isolates and all four agr types were identified based on the agr product size (Figure 4.2). Agr type I was the most common type, identified in 99 (49.7%) isolates, followed by agr type II in 57 (28.6%) isolates and type III in 37 (18.6%) isolates. Only six isolates (3%) were identified as agr type IV. The distribution of agr types amongst MRSA and methicillin susceptible *S. aureus* (MSSA) isolates was similar, with agr type I being the dominant type followed by agr types II, III and IV (Table 4.2).

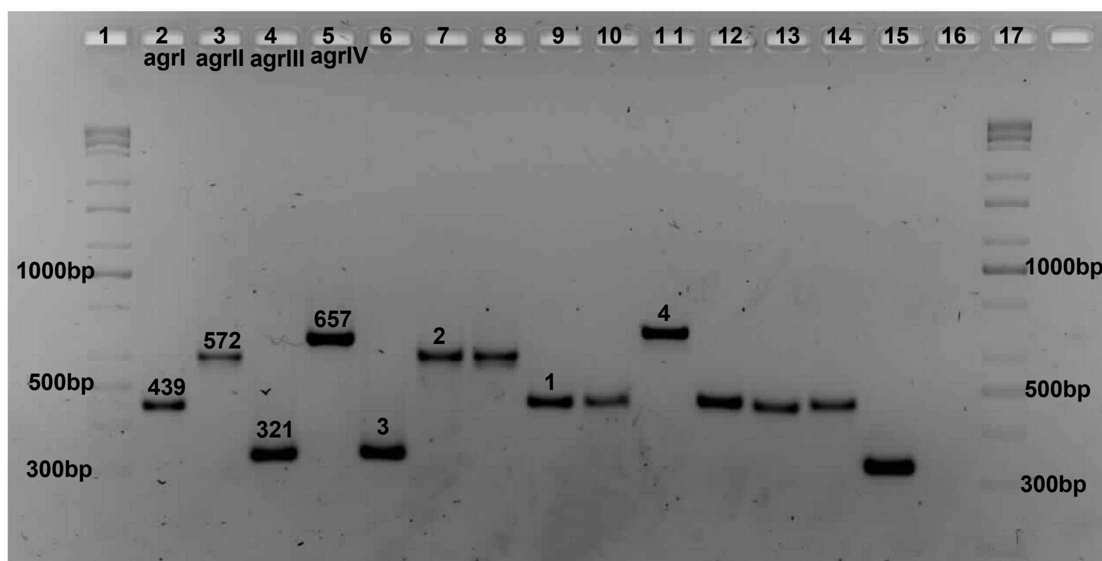

Figure 4.2: Representative agr typing gel. 2.5% agarose gel for the assignment of agr types to isolates. Lane 1 and 17 contain the KAPA Universal Ladder; lanes 2-5 contain the positive controls for agr type I-IV respectively; lanes 6-15 contain ten isolates and lane 16 contains a no template PCR control. Isolates in lanes 9, 10 and 12-14 were identified as agr type I; lanes 7 and 8 as agr type II; lanes 1 and 15 as agr type III and lane 11 as agr type IV.

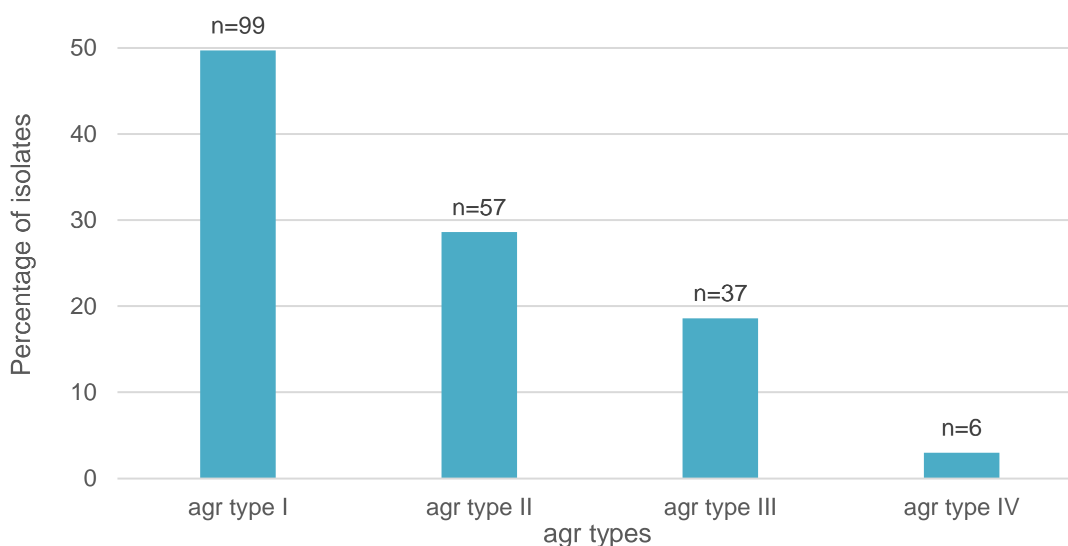

Figure 4.3: Distribution of agr types. The data labels indicate the number of isolates within each agr type.

Table 4.2: Distribution of agr types among MRSA and MSSA isolates.

	Total No (%)	**MSSA No (%)**	**MRSA No (%)**	***p* value***
agr I	99 (49.7)	72 (49.7)	27 (50)	-
agr II	57 (28.6)	37 (25.5)	20 (37)	0.31
agr III	37 (18.6)	30 (20.7)	7 (13)	0.32
agr IV	6 (3)	6 (4.1)	0 (0)	0.57

**p* value <0.05 was interpreted as significant.

Agr functionality

Phenotypic synergistic agr functionality assay

The agr functionality assay, based on the phenotypic detection of δ-haemolysis, identified 25 (12.6%) of the isolates as agr dysfunctional based on the lack of enhanced haemolysis within the β-haemolysis zone of the RN4220 control strain, indicating the absence of δ-haemolysin production (Figure 4.4). Enhanced haemolysis, indicating the production of δ-haemolysin, was detected for the remaining 174 isolates; which were classified as agr functional.

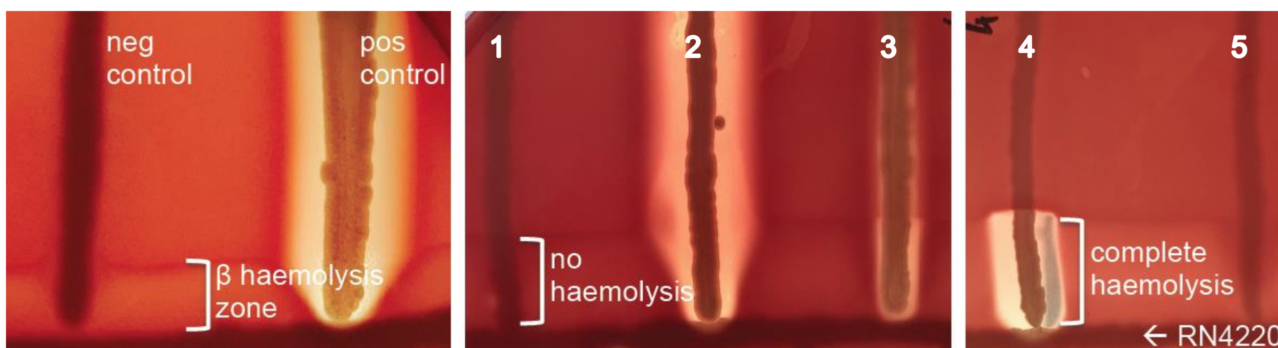

Figure 4.4: Determining agr functionality using the synergistic activity of β- and δ-haemolysin. The negative (NRS155) and positive (NRS149) strains are displayed on the left. Isolates 1 and 5 were classified agr dysfunctional based on of the absence of a complete haemolysis zone within the β-haemolytic zone of RN4220. Isolates 2, 3 and 4 were classified as agr functional, as indicated by the complete haemolysis of the blood cells within the β-haemolytic zone of RN4220.

Screening for δ-haemolysin toxin by MALDI-TOF MS

All isolates were also tested for the presence of the δ-haemolysin toxin using MALDI-TOF MS, as previously described by Gagnaire *et al.* (2012). One hundred and fifty isolates (75.4%) had the $3005{\pm}5^{WT}$ δ-haemolysin toxin peak, five (2.5%) had the $3035{\pm}5^{G10S}$ peak, and an additional 32 (16.1%) isolates had both the $3005{\pm}5^{WT}$ and $3035{\pm}5^{G10S}$ peaks (Figure 4.5, Table 4.3). All 187 (94%) isolates in which peaks were detected were classified as agr functional by MALDI-TOF MS. Twelve (6%) isolates had no peak and were classified as agr dysfunctional by MALDI-TOF MS (Table 4.3).

Figure 4.5: Determining agr functionality by the detection of δ-haemolysin toxin peaks by MALDI-TOF MS. Graphs (a), (b) and (c) represent agr functional isolates. Graph (a) indicates an isolate containing the wild type peak ($3005{\pm}^{WT}$Th). Graph (b) shows an isolate expressing the variant δ-haemolysin toxin ($3035{\pm}5^{G10S}$

Th), while graph (c) shows a strain with both peaks (3005±^{WT} Th and 3035±5^{G10S} Th). The isolate presented in graph (d) is δ-haemolysin negative and contains no peak.

Table 4.3: MALDI-TOF MS agr functionality results.

	MALDI-TOF MS detected δ-haemolysin peaks	Number of isolates
Agr functional	3005±5^{WT}	150 (75.4%)
	3035±5^{G10S}	5 (2.5%)
	Both peaks	32 (16.1%)
Agr dysfunctional	No peak	12 (6%)

Phenotypic synergistic assay vs MALDI-TOF MS

Twenty-five (12.6%) isolates were dysfunctional by the phenotypic synergistic assay and 12 (6%) dysfunctional by MALDI-TOF MS. Ten isolates were dysfunctional by both assays (Table 4.4). Two isolates had no peaks and were dysfunctional by MALDI-TOF MS, but functional by the phenotypic assay, while 15 were phenotypically dysfunctional but still showed peaks by MALDI-TOF MS (Table 4.4). Altogether there were 17 isolates with discrepant results after repeating the phenotypic synergistic assay for the discrepant isolates.

Table 4.4: Comparison of agr functionality results from the phenotypic synergistic and MALDI-TOF MS assays.

		MALDI-TOF MS			
		3005±5^{WT}	3035±5^{G10S}	Both peaks	No peak/dysfunctional
Phenotypic assay	Functional	139	2	31	**2**
	dysfunctional	**11**	**3**	**1**	10

*The numbers in bold are the number of isolates with discrepant results even after the phenotypic assay was repeated**.**

The prevalence of phenotypic agr dysfunctionality was higher among MRSA isolates (18.5%; n=10/54) compared to MSSA isolates (10.3%; n=15/145). In contrast to this, for the MALDI-TOF MS assay, agr dysfunctionality was slightly higher among MSSA (6.2%; n=9/145) isolates compared to MRSA (5.6%; n=3/54) isolates; however neither result was statistically significant (Table 4.5).

Table 4.5: Distribution of agr dysfunctionality among MRSA and MSSA isolates.

	Total No(%)	**MSSA No (%)**	**MRSA No (%)**	***p* value***
Phenotypic dysfunction	25 (12.6)	15 (10.3)	10 (18.5)	0.14
MALDI-TOF MS dysfunction	12 (6)	9 (6.2)	3 (5.6)	0.71

**p* value <0.05 was interpreted as significant

Correlation between agr type and agr functionality

The prevalence of agr dysfunctionality was highest among isolates typed as agr type IV (33.3%; n=2/6), followed by agr type III (32.4%; n=12/37), II (8.8%; n=5/57) and I (6.1%; n=6/99) using the phenotypic test (Table 4.6). However, for the MALDI-TOF MS defined agr dysfunctional isolates, agr type III (10.8%; n=4/37) and agr type II (8.8%; n=5/47) showed the highest dysfunctionality rates (Table 4.6). Only 3% (n=3/99) of the isolates typed as agr type I were agr dysfunctional by MALDI-TOF MS. All agr type IV isolates were agr functional with detectable *hld* peaks by MALDI-TOF MS.

Table 4.6: Proportion of agr types classified as agr dysfunctional by either the phenotypic synergistic assay or MALDI-TOF MS.

	Phenotypic assay	**MALDI-TOF MS**	**Both assays**
Agr I (n=99)	6 (6.1%)	3 (3%)	2 (2%)
Agr II (n=57)	5 (8.8%)	5 (8.8%)	5 (8.8%)
Agr III (n=37)	12 (32.4%)	4 (10.8%)	3 (8.1%)
Agr IV (n=6)	2 (33.3%)	0 (0%)	0 (0%)

Agr related virulence characteristics of strain types

For the purpose of this analysis, the agr typing and agr functionality data were combined with the *spa* typing, SCC*mec* typing and MLST results from Chapter 3, on the neighbour-joining phylogenetic tree generated based on *spa* type, and originally presented as Figure 3.5.

Twelve *spa*-CCs were identified of which six consisted of only agr type I isolates (*spa*-CC 701/2360, *spa*-CC 015/073, *spa*-CC 032/578, *spa*-CC NF9, *spa*-CC NF11, *spa*-CC NF12), two CCs contained only agr type II isolates (*spa*-CC 002, *spa*-CC 084), one *spa*-CC had only agr type III isolates (*spa*-

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CC 174) and one *spa*-CC belonged to agr type IV (*spa*-CC NF10) (Figure 4.6). Two *spa*-CCs (*spa*-CC 012 and *spa*-CC 5916) were mixed and contained isolates belonging to agr types I and III (Figure 4.6).

The dominant strain type among agr type I was t037-ST239-MRSA-IV (n=7) and the most common strain types among agr types II and III were t045-ST5/STNT-MRSA-NV/NT (n=22) and t318-ST865 (n=15) respectively (Table 4.7).

Of the twenty-five isolates that were dysfunctional by the phenotypic synergistic assay, five were t012-MRSA-II (3 MRSA, 2 MSSA), while t045-ST5-MRSA-NV (2 MRSA, 1 MSSA) and t037-ST239-MRSA-III (3 MRSA) each accounted for three isolates. Two isolates were t399 (2 MSSA) and the remaining 12 phenotypically dysfunctional isolates each belonged to a different *spa* type. Three out of the 12 dysfunctional isolates by MALDI-TOF MS were t045-ST5-MRSA-NV (n=3; 2 MRSA, 1 MSSA). One of the isolates was non-typeable and the remaining 8 each belonged to a different *spa* type (Figure 4.6).

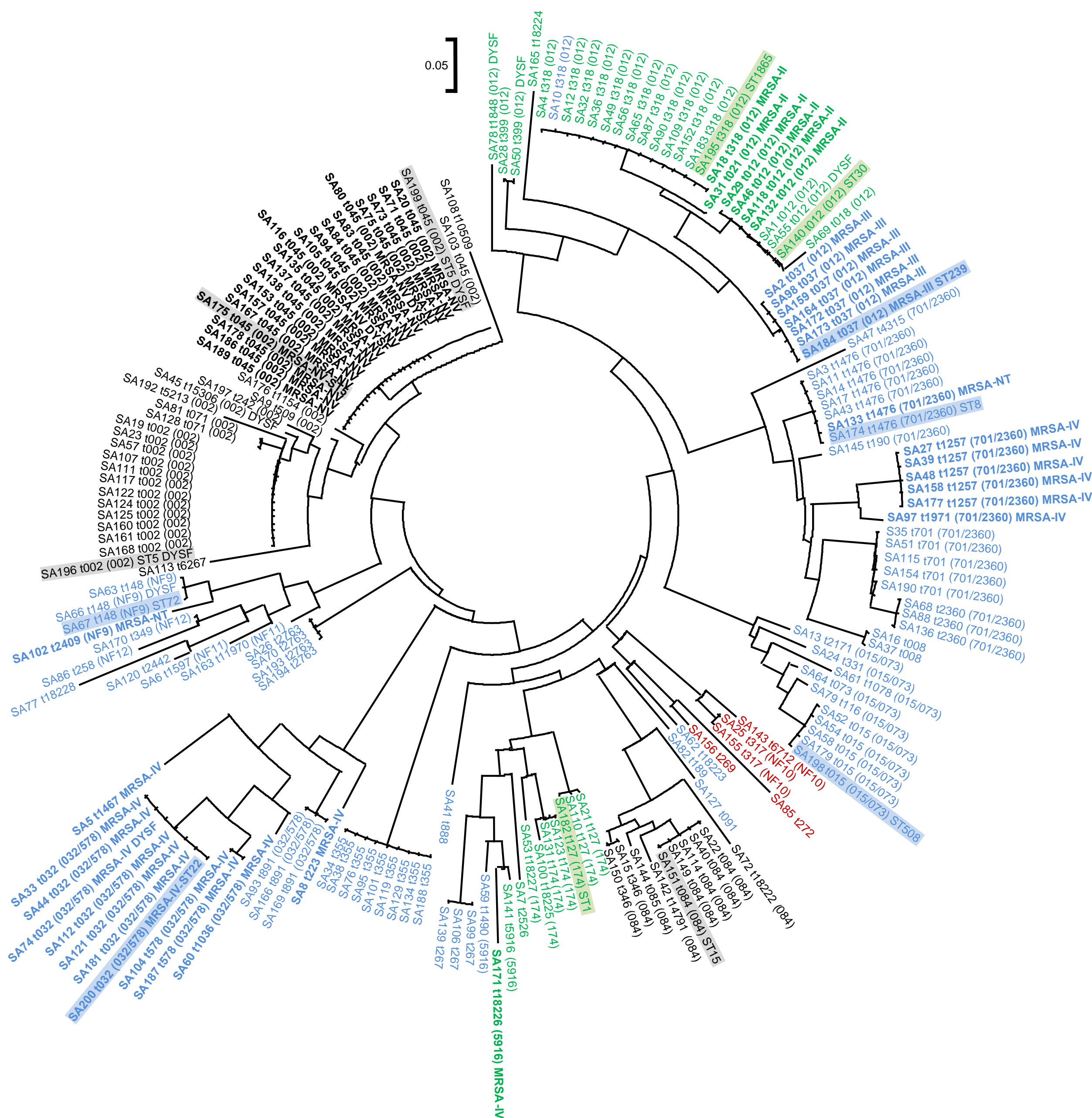

Figure 4.6: Phylogeny of the *S. aureus* isolates based on *spa* type, with reference to agr type, agr functionality, SCC*mec* type and multi-locus sequence type (MLST, where relevant). The *spa* typing results identified 12 *spa*-CCs using the BURP algorithm in Ridom StaphType software. The numbers on the end on each branch represent the strain's *spa* type and the *spa*-CC. The strains in blue are agr type I, black are agr type II, green belongs to agr type III and red agr type IV. The strains in bold are MRSA isolates while the highlighted strains represent isolates selected for MLST. "DYSF" indicates isolates with a dysfunctional agr locus based on both assays.

Table 4.7: Agr type assignment for the dominant strain types as described by *spa* typing, MLST and SCC*mec* typing.

Agr type	Dominant strain types (n)	MSSA/MRSA/Mixed
Agr type I	t037-ST239-MRSA-IV (7)	MRSA
	t032-ST22-MRSA-IV (7)	MRSA
	t1257-MRSA-IV (5)	MRSA
	t015-ST508 (5)	MSSA
	t701 (5)	MSSA
	t355 (9)	MSSA
	t1476-ST8 (7)	6 MSSA, 1 MRSA
Agr type II	t045-ST5/STNT-MRSA-NV/NT (22)	2 MSSA, 20 MRSA
	t002-ST5 (13)	MSSA
	t084-ST15 (5)	MSSA
Agr type III	t318-ST865 (15)	14 MSSA, 1 MRSA
	t012-ST36-MRSA-II (7)	3 MSSA, 4 MRSA

Discussion

In the present study, the agr types of 199 *S. aureus* isolates obtained from patients with bacteraemia at Tygerberg Hospital were determined. Agr type I is reportedly the most prevalent among clinical isolates and agr type IV represents the minority of strains isolated (Traber *et al.*, 2008). Similarly in our study the dominant agr type was agr type I, followed by agr type II, III and IV. This is consistent with studies from the United States and the United Kingdom which also report agr type I as the dominant type followed by agr type II, III and IV (Moore and Lindsay, 2001; Traber *et al.*, 2008; Schweizer *et al.*, 2011). However, this pattern differed in some other studies. For example; a study performed in Spain reported the dominant agr type to be II, followed by I, III and IV (Vindel *et al.*, 2009). Another from France reported agr type I as the dominant type, but this was followed by II, IV and III (Jarraud *et al.*, 2002). In the United States two studies reported agr type I followed by agr type III, II and IV (Shopsin *et al.*, 2003; Wright *et al.*, 2005).

This difference in dominant agr types could be explained by the difference in dominant strain types circulating in that setting. Also, the type of clinical infection from which *S. aureus* was identified might explain these differences, since certain agr types have been shown to be associated with specific types of infections (Robinson *et al.*, 2005). Identical strain types and those closely related to each

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other have been shown, in most cases, to fall within the same agr type. Therefore, dissimilarities in local dominant strain types, outbreaks as well as study design could account for the widely varying distributions of agr types (Dufour *et al.*, 2002; Shopsin *et al.*, 2003; Traber *et al.*, 2008). This can be seen in a study performed on CA-MRSA isolates in Egypt where agr type III and IV were the only reported types (Enany *et al.*, 2010). Furthermore, the dominant strains in the Egyptian study were identified as ST30 and ST80 which are associated with agr type III, explaining the dominance of this type (Ramdani-bouguessa *et al.*, 2006; Enany *et al.*, 2010; Breurec *et al.*, 2011). Agr type III strains are also known to be associated with CA-MRSA which further explains agr type III dominance of agr type III in this Egyptian study, which only investigated CA-MRSA (Robinson *et al.*, 2005).

Two previous studies done at Tygerberg Hospital have also performed agr typing on *S. aureus* isolates; one from clinical specimens other than blood cultures (2009) and another from blood cultures and nasal swabs of the same patient (2010-2012) (Oosthuysen *et al.*, 2013; Karayem, 2014). Agr type distributions similar to ours were reported by all three studies done in this setting. In all three studies, agr type I was dominant among MSSA and MRSA isolates, however, agr type II was the second most prevalent in the 2010-2012 study as oppose to type III amongst MSSA isolates in the 2009 study (Oosthuysen *et al.*, 2013; Karayem, 2014). This difference may be attributed to the difference in dominant strain among the MSSA isolates, which was t891 and agr type III, causing a higher prevalence of agr type III in among these isolates (Oosthuysen *et al.*, 2013). An association between agr type I and invasive infections, especially bacteraemia, has previously been described, while agr type III was linked to non-invasive infections (Ben Ayed *et al.*, 2006). This further explains the higher prevalence of agr type III in the 2009 study, since all clinical sources apart from blood culture were included. Agr type IV was the least common agr type present among MSSA and MRSA isolates for this study as well as the 2009 collection, while no agr type IV isolates were detected among isolates collected in the 2010-2012 study.

Ten of the twelve *spa*-CCs identified in this study contained isolates of only one agr type (Figure 4.6). *spa*-CC 012 and *spa*-CC 5916, however, contain isolates belonging to agr type III and agr type I, suggesting that recombination of agr could have occurred. Two models have been proposed for the evolution of agr and strain types (Wright *et al.*, 2005; Robinson *et al.,* 2005). The first, by Wright *et al.* (2005), suggests that the divergence of agr types preceded the development of mutations that are currently used to distinguish between strains and states that strains are phylogenetically structured according to agr type. This hypothesis is based on the observation that related or corresponding strain types (typed by any strain typing method) were present in only one agr type. Wright *et al.* (2005) noted that a megabase separates the *spa*, multi-locus sequence typing (MLST), *coa* and SCC*mec* loci from the agr locus and speculated that recombination between strains could affect these loci while leaving the agr locus intact. However, a few cases have been reported where strains from a single lineage or CC have different agr types due to intrastrain rearrangements and interstrain recombination (Wright *et al.* 2005; Robinson *et al.* 2005), and this is not accounted for by

the Wright model. Robinson *et al.* (2005) refuted the first hypothesis and suggested that recombination of the agr locus between CCs could result in more than one agr type being present within a CC (Robinson *et al.,* 2005). Genetically identical strains as well as CCs are expected to have the same agr type since variations in housekeeping genes occur approximately 15 times more often by mutations than by recombination. Therefore, because of these exceptions, Robinson *et al.* (2005) presented a model that takes this recombination into account, making it the more acceptable model. They also reported that agr type I and III strains could cluster together, based on visual inspection of phylogenetic trees generated from nucleotide sequences of the hypervariable region of the agr locus (*agrB*, *agrD* and *agrC*) (Robinson *et al.* 2005). This could result in novel, advantageous patterns of agr-mediated gene expression causing different disease presentations within a CC (Robinson *et al.*, 2005). Our results are consistent with the model described by Robinson *et al.* (2005). The relationship between agr type and various clinical features and outcome are interrogated in chapter 5.

Of the 199 isolates, 25 (12.5%) were classified as agr dysfunctional by the phenotypic synergistic assay, which is lower than previously reported at Tygerberg Hospital, using the same method. Karayem (2014) investigated agr functionality amongst isolates from blood cultures and nasal swabs and documented 29.3% of the isolates collected as agr dysfunctional (Karayem 2014). This previous study investigated nasal and blood samples of which 79% of isolates where pairs and 57.4% of all bacteraemia cases were endogenous (same strain in nasal and bacteraemia). The difference in agr dysfunctionality between this and Karayem's (2014) study is not likely due to the difference in clinical samples used since agr dysfunctionality rates among blood cultures and nasal swabs has previously been reported to be similar (Smyth *et al.*, 2012). The difference in the prevalence of agr dysfunctionality may be attributed to the subjectivity of the phenotypic synergistic test; what one reader may call dysfunctional may be called functional or weak functional by another. In our study we tried to overcome this by repeating the phenotypic synergistic test for all isolates in triplicate and having three people read the results. Where discrepancies were identified the phenotypic synergistic test was repeated.

Other studies investigating agr dysfunctionality, from multiple infection sites, using the phenotypic synergistic assay have reported agr dysfunctionality rates of 9% (Sakoulas *et al.*, 2009), 13% (Ferreira *et al.*, 2013), 15% (Tsuji *et al.*, 2011) and 22% (Schweizer *et al.*, 2011); which are more comparable with the findings of this study. Thirteen (6.5%) isolates were dysfunctional by MALDI-TOF MS compared to the 10.1% dysfunctional isolates described in the study performed by Gagnaire *et al.* 2012.

Agr dysfunctionality has been described to be associated with MRSA strains (Sakoulas *et al.*, 2009), however this was not the case in our study. Although not statistically significant, agr functionality was slightly higher among MRSA isolates compared to MSSA isolates using the phenotypic synergistic assay; but lower using MALDI-TOF MS. In addition, there was no association between agr

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dysfunctionality and any specific *spa* type. The 25 agr dysfunctional isolates belonged to 16 different *spa* types and seven different *spa*-CC; and two dysfunctional isolates were singletons. Therefore, dysfunctionality of the agr locus was not clone specific, which is in agreement with results from Shopsin *et al.* (2008). In contrast to this, agr dysfunction has previously been reported to be significantly associated with CC8 and CC30 (Holmes *et al.*, 2014).

In this study, both the phenotypic synergistic assay and MALDI-TOF MS were used to determine agr functionality. The results of the two methods were comparable with results previously reported by Gagnaire *et al.* (2012), who reported a 100% concordance between the results of the two tests. In our study 171 isolates were functional by both methods and 10 were dysfunctional by both, thus 181 isolates had the same result from both methods representing a correlation of 91% between the two tests. There were, however, some discrepant results between the two tests.

Fifteen isolates were identified which showed no δ-haemolysis on sheep blood agar, but had a δ-haemolysin peak on MALDI-TOF, while thirty-two isolates reported as functional by MALDI-TOF MS had both the wild type and variant δ-haemolysin peak (1 dysfunctional and 31 functional by the synergistic assay). A previous study reported cases where different haemolysis patterns were observed in isolates from the same specimen; both agr functional and dysfunctional isolates were present in the same patient (Traber *et al*., 2008). The isolates where shown to be the same strain type. Since the isolates were obtained directly from the infected material, the authors believed that the difference in the δ-haemolysis patterns was not because of post-isolation loss of activity, but rather from a mixed strain population in the patient (Traber *et al.,* 2008). This might in part explain the cases where both the wild type δ-haemolysin peak as well as the variant δ-haemolysin peak were observed, since separate colonies with different agr functionalities from the already mixed population might have been used during these tests. This can be investigated by sequencing the gene encoding δ-haemolysin from multiple colonies from the isolates to confirm whether both wild type and the variant are present.

Three of the agr functional isolates identified by the phenotypic synergistic assay did not have a detectable δ-haemolysin peak on MALDI-TOF MS. It has been reported that synergistic haemolysis with the β-haemolytic strain RN4220 is not exclusively due to δ-haemolysin, but that a series of phenol soluble modulins (PSMS), produced in almost all *S. aureus* strains, also show synergistic haemolysis capabilities on sheep blood agar (Cheung *et al*., 1994). The study concluded that the phenotypic synergistic assay should not be used for detecting the presence of δ-haemolysin, but it is still reliable for determining whether the agr locus is functional since all PSMs are controlled by the agr locus (Cheung *et al*., 1994). This could explain why some isolates from our study have no δ-haemolysin peak by MALDI-TOF MS, but still indicate haemolysis on sheep blood. It could also be speculated that there are variants, other than the G10S variant, resulting in the production of a protein that is not represented by the known peak sizes currently described and was therefore not

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screened for when looking at peaks by MALDI-TOF MS. To confirm this, further investigation is required. Further research may include sequencing of the agr locus, specifically *agrA, B, C* and *D,* to identify whether there are mutations, other than the ones already described, responsible for agr dysfunctionality. The gene encoding δ-haemolysin can be sequenced to determine whether there are alternative mutations resulting in a different MALDI-TOF MS peak that was not detected. Investigation of the expression levels of the agr effector molecule RNAIII may also be used to further investigate agr functionality.

Conclusion

In conclusion, this chapter describes the prevalence of the four different agr types and agr dysfunctionality amongst the *S. aureus* strains isolated from blood cultures from patients at Tygerberg Hospital. Agr type I was the most common agr type and there was no significant difference in the distribution of agr types in MRSA and MSSA isolates. The dominant strain in this study was agr type II (t045-ST5-MRSA-NV) especially among MRSA isolates. The most prevalent strain type among MSSA isolates was agr type III (t318-ST1865). Two assays were used to determine the rate of agr dysfunctionality among the isolates and the results of the two assays were, for the most part, comparable; however, some discrepancies were identified. Agr dysfunctionality was not common in this study and it was not significantly different between MRSA and MSSA isolates. Agr dysfunctionality was not associated with any specific clone. This study provides the basis for further studies that aim to determine the effects of agr type and agr functionality/dysfunctionality on *S. aureus* virulence and clinical disease (Chapter 5).

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CHAPTER 5: The impact of strain characteristics on disease presentation and clinical outcome

Introduction

Staphylococcus aureus is an opportunistic and highly virulent pathogen, able to cause life-threatening infections. Many clinical settings are dedicating a substantial amount of resources to screen for methicillin-resistance (through *mec*A gene detection) to ultimately improve patient outcome, since an association between increased mortality and methicillin-resistant *S. aureus* (MRSA) isolates, especially in the context of bacteraemia, has been described (Cosgrove *et al.*, 2003; Schweizer *et al.*, 2011). There are, however, other microbiological characteristics that may be equally important to detect in the pursuit to improve patient outcome.

Certain strain types and agr related virulence characteristics may be predictors or risk factors for clinical outcomes such as mortality and increased length of stay. Agr dysfunctional strains, for example, have been linked to persistent bacteraemia, reduced susceptibility to vancomycin as well as an increased mortality (Fowler, Jr. *et al.*, 2004; Sakoulas *et al.*, 2005; Schweizer *et al.*, 2011). Therefore, *S. aureus* strain typing and virulence profiling are essential to track and reduce the spread of infection (Earls *et al.*, 2017).

Determining the exact role of the genetic background and virulence profile of *S. aureus* isolates on patient outcome has become challenging because of the host specificity of virulence profiles as well as the high diversity of these isolates (Pérez-Montarelo *et al.*, 2018). Because of these difficulties, data comparing strain types and agr related virulence characteristics with clinical outcome and disease presentation in our setting are limited. Insight into this may provide progress in treatment of staphylococcal disease. The purpose of this chapter was to assess associations between strain types and agr related virulence characteristics and clinical presentation and patient outcome.

Methodology

This study included 199 patients of all ages with *S. aureus* bacteraemia at Tygerberg Hospital between January 2015 and March 2017, from whom non-duplicate *S. aureus* isolates were collected, as described in Chapter 2. Clinical and demographical data were documented by the Group for Enteric, Respiratory and Meningeal Disease Surveillance in South Africa (GERMS-SA). Isolates were classified as MRSA using the Vitek®2 system (bioMérieux, France).

Logistic regression models for categorical variables were used to assess associations between the final outcomes, mortality and methicillin resistance; and various patient- and isolate-related variables, using both univariable and multivariable analyses. The variables included were age group, gender, diagnosis, place of onset of infection (HA, HCA and CA) (Chapter 2), *spa*-CC (Chapter 3),

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and agr type and agr functionality (Chapter 4). For the purpose of this analysis, the *spa*-CCs were grouped into five groups, namely, *spa*-CC002, *spa*-CC012, *spa*-CC701/2360, singletons and other (*spa*-CC 015/037, *spa*-CC 084, *spa*-CC 032/578, *spa*-CC 174, *spa*-CC 5916, *spa*-CC NF9, *spa*-CC NF10, *spa*-CC NF11 and *spa*-CC NF12). In the multivariable analysis, stepwise elimination was done for all variables with *p*-values of <0.1. Length of stay was analysed as a continuous variable; therefore, the two-sample Wilcoxon rank-sum (Mann-Whitney) test and survival analysis were used for univariable and multivariable analysis, respectively. In the survival analysis, the final outcome was length of stay and discharge was used as the failure event. Statistical analysis was done on Stata V15 (StataCorp LLC, USA) and *p*-values of <0.05 were interpreted as significant.

Results

Mortality

The univariable analysis showed an association between age group and mortality, with a significant increase in mortality in the elderly age group compared to neonates (*p*=0.007) (Table 5.1). This was confirmed by the multivariable analysis which showed a significant increase in mortality in adults (OR=2.28; 95% CI: 0.92 – 5.62; *p*=0.073) and elderly patients (OR=7.48; 95% CI: 2.82 – 19.89; *p*<0.001), in comparison to neonates (Figure 5.1). No significant associations between mortality and gender, diagnosis, place of onset of infection, *spa*-CC, and agr type or dysfunctionality were identified in the univariable analysis (Table 5.1). However, HA infections were associated with higher mortality compared to CA infections, based on the multivariable analysis (OR= 2.26; 95% CI: 1.12 – 4.55; *p*=0.023).

MRSA

There is an association between MRSA infection and age based on the univariable analysis, with neonates being more likely to have an MRSA infection than other age groups (Table 5.1). This was however not the case in the multivariable analysis, where there was no significant association between MRSA and age (Figure 5.1). MRSA was associated with HA infections in both the univariable and multivariable analyses (Table 5.1, Figure 5.1). *spa*-CC 002 was associated with MRSA in both analyses, with a lower MRSA prevalence amongst the singletons and *spa*-CCs grouped as “other” (Table 5.1, Figure 5.1). There were no associations between MRSA and gender, diagnosis, agr type or dysfunctionality.

Length of stay

There were significant associations between length of stay and age, place of onset of infection, *spa*-CC and agr types, based on the univariable analysis (Table 5.1). However, for the multivariable analysis, only place of onset of infection was significantly associated with the length of stay (Figure

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5.1). HA infections were associated with a shorter length of stay compared to CA infections (OR=0.48; 95% CI: 0.30 – 0.76; p=0.002), while HCA infections are associated with an increased length of stay (OR=1.97; 95% CI: 1.13 – 3.42; p=0.017). There was no significant association between length of stay and agr functionality in the univariable analysis. However, a borderline significant association between agr functionality and an increased length of stay was identified in the multivariable analysis (OR=1.66; 95% CI: 0.93 – 2.99; p=0.089) (Figure 5.1). There were no associations between length of stay and gender or diagnosis in either the univariable or multivariable analysis.

Table 5.1: Univariable analysis of age group, gender, diagnosis, place of onset of infection, strain type, agr type and functionality with mortality, MRSA infections and length of stay.

Variable	Clinical outcome						
	Mortality			MRSA			Length of stay
	OR	**95% CI**	***p*-value**	**OR**	**95% CI**	***p*-value**	***p*-value**
Age group							**0.002**
Neonates	-	-	-	-	-	-	-
Children	0.98	0.24 – 4.00	0.978	0.25	0.08 – 0.81	**0.021**	-
Adults	2.55	0.80 – 8.17	0.115	0.26	0.10 – 0.70	**0.008**	-
Elderly	5.41	1.59 – 18.39	**0.007**	0.16	0.05 – 0.51	**0.002**	-
Gender							0.901
Male compared to Female	1.12	0.61 – 2.04	0.717	0.62	0.33 – 1.18	0.145	-
Diagnosis							0.169
Bacteraemia without focus compared to with focus	1.76	0.87 – 3.55	0.114	1.81	0.85 – 3.87	0.124	-
Place of onset of infection							**<0.001**
CA	-	-	-	-	-	-	-
HA	1.28	0.59 – 2.77	0.530	12.83	2.94 – 56.09	**0.001**	-
HCA	0.71	0.27 – 1.80	0.466	3.32	0.65 – 17.03	0.150	-
***spa*-CC**							**0.045**
spa-CC002	-	-	-	-	-	-	-
spa-CC012	1.11	0.44 – 2.84	0.821	0.68	0.27 – 1.71	0.413	-
spa-CC701/2360	1.65	0.59 – 4.64	0.342	0.48	0.16 – 1.41	0.182	-
Singletons	0.94	0.36 – 2.47	0.905	0.07	0.02 – 0.35	**0.001**	-
Other *spa*-CCs	0.98	0.44 – 2.19	0.964	0.24	0.10 – 0.56	**0.001**	-
agr type							**0.044**
I	-	-	-	-	-	-	-
II	0.73	0.37 – 1.44	0.368	1.44	0.72 – 2.91	0.307	-
III	0.50	0.22 – 1.15	0.103	0.62	0.24 – 1.58	0.319	-
IV	0.68	0.12 – 3.88	0.663	1.00	-	-	-
Phenotypic synergistic agr assay							0.451
Functional compared to dysfunctional	0.72	0.31 – 1.67	0.452	0.51	0.21 – 1.21	0.127	-

*OR – Odds Ratio, CI – Confidence Interval, HR – Hazards Ratio, Other *spa*-CC – *spa*-CC 015/037, *spa*-CC 084, *spa*-CC 032/578, *spa*-CC 174, *spa*-CC 5916, *spa*-CC NF9, *spa*-CC NF10, *spa*-CC NF11, *spa*-CC NF12. The statistically significant results are highlighted in bold.

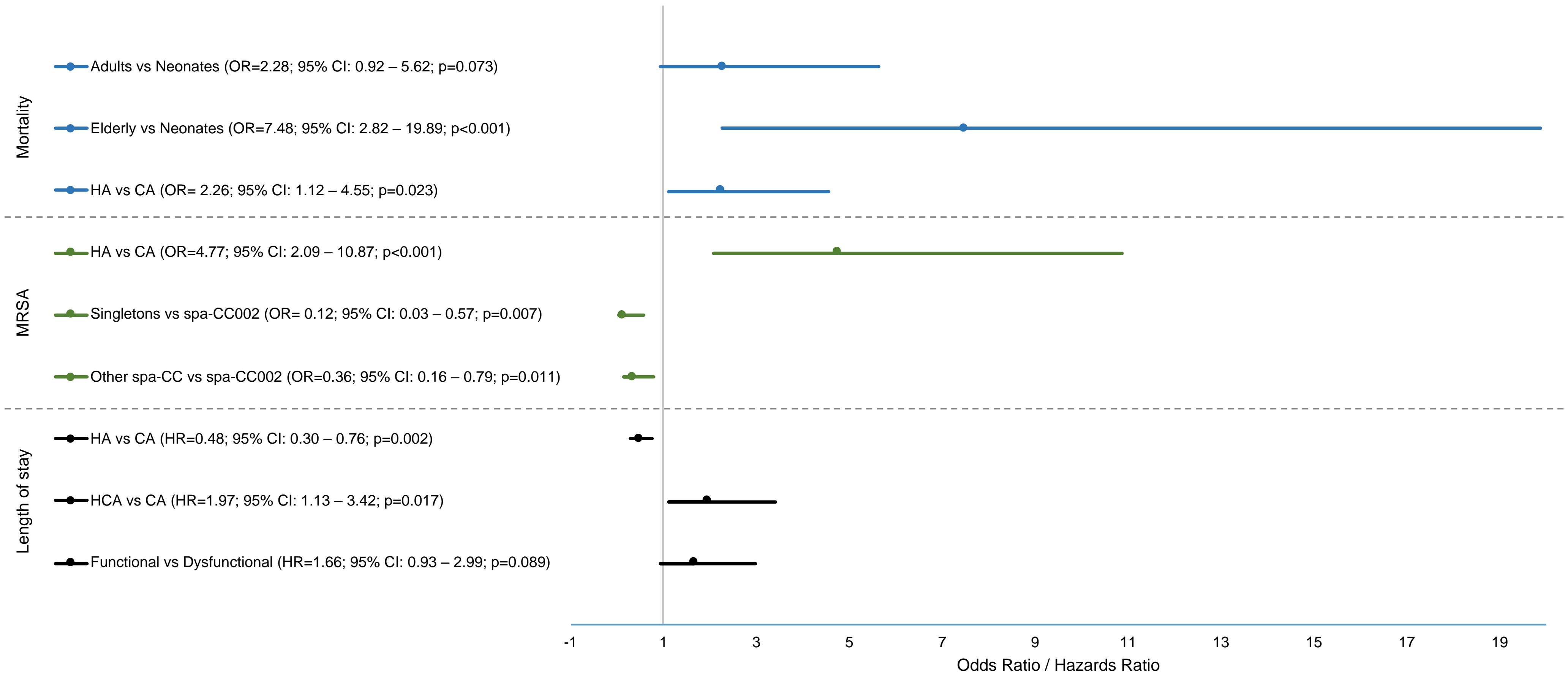

Figure 5.1: Independent risk factors for the clinical outcomes mortality, MRSA infection and length of stay presented in forest plot form, following the multivariable analysis. Variables associated with mortality were highlighted in blue, while variables associated with MRSA infections are in green. Black are those associated with length of stay when the variable "discharged" is considered as failure for the survival analysis. Mortality and MRSA infection results were reported as odds ratio, and length of stay was reported as hazards ratio.

Discussion

Complicated bloodstream infections caused by *S. aureus* are difficult to treat and have a mortality rate ranging from 29-63% (Perovic *et al.*, 2015). Specific host and pathogen factors are known to predict poor outcomes and less effective response to vancomycin (Schweizer *et al.*, 2011). This chapter discusses possible associations between strain characteristics (strain type and agr related virulence) and clinical presentation and patient outcome.

In our study, older age was shown to be a risk factor for mortality. This is inconsistent with a previous study performed on *S. aureus* bacteraemia isolates from patients in Johannesburg, which found no association between older age and mortality (Perovic *et al.*, 2006). This difference may be attributed to the difference in the definition of age groups and mortality; in our study crude in-hospital mortality was used, whereas the Perovic study used 14-day mortality and also reported a low median age. However, our results are consistent with a previous study performed in Gauteng and the Western Cape, which compared HA-MRSA and CA-MRSA with crude in hospital mortality data collected by GERMS-SA (Perovic *et al.*, 2017); and increased mortality (within 12 weeks of first blood culture) among elderly patients was also reported by a study performed in the USA (McClelland *et al.*, 1999). Interestingly, they also reported that elderly patients were three times more likely to not present with a fever prior to diagnosis, which may have resulted in a delayed diagnosis and treatment. Other studies have reported that poor outcomes in bacteraemia are related to delays in starting treatment with the correct antibiotics (McClelland *et al.*, 1999). The increased mortality among elderly patients may also be attributed to the fact that older patients have a higher risk of HA infections because of immunosenescence, impaired wound healing and multiple comorbidities (McGarry *et al.*, 2004). In this study, HA infections were also associated with an increased mortality, which further explains the association between age and mortality.

Another factor that may contribute to poorer outcomes in elderly patients is the commonly described association between elderly patients and MRSA infections (Soriano *et al.*, 2000; McGarry *et al.*, 2004; Cosgrove *et al.*, 2005; Struelens *et al.*, 2009). This was however not the case in our study. Elderly patients were less likely to have MRSA infections, based on the univariable analysis, but this association was not confirmed in the multivariable analysis. The MRSA outbreak in the paediatric and neonatal wards during 2015 and 2016 may have influenced this result.

HA infections were associated with all three outcomes namely, mortality, MRSA infection and length of stay. The association between HA infections and MRSA is not surprising since the antibiotic selective pressure is higher in hospitals than in the community. Isolates that have acquired mobile genetic elements that offer advantages are selected for in hospitals resulting in a higher MRSA prevalence in hospitals compared to the community (Stefani *et al.*, 2012). The association between HA infections and an increased mortality may be linked to the association between HA infections and MRSA, since previous studies, performed in different settings, have reported higher mortality

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due to the complications associated with MRSA bacteraemia compared to MSSA bacteraemia (Soriano *et al.*, 2000; Topeli *et al.*, 2000; Cosgrove *et al.*, 2003; Perovic *et al.*, 2006). Although there was no significant difference in mortality between MRSA and MSSA infections in this study, a meta-analysis performed on 31 studies comparing mortality in patients with MRSA infections to MSSA infections reported an overall increase in mortality among patients with MRSA infections. Interestingly, this association reached significance only in 7 out of the 31 studies included in the meta-analysis, the reason being that many of the studies were small and didn't have power to detect significant associations. However, the pooled analysis showed an apparent association between increased mortality and MRSA infections (Cosgrove *et al.*, 2003).

HA infections were also associated with a shorter length of stay. This is inconsistent with a previous study which investigated the effects of *S. aureus* bacteraemia on patient outcomes. The study reported increased length of stay among patients with HA infections (Cosgrove *et al.*, 2005). The paradoxical reduction in length of stay among patients with HA infections observed in this study may be due to the paediatric and neonatal MRSA outbreak which may have resulted in cases of less complicated bacteraemia amongst neonates, which would have "resolved" more rapidly than complicated *S. aureus* bacteraemia.

Our molecular epidemiology results (Chapter 3) suggest a high diversity among *S. aureus* strains causing bacteraemia in our setting. However, there was a noteworthy difference in clonality between MRSA and MSSA, with MSSA isolates being more genetically diverse, which is consistent with previous studies performed in the USA and Europe (Miko *et al.*, 2013; Grundmann *et al.*, 2014; Park *et al.*, 2017; Pérez-Montarelo *et al.*, 2018). CC5 was the most prevalent lineage among MRSA strains, accounting for 64% of the MRSA strains, with more than half of these belonging to *spa*-CC002. *spa*-CC 002 (CC5) was identified as a risk factor of MRSA infections. It is worth noting that the outbreak strain (t045) which forms part of *spa*-CC 002, represented the majority of the MRSA strains, possibly contributing to this association. A recent study performed in Spain, also reported a significant association between CC5 and MRSA infections, while CC30, CC45 and CC15 were associated with MSSA infections (Pérez-Montarelo *et al.*, 2018). Although we cannot report a direct association between CC30, CC45 and CC15 with MSSA isolates, these CCs were present among the singletons and the *spa*-CCs grouped in "other", which were negatively associated with MRSA.

The agr locus is known to be involved in the regulation of 23 virulence genes (George and Muir, 2007). This observation has led to the assumption that the agr locus is important in staphylococcal pathogenesis (Kong *et al.*, 2006). However, there are many studies with contradicting results regarding the importance of a functional agr locus in staphylococcal pathogenesis. Our multivariable analysis identified a borderline significant association between agr functionality and increased length of stay, while there were no associations between agr functionality and mortality or MRSA infections. However, the number of dysfunctional isolates in our study is very small, which may have affected our ability to detect significant differences. within contrast to our results, a study performed in the

USA reported significantly increased mortality among bacteraemia patients infected with agr dysfunctional strains (Schweizer *et al.*, 2011). The study reported a high prevalence of MRSA infections and therefore a large proportion (86%) of the patients were treated with vancomycin. According to pharmacodynamic models, vancomycin has reduced bactericidal activity against agr dysfunctional strains (Sakoulas *et al.*, 2005), therefore it was concluded that the association between agr dysfunction and mortality may have been linked to the reduced bactericidal activity of vancomycin against agr dysfunctional strains (Schweizer *et al.*, 2011). While our results suggest a shorter length of stay among patients infected with agr dysfunctional strains, other studies have reported associations between agr dysfunctionality and persistent bacteraemia, which could result in an increase in hospital stay (Fowler, Jr. *et al.*, 2004; Chong *et al.*, 2013).

There was also no significant association between agr dysfunction and MRSA infections in our study, which is contradictory to previous studies which reported a high prevalence of agr dysfunctionality among MRSA isolates (Gagnaire *et al.*, 2012; Jang *et al.*, 2012). Agr dysfunctionality has previously been described to be less prevalent among SCC*mec* IV isolates compared to SCC*mec* I-III (Jang *et al.*, 2012). SCC*mec* IV was the dominant SCC*mec* type present among our MRSA isolates, which might account for the lower prevalence of agr dysfunction in our study, and may explain why no association between agr dysfunction and MRSA was identified.

A limitation to this study is that many patients had incomplete or missing data profiles, especially with regards to the focus of the bacteraemia. The diagnosis assigned to the majority of the patients was bacteraemia without focus, however it is possible that the focus of the bacteraemia was unknown at the time of data collection by the GERMS-SA surveillance officer or that thorough investigation of the focus was not done; and this may have overestimated the number of patients with this diagnosis. In a previous study, bacteraemia with respiratory infection as the focus was associated with MRSA and increased mortality, while bacteraemia as a result of skin and soft tissue infections was associated with a lower mortality and a shorter length of stay (Cosgrove *et al.*, 2005). Different agr types have also been associated with certain disease types, such as agr types I and II with endocarditis (Jarraud *et al.*, 2002). We were unable to investigate similar associations, since the majority of the patients included in our study were diagnosed with bacteraemia without focus.

Conclusion

This section investigated associations between strain types and agr related virulence characteristics identified in Chapters 3 and 4 and the clinical outcomes and presentations described in Chapter 2. Adults and elderly patients were at higher risk for mortality, which is consistent with previous studies. HA infections were associated with MRSA, higher mortality as well shorter length of stay.. A previously described association between *spa*-CC 002 (CC5) and MRSA was also identified in our setting, however, this association may have been driven by an outbreak since *spa*-CC 002 included the outbreak strain t045. Identifying associations between strain characteristics and clinical data may

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lead to a better understanding of how different strain types and the agr regulator contribute to *S. aureus* virulence; and may ultimately aid in the development of alternative treatment plans or prevention of staphylococcal disease.

CHAPTER 6: Concluding remarks

This study sought to describe the molecular epidemiology of *S. aureus* isolates identified from bacteraemic patients at Tygerberg Hospital, and to investigate the possible associations between microbial characteristics and clinical outcome and presentation. In South Africa, data describing the link between epidemiology and virulence characteristics, and clinical outcome are limited. Therefore, our study may provide a baseline overview for future investigations.

We noted that MRSA accounted for 27% of the 199 *S. aureus* isolates included in this study, which was lower than the 33% and 24% documented in 2015 and 2017 respectively in Gauteng and the Western Cape by GERMS-SA (Crowther-Gibson & Quan 2016; Crowther-Gibson & Quan 2017). *Spa* typing data suggested a highly diverse *S. aureus* population circulating within Tygerberg Hospital, this is reflected by the 73 different *spa* types identified. This genetic diversity was driven by MSSA strains, since MRSA strains were more clonal; which is consistent with literature (Miko *et al.*, 2013; Grundmann *et al.*, 2014; Park *et al.*, 2017; Pérez-Montarelo *et al.*, 2018). Both locally emerged and pandemic clones were identified in this study; t045-ST5-MRSA-NV (novel variant) was the most dominant MRSA clone and it seems to be circulating in hospital settings within South Africa as shown by previous studies. This clone was associated with a paediatric and neonatal outbreak at Tygerberg Hospital in 2015 and 2016. t1257-ST612-MRSA-IV is also a local dominant clone which also seems to be well adapted to health care settings nationwide. Pandemic clones such as t037-ST239-MRSA-III, t032-ST22-MRSA-IV and t012-ST36-MRSA-II were also identified in this study. Statistical analysis showed that MRSA strains were associated with hospital acquired (HA) infections, which is not surprising considering antibiotic selective pressure is higher in hospitals compared to the community and spread of resistant strains in hospitals is common (Stefani *et al.*, 2012).

The overall crude mortality rate was 29% and older patients were found to be the most affected. This is in agreement with previous studies (McClelland *et al.*, 1999; Perovic *et al.*, 2017). Mortality was also associated with HA infections, which could be explained by the complicated nature of these infections leading to death (Soriano *et al.*, 2000; Topeli *et al.*, 2000; Cosgrove *et al.*, 2003; Perovic *et al.*, 2006).

Agr typing revealed that agr type I was the most common type and there was no significant difference in the distribution between MSSA and MRSA isolates. We compared the agr functionality of the included isolates using two assays, the phenotypic synergistic assay and matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS). This was done due to subjectivity linked to the phenotypic synergistic assay. However, the findings from these assays were comparable, with limited discrepancies. The discrepancies may be due to the fact that phenol soluble modulins (PSMs), which are also controlled by the agr locus, can also result in synergistic haemolysis with the β-haemolytic strain RN4220 on sheep blood agar (Cheung *et al.*, 1994). Therefore, the phenotypic synergistic assay investigates crude activation of the agr locus, while

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MALDI-TOF MS investigates only the presence of δ-haemolysin. Nevertheless, the phenotypic assay identified 12.6% dysfunctional isolates as opposed to 6% identified by MALDI-TOF MS. Overall, this dysfunctionality rate was low and did not show any clonal preference, in keeping with results from Shopsin *et al.* (2008); whereas some studies have shown that agr dysfunctionality was linked with CC8 and CC30 (Holmes *et al.*, 2014). The association between agr functionality status and clinical outcome is controversial. We observed a trend towards a significant association between agr dysfunctionality and a shorter length of hospital stay, however the number of dysfunctional strains in this study is small and ascertaining the reason for this association would require further investigation.

There were no associations between patient diagnosis and mortality, MRSA or length of stay. This may be because majority of the patient diagnoses were documented as bacteraemia without focus; possibly because a thorough investigation of the focus was not done or the focus of the bacteraemia was unknown at the time of collection by the GERMS-SA surveillance officer.

This study provided insights into the associations between microbial characteristics and clinical outcome despite the limited clinical data used. This should be overcome in planning future studies. Future directions may include investigating additional associations between agr-related virulence factors and clinical features such as treatment, severity of illness or diagnosis. Different disease types have been associated with different outcomes; for example respiratory infections are associated with increased mortality, while skin and soft tissue infections have a lower mortality (Cosgrove *et al.*, 2005). Therefore, this study could be extended to isolates from all clinical specimens, to allow the identification of associations between the type of infection and outcome, as well as agr type, agr functionality and strain type. Further research may also include sequencing of the agr locus to identify possible mutations responsible for agr dysfunctionality, and reverse transcription quantitative PCR to investigate RNAIII expression; to further investigate discrepancies between the phenotypic synergistic assay and MALDI-TOF MS. Furthermore, experiments to describe the effect of agr dysfunction on the physiology of *S. aureus* isolates may also provide insight into disease presentation among agr dysfunctional strains. Altogether these investigations could add to the understanding of specific agr-related virulence profiles and may ultimately improve patient outcome.

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