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Molecular characterisation of methicillin-resistant *Staphylococcus aureus* isolates associated with outbreaks in burn wound and neonatal ward patients at healthcare centres in Gauteng, South Africa

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Molecular characterisation of methicillin-resistant *Staphylococcus aureus* isolates associated with outbreaks in burn wound and neonatal ward patients at healthcare centres in Gauteng, South Africa

by

KEITUMETSE BOGATSE GAMA

Submitted in fulfilment of the requirements for the degree

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DECLARATION

I, the undersigned, declare that the dissertation hereby submitted to the University of Pretoria for the degree MSc (Medical Microbiology) and the work contained herein is my own original work and has not previously, in its entirety or in part, been submitted to any university for a degree. I further declare that all sources cited are acknowledged by means of a list of references.

Signed __________this__________ day of____________2019

“Stay far from timid, only make moves when your heart’s in it and live the phrase sky’s the limit”

-The Notorious B.I.G

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LIST OF ABBREVIATIONS

AIDS	:	Acquired immunodeficiency syndrome
CA	:	Community-associated
CC	:	Clonal complex
CCR	:	Cassette chromosome recombinase
CHIPS	:	Chemotaxis inhibitory protein of staphylococci
CLSI	:	Clinical Laboratory Standards Institute
CONS	:	Coagulase negative staphylococci
DNA	:	Deoxyribonucleic acid
ESKAPE	:	*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* species
EUCAST	:	European Committee on Antimicrobial Susceptibility Testing
G+C	:	Guanine-cytosine
HA	:	Healthcare-associated
HAI	:	Healthcare-associated infection
HCW	:	Health care workers
IEC	:	Immune evasion complex
IS	:	Insertion sequence
J-region	:	Joining region
kDa	:	Kilo Dalton
MALDI-TOF	:	Matrix assisted laser desorption ionization-time of flight
Mbp	:	Mega base pair/s

MGE	:	Mobile genetic element
MBC	:	Minimum bactericidal concentration
MIC	:	Minimum inhibitory concentration
MLST	:	Multilocus sequence typing
M-PCR	:	Multiplex polymerase chain reaction
MRSA	:	Methicillin-resistant *Staphylococcus aureus*
MSCRAMMS	:	Microbial surface components recognizing adhesive matrix molecules
MSSA	:	Methicillin-susceptible *Staphylococcus aureus*
NaCl	:	Sodium chloride
NHLS	:	National Health Laboratory Service
NICD	:	National Institute for Communicable Diseases
NICU	:	Neonatal intensive care unit
*orf*X	:	Open reading frame X
PCR	:	Polymerase chain reaction
PBP	:	Penicillin binding protein
PFGE	:	Pulsed-field gel electrophoresis
PVL	:	Panton-Valentine Leukocidin
RNA	:	Ribonucleic acid
S. aureus	:	*Staphylococcus aureus*
SaPI	:	Staphylococcal pathogenicity island
SCC	:	Staphylococcal cassette chromosome
SCIN	:	Staphylococcal complement inhibitors

SCV	:	Small colony variant
SERAM	:	Secretable expended repertoire adhesin molecules
SIRS	:	Systemic inflammatory response syndrome
*Sma*I	:	*Serratia marcescens* I endonuclease
Spa	:	Staphylococcal protein A
SSSS	:	Staphylococcal scalded skin syndrome
SSTI	:	Skin and soft tissue infections
ST	:	Sequence type
TAD	:	Tshwane Academic Division
TSS	:	Toxic shock syndrome
TSST	:	Toxic shock syndrome toxin
UPGMA	:	Unweighted pair group method with arithmetic mean
UV	:	Ultraviolet
VISA	:	Vancomycin intermediate *Staphylococcus aureus*
VRE	:	Vancomycin resistant enterococci
VRSA	:	Vancomycin resistant *Staphylococcus aureus*
WGS	:	Whole genome sequencing
WHO	:	World Health Organization

LIST OF PUBLICATIONS AND CONFERENCE CONTRIBUTIONS

CONFERENCE PRESENTATIONS

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MOLECULAR CHARACTERISATION OF METHICILLIN-RESISTANT *Staphylococcus aureus* ISOLATES ASSOCIATED WITH OUTBREAKS IN BURN WOUND AND NEONATAL WARD PATIENTS AT HEALTHCARE CENTRES IN GAUTENG, SOUTH AFRICA

by

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SUMMARY

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the leading causes of nosocomial infections worldwide. It is an ESKAPE pathogen and is known for causing difficult-to-treat infections due to its antibiotic resistance. In addition to its antibiotic resistance, this bacterium has a large arsenal of virulence factors that allow this pathogen to cause disease and to evade the host immune system. An increase in the number of reports of MRSA isolates from the burn unit and neonatal wards from various hospitals across the Gauteng province prompted this study. The aim of this study was to molecularly characterise the MRSA isolates associated with outbreaks in the burn and neonatal wards at four hospitals in Gauteng, South Africa using multiplex polymerase chain reaction (M-PCR) assays, pulsed-field gel electrophoresis (PFGE) and whole genome sequencing (WGS). The study also aimed to determine the antibiotic and virulence gene profiles associated with these MRSA strains.

To identify MRSA, a M-PCR assay was performed to confirm the presence of the *Staphylococcus* 16S rRNA gene, the *S. aureus*-specific nuclease (*nuc*) gene and the methicillin A (*mecA*) gene that confers resistance to beta-lactam antibiotics. The isolates were also screened for the Panton-Valentine Leukocidin (*pvl*) gene, which encodes a pore forming toxin associated with severe disease. All 85 isolates were confirmed to be MRSA and none of the isolates were *pvl*-positive. Susceptibility testing of the MRSA isolates revealed that the isolates were resistant to antibiotics such as penicillin (100%), cloxacillin (100%), gentamicin (98%), clindamycin (97%), erythromycin (97%), ciprofloxacin (91%) and tetracycline (84%). Susceptibility to vancomycin, teicoplanin, linezolid and fusidic acid was observed.

The dendrogram constructed based on the PFGE banding profiles revealed that the MRSA isolates clustered into three major pulsotypes. The largest pulsotype, Pulsotype A, consisted of 32 MRSA isolates that were recovered from burn and neonatal wards. Pulsotypes B and C were made up of five isolates each and only consisted of isolates from the neonatal wards. All three pulsotypes were composed of MRSA isolates from different hospitals, recovered between 2015 and 2019. Five representative isolates were selected based on their clustering and antibiotic resistance and sent for WGS. Three clones, ST239-MRSA-III, ST5-MRSA-I and ST612-MRSA-IV were identified using WGS data. These clones were associated with *spa* types t037, t045 and t1257 respectively. The clone ST239-MRSA-III-t037 was detected in three different hospitals. The virulence factors detected in the five isolates included staphylococcal enterotoxins A (SEA), SEB, SEG, SEK, SEN, SEO, and SEQ and the bi-component pore-forming leukocidins, gamma-hemolysin and leucocidin ED. The immune evasion complex (IEC) genes identified were the staphylococcal complement inhibitor, staphylokinase and SEA.

The movement of patients and healthcare workers between wards and hospitals may have resulted in intra- and inter-hospital spread of MRSA. The study emphasises the importance of having infection control programs in place and adhering to them. The importance of continuous surveillance is also emphasised.

Keywords: MRSA, neonates, burn wound patients, antibiotic resistance, virulence, STs

CHAPTER 1

1.1 Introduction

Staphylococcus aureus (*S. aureus*) is a Gram-positive bacterium belonging to the family *Staphylococcaceae* that colonises the skin, mucous membranes and anterior nares of healthy individuals (Green *et al*., 2012). Despite its ability to asymptomatically colonise humans, this opportunistic pathogen has the ability to cause serious disease in individuals at risk such as those with a compromised immune system due to diabetes mellitus, cancer therapy or chronic kidney failure, patients with indwelling catheters, infants and burn patients (Green *et al*., 2012; Mahon *et al*., 2015; Santosaningsih *et al*., 2017). The staphylococcal genome consists of a number of virulence factors that are either acquired or intrinsic, which contribute to pathogenicity of *S. aureus* (Malachowa and DeLeo, 2010). These virulence factors include toxins, enzymes and cellular components and are largely responsible for the bacterium's success as a pathogen due to their ability to facilitate infection and colonisation of the host (Hiramitsu *et al*., 2014; Mahon *et al*., 2015).

The mortality rate of patients with staphylococcal infection in the pre-antibiotic era was as high as 80% (Lowy, 2003). The introduction of penicillin in the 1940s resulted in a better prognosis (van Hal *et al*., 2012). However, resistance to penicillin was observed shortly after its introduction (Grema *et al*., 2015). To overcome the problem of penicillin resistance, a semi-synthentic derivative of penicillin called methicillin was introduced in the late 1950s (Chongtrakool *et al*., 2006). Methicillin was used to successfully treat staphylococcal infections caused by staphylococci that were resistant to penicillin. However, two years after the introduction of this antibiotic, methicillin-resistant *S. aureus* (MRSA) strains were observed (Zhang *et al*., 2005; Al-Mebairik *et al*., 2016).

Methicillin-resistant *S. aureus* is of importance because it has proven to be a persistent pathogen in healthcare settings as well as in the community (Green *et al*., 2012). This pathogen has elevated the cost of healthcare because infections caused by MRSA are more expensive to treat than those caused by methicillin-susceptible *S. aureus* (MSSA) due to the increased length of hospitalisation and the need for alternative treatment owing to resistance

to beta-lactam antibiotics (David and Daum, 2010; Méric *et al*., 2015). Methicillin-resistant *S. aureus* was thought to be restricted to hospitals and people in contact with healthcare settings prior to the 1990s and was referred to as Healthcare-associated MRSA (HA-MRSA) (David *et al.*, 2011; Batabyal *et al*., 2012; Tomoda, 2016). However, increasing reports emerged of MRSA infections in people lacking the traditional risk factors associated with exposure to the healthcare settings since the mid 1990s (David and Daum, 2010). This type of MRSA was referred to as Community-associated MRSA (CA-MRSA) (David and Daum, 2010). Community-associated MRSA strains cause tissue destructive infections that were rarely seen before the emergence of CA-MRSA (Chambers and De Leo, 2009). Epidemiological studies have shown that the Panton-Valentine leukocidin (*pvl*) genes are carried mainly by CA-MRSA but have also been described in HA-MRSA (Hu *et al*., 2015). Although the PVL toxin is associated with MRSA, it is also common in MSSA strains (Brown *et al*., 2012; Schaumberg *et al*., 2014; Abdulgader *et al*., 2015; Mahon *et al*., 2015).

The PVL gene encodes an exotoxin associated with severe skin infections and necrotising pneumonia (Moroney *et al*., 2007). This cytolytic toxin has the ability to lyse the cell membrane of human neutrophils resulting in haemorrhage, necrosis and destruction of cells (David and Daum, 2010; Bonesso *et al*., 2011). This toxin has the ability to suppress phagocytosis and therefore imparts a survival advantage to *S. aureus* and contributes to the invasiveness of the strains that carry the *pvl* gene (Mahon *et al*., 2015).

Staphylococcus aureus harbours a battery of virulence factors that promote tissue colonisation and damage, persistence, evasion of host defences and dissemination of the bacteria (Bien *et al*., 2011). These virulence factors include hemolysins, leukocidins, proteases, enterotoxins, exfoliative toxins and immune modulatory factors (Oogai *et al*., 2011). The full set of virulence factors (virulome) and antibiotic resistance genes (resistome) carried by a *S. aureus* bacterium play a significant role in the type and severity of infection it will cause in the host (Jiménez *et al*., 2011). Most virulence factors and antibiotic resistance genes are found on mobile genetic elements (MGEs) and the consequence of this is that these virulence and antibiotic resistance genes can be disseminated to other bacteria (Imanishi *et al*., 2019).

Methicillin-resistant *S. aureus* outbreaks are frequently reported in burn trauma units and neonatal intensive care units (NICU) (Milstone *et al*., 2010). Neonates are exposed to MRSA shortly after birth and can become colonised after contact with adult skin or their immediate

environment (Nelson and Gallagher, 2012). Common sites for colonisation include the umbilical cord, skin, nasopharynx, anterior nares and gastrointestinal tract (Nelson and Gallagher, 2012). There are a number of factors that make neonates especially susceptible to colonisation and infection and these include: immaturity of the immune system, low birth weight, mechanical ventilation, overcrowding, prematurity and surgical procedures (Heinrich *et al*., 2011; Nelson and Gallagher, 2012; Nelson *et al*., 2015). Patients in burn trauma units also have a high risk for acquiring nosocomial infection such as MRSA due to increased hospital stay and suppressed immunity (Schweizer *et al*., 2012). Patients with extensive burn injuries are susceptible to infection due to disruption of the normal skin barrier (Cook, 1998).

The evolution and the epidemiology of MRSA have been studied using various molecular typing methods (Schwalm *et al*., 2011). Typing of MRSA isolates is important for investigations pertaining to the spread and transmission of MRSA within and among hospitals (Nübel *et al*., 2013). There are many typing methods used for the characterisation of bacterial isolates some of which include typing, multi-locus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), staphylococcal cassette chromosome *mec* (SCC*mec*) typing, staphylococcal protein A (*spa*) typing and whole genome sequencing (WGS) (Enright *et al.*, 2000; Schwalm *et al*., 2011). Typing methods such as SCC*mec* typing are PCR-based and provide a rapid means of characterising MRSA isolates (Adzitey *et al*., 2013). Multi-locus sequence typing and *spa* typing are sequence based typing methods that have a good discriminatory power but the high costs associated with these typing methods is a limiting factor for most laboratories (Sabat *et al*., 2006). The most widely used molecular-based typing method for the study of local and global epidemiologies is PFGE, which has long been considered the gold standard for molecular typing of *S. aureus* (Enright *et al*., 2000; Sabat *et al*., 2017). This typing technique relies on restriction digestion of chromosomal DNA with the use of an enzyme such as *Sma*I followed by separation with the use of agarose gel electrophoresis (Deurenberg *et al*., 2007). Despite its usefulness, PFGE is labour intensive, time-consuming and inter-laboratory comparisons are difficult (Li *et al*., 2013). While still widely used, these techniques cannot always discern two closely related bacteria and provide no information regarding the antibiotic resistance and virulence profiles of the bacteria (Price *et al*., 2013).

Whole genome sequencing (WGS) has a higher discriminatory power than all the abovementioned techniques and has the ability to replace these conventional typing methods

that are used for characterisation (Rumore *et al*., 2018). Since the entire genomic data is available, WGS can provide all the information of a bacterium including but not limited to the identity, data on MLST, resistome, virulome, all the mobile genetic elements (MGEs) such as plasmids, phages and transposons (mobilome), serotypes and it allows for genomic comparison (Bezdicek *et al*., 2019). Due to the vast amount of genomic information that WGS provides, this technique is considered the new gold standard for molecular typing (Sabat *et al*., 2017). In order to have efficient infection control, it is necessary to have accurate typing methods with a high discriminatory power (Sabat *et al*., 2017). Despite the expenses associated with WGS, this method still remains relevant during outbreak investigations because it provides the ultimate resolution power, allowing for the accurate molecular typing of isolates and it has the ability to correctly exclude epidemiologically unrelated isolates (Sabat *et al*., 2017).

The distribution, emergence and evolution of MRSA in Africa is poorly understood (Kpeli *et al*., 2017). Outbreaks of MRSA often occur in healthcare centres if the proper surveillance systems and infection control programmes are not implemented or followed. Due to the detection of an increased number of MRSA isolates from burn and neonatal wards from four tertiary academic hospitals in the Gauteng province, this study was proposed. Molecular characterisation of MRSA clones causing outbreaks in neonatal wards and burn units can shed light on whether it is the same sequence type causing outbreaks in the different healthcare centres during the period of 2015 to 2019 or whether the outbreaks were caused by different clones. This information has implications for surveillance and infection control programmes within the healthcare centres.

1.2 Aim

The aim of this study was to characterise MRSA isolates causing outbreaks in burn wound and neonatal ward patients at healthcare centres in Gauteng, South Africa with the use of PFGE and WGS and to determine the antibiotic and virulence gene profiles associated with these outbreak strains.

1.3 Objectives:

The objectives of this study were:

1. To select 85 clinical MRSA isolates from the culture bank of the Department of Medical Microbiology, associated with outbreaks in burn wound and neonatal ward patients collected between 2015 and 2018
2. To obtain antibiotic resistance profiles of the MRSA isolates following routine analysis by the NHLS/TAD laboratory using the Vitek® 2 automated system (bioMèrieux, France)
3. To perform total genomic DNA extraction of the isolates using the ZR Fungal/Bacterial DNA Miniprep™ commercial kit (Zymogen Fermentas, USA)
4. To confirm the identity of the MRSA isolates and detect the *mec*A and *pvl* genes using an identification multiplex polymerase chain reaction (M-PCR) assay
5. To perform genotyping of the 85 MRSA isolates using pulsed-field gel electrophoresis (PFGE) and to construct a dendrogram
6. To perform whole genome sequencing on selected MRSA isolates in collaboration with the National Institute of Communicable Diseases (NICD) to characterise these isolates

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Staphylococcus aureus (*S. aureus)* is a Gram-positive bacterium that forms part of the normal flora of the skin, nose and mucous membranes of healthy individuals (Ryan *et al*., 2014). Although this bacterium is a commensal of humans, it is an opportunistic pathogen capable of causing infection in people with a compromised immune system (David and Daum, 2010). This bacterium has the ability to cause a wide scope of infections that range in severity from mild skin and soft tissue infections to more severe, life-threatening infections such as bloodstream infections and osteomyelitis (David and Daum, 2010; Batabyal *et al*., 2012; Wang *et al*., 2016).

Staphylococcus aureus is a common cause of nosocomial infections such as intravenous catheter associated infections, surgical site infections and ventilator associated pneumonia (Uhlemann *et al*., 2014). Infections caused by methicillin-resistant *S. aureus* (MRSA) are more expensive to treat than infections caused by methicillin-sensitive *S. aureus* (MSSA) due to the increased length of hospitalisation and the need for alternative treatment owing to the widespread resistance to beta-lactam antibiotics (David and Daum, 2010; Merić *et al*., 2015). Methicillin-resistant *S. aureus* is not only a problem in healthcare settings but it is also established in the community (Uhlemann *et al*., 2014).

Staphylococcus aureus is the most clinically significant species of the staphylococci due to its large arsenal of virulence factors that are associated with its pathogenicity (Stefani and Goglio, 2010; Green *et al*., 2012). Virulence factors include exotoxins, cytolytic toxins as well as cell wall associated proteins, which assist in mediating adhesion to host cells or evading the immune system (Hiramitsu *et al*., 2014; Mahon *et al*., 2015). With the use of these cell wall associated proteins, *S. aureus* has the ability to form biofilms, which is considered an important virulence mechanism leading to chronic infections (Watkins *et al*., 2012; McCarthy *et al*., 2015). These virulence factors together with increasing antibiotic resistance contribute to this bacterium’s success as an infective agent (Green *et al*., 2012).

There has been an increase in the number of reports of MRSA from the burn wound and neonatal ward patients between 2015 and 2018 at four tertiary academic hospitals in Gauteng. This study was proposed to investigate the molecular characteristics of MRSA isolates recovered from the four different healthcare settings. This could indicate a common source and clone of MRSA that can be associated with an increased number of infections that are difficult to treat due to antibiotic resistance within these patient populations. The aim of this study was to molecularly characterise MRSA isolates associated with outbreaks in burn wound and neonatal ward patients at healthcare centres in Gauteng, South Africa with the use of pulsed-field gel electrophoresis (PFGE) and whole genome sequencing (WGS) on selected representative strains and to determine the antibiotic and virulence gene profiles associated with these outbreak strains.

2.2 Classification of *Staphylococcus aureus*

Staphylococci are members of the family *Staphylococcaceae* (Mahon *et al*., 2015). Historically, the genera *Staphylococcus* and *Micrococcus* were both placed in the family *Micrococcaceae* due to similar morphological traits (Steinhauer, 2010; Becker *et al*., 2014). However, recent studies based on molecular and phylogenetic analysis revealed that the two genera are not as closely related as previously thought and consequently, *Staphylococcus* was reclassified (Gherardi *et al*., 2018). *Staphylococcus* has been classified in the *Bacillus-Lactobacillus-Streptococcus* cluster, which encompasses Gram-positive bacteria with a low guanine+cytosine (G+C) content (<60%) (Ventura *et al*., 2006; Foster and Geoghegan, 2015). To date, there are more than 50 species and subspecies that belong to the genus *Staphylococcus* (Hennekinne *et al.,* 2012). *Staphylococcus aureus* is the most clinically significant member of the *Staphylococcus* genus due to its ability to cause infections that range from mild to life threatening (Mahon *et al*., 2015). The classification of *S. aureus* is shown below in Table 2.1 (Euzéby, 2015; Liu, 2015).

Table 2.1: Classification of *Staphylococcus aureus* (Euzéby, 2015; Liu, 2015)

Domain	*Bacteria*
Kingdom	*Eubacteria*
Phylum	*Firmicutes*
Class	*Bacilli*
Order	*Bacillales*
Family	*Staphylococcaceae*
Genus	*Staphylococcus*
Species	*Staphylococcus aureus*

Other members of the genus of clinical relevance include *S. epidermidis*, *S. haemolyticus*, *S. lugdunensis* and *S. saprophyticus* (Cornelissen *et al*., 2013; Mahon *et al*., 2015). Members such as *S. capitis*, *S. hominis*, *S. schleiferi, S. simulans* and *S. warneri* are less common but are recognised as opportunistic pathogens (Mahon *et al*., 2015).

2.3 Characteristics of *Staphylococcus aureus*

The bacteria forming part of the genus *Staphylococcus* were described and classified in 1882 by a Scottish surgeon by the name of Sir Alexander Ogston (Stryjewski and Corey, 2014). The word *Staphylococcus* stems from the word '*staphylos*', which is Greek for grape and '*kokkos*', which means berry or seed (Thurlow *et al*., 2012). *Staphylococcus aureus* has a typical Gram-positive cell wall consisting of peptidoglycan and teichoic acid (Ryan *et al*., 2014). Peptidoglycan protects the bacterium from osmotic lysis and it serves as a scaffold to which surface proteins can attach themselves (Becker *et al*., 2014). On stained smears, the bacteria appear spherical, with a diameter of 0.5 to 1.5 μM and are present as pairs, clusters and single cells (Mahon *et al*., 2015). *Staphylococcus aureus* is non-motile, non-spore forming and can grow in the presence or absence of oxygen, making them facultative anaerobic (Mahon *et al*., 2015). *Staphylococcus aureus* is a chemoorganotroph with the ability to undergo both respiratory and fermentative metabolism (Schelin *et al*., 2011).

Staphylococci grow easily on blood agar plates and in nutrient broth (McPherson and Pincus, 2011). The bacteria produce white, cream coloured or gold colonies following overnight incubation on blood agar plates, with some strains showing clear beta-hemolysis around the colony (Ryan *et al*., 2014). The gold colour of the colonies is due to the presence of a carotenoid, which is a virulence factor that protects the pathogen from oxidative killing by the host immune system, in response to infection (Plata *et al*., 2009). This pathogen produces catalase, which is an enzyme that has the ability to convert hydrogen peroxide into hydrogen and water (Brooks *et al*., 2013). The growth of *S. aureus* needs to be supplemented with various amino acids such as arginine, cysteine, proline and valine, which are important sources of nitrogen and growth factors such as B vitamins and inorganic salts (Medvedova and Valik, 2012; Cornelissen *et al*., 2013).

Staphylococcus aureus is a mesophile and has an optimum growth temperature ranging from 37°C to 40°C (Medvedova and Valik, 2012). *Staphylococcus aureus* can also grow in a wide

pH range (4.2 to 9.3 with an optimum of 7.0 to 7.5) and can survive a sodium chloride (NaCl) concentration up to 15% (Le Loir *et al*., 2003). The production of catalase is used to differentiate staphylococci from streptococci, which are catalase negative (Brooks *et al*., 2013). *Staphylococcus aureus* is coagulase-positive, which means it is capable of clotting plasma (McPherson and Pincus, 2011). Coagulase is an enzyme that has the ability to bind prothrombin in a manner that allows for the cleavage of fibrinogen to fibrin (Ryan *et al*., 2014). This phenomenon can be demonstrated by incubating staphylococci in plasma for a few hours, which will result in the formation of a fibrin clot (Ryan *et al*., 2014). Coagulase may deposit fibrin on the surface of staphylococci and alters the ability of the staphylococci to be ingested by phagocytic cells or to be destroyed within these cells (Brooks *et al*., 2013). The ability of *S. aureus* to produce coagulase is used to separate it from other staphylococci such as *S. epidermidis* and *S. haemolyticus*, which are known as coagulase negative staphylococci (CONS) (Cheng *et al*., 2010; Mahon *et al*., 2015). Coagulase negative staphylococci were previously considered less pathogenic but have been recognised as major nosocomial pathogens (Saber *et al*., 2017). *Staphylococcus aureus* has a fibrin coat on its outer surface, which may facilitate immune evasion (Otto, 2013). This pathogen is hardy and is resistant to heat and desiccation, allowing it to persist on fomites and thus resulting in its spread (Cornelissen *et al*., 2013).

2.4 Genomic characteristics of *Staphylococcus aureus*

The genome of *S. aureus* is a circular chromosome consisting of approximately 2.8 Mbp and a G+C content of about 33% (Plata *et al*., 2009; Chua *et al*., 2013). Comparative analysis of a number of *S. aureus* genomes has revealed that most regions of the staphylococcal genome are well conserved and others exhibit variability (Baba *et al*., 2008). The part of the genome, which is well conserved is referred to as the core genome and it encodes all the housekeeping genes that are vital for the survival and growth of the bacteria such as those involved in metabolism, nucleic acid synthesis and replication (Malachowa and DeLeo, 2010; Chua *et al*., 2013). The core genome makes up 75% of the staphylococcal genome and is present in 95% of *S. aureus* isolates (Plata *et al*., 2009; Fuchs *et al*., 2018). The core genome of *S. aureus* is shielded from high recombination or mutation owing to the fact that this part of the genome is essential for function and any changes or mutations in the core genome could have an adverse effect on the bacterium (Croll and McDonald, 2012). The part of the genome that shows high variability is referred to as the accessory genome and it encodes adaptive traits that confer an

advantage to *S. aureus* under certain environmental conditions (Juhas *et al*., 2008). The accessory genome accounts for 25% of the staphylococcal genome (Plata *et al*., 2009). In addition to facilitating rapid adaptation, the accessory genome is a source of variation between strains and is responsible for the genetic plasticity of *S. aureus* (Deghorain and Van Melderen, 2012; Everitt *et al*., 2014). A diagrammatic representation of the *S. aureus* genome is shown in Figure 2.1 (Turner *et al*., 2019).

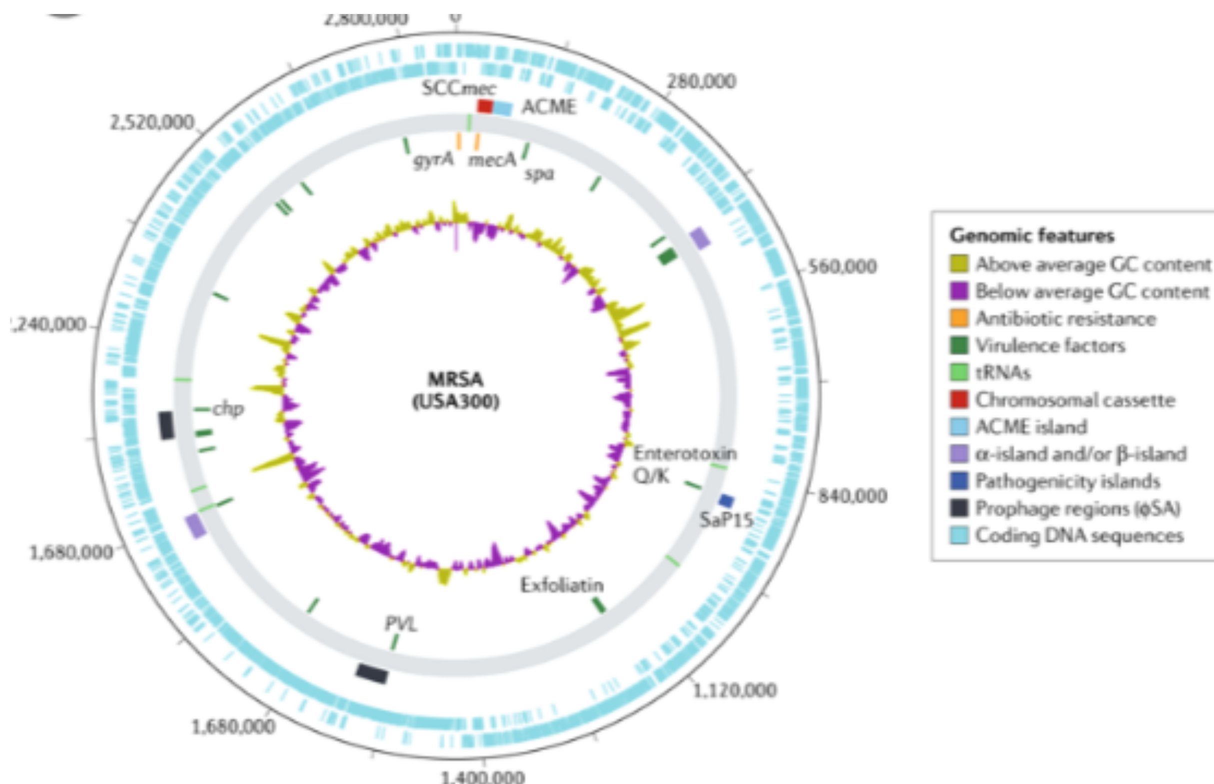

Figure 2.1: Diagrammatic representation of the *S. aureus* genome (Turner *et al*., 2019)

The accessory genome offers the benefit of possibly expanding host range due to the potential to acquire new virulence factors (Croll and McDonald, 2012). The G+C content of the accessory genome is usually different from that of the core genome due to the accessory genome being obtained from different bacteria (Malachowa and DeLeo, 2010). *Staphylococcus aureus* mobile genetic elements (MGEs) include bacteriophages, pathogenicity islands, plasmids, staphylococcal cassette chromosome *mec* (SCC*mec*) and transposons (Wan *et al*., 2017). In addition to impacting the genomic plasticity and evolution of the *S. aureus* genome, MGEs play a role in the dissemination of antibiotic resistance and virulence genes and catabolic pathways (Juhas *et al*., 2008).

2.4.1 Bacteriophages of *Staphylococcus aureus*

A bacteriophage or a phage is a virus that infects and replicates inside a bacterial cell (Cornelissen *et al*., 2013). Integrated phage genomes are known as prophages and the bacteria that contain these prophages are known as lysogens or lysogenic bacteria (Kasman and Porter, 2018). Staphylococcal phages belong to the order *Caudovirales*, which encompasses phages with an icosahedral capsid containing double-stranded DNA and a thin filamentous tail (Veesler and Cambillau, 2011; Xia and Wolz, 2014). This order is further divided into three families based on the morphology of the tail namely: i) *Myoviridae* with long contractile tails, ii) *Podoviridae* with a small capsid and a short, non-contractile tail and iii) *Siphoviridae* with long non-contractile tails (Kutter, 2001). Most of the *S. aureus* phages belong to the family *Siphoviridae* (Deghorain *et al*., 2012; Melo *et al*., 2014). The accessory genes found on the phage genome encode staphylococcal virulence factors (Xia and Wolz, 2014). Bacteriophages allow for the mobility of genetic material such as the helper phage 80alpha, which mediates the excision and transfer of pathogenicity islands to other staphylococci (Malachowa and DeLeo, 2010) in a process known as transduction (Kasman and Porter, 2018). Phages shape the bacterial genome architecture and play an important role in bacterial evolution (Deghorain and van Melderen, 2012).

2.4.2 Pathogenicity islands in *Staphylococcus aureus*

Staphylococcus aureus pathogenicity islands (SaPIs) are a family of mobile phage-related genes with sizes that range between 10 kb to 200 kb and are widely distributed among *S. aureus* strains (Schmidt and Hensel, 2004; Tormo *et al*., 2008; Malachowa and DeLeo, 2010; Mir-Sanchis *et al*., 2012). These MGEs are integrated at specific sites in the staphylococcal chromosome designated att_c and are in a quiescent state due to the action of a master repressor called Stl (Malachowa and DeLeo, 2010; Argudin *et al*., 2010). The SaPIs need to have an att_s site and require a functional integrase gene in order to integrate into the genome (Mir-Sanchis *et al*., 2012). Staphylococcal pathogenicity islands share common features, which include: i) base composition (G+C content) as well as the codon usage that differs from that of the core genome (Schmidt and Hensel, 2004; Gal-Mor and Finlay, 2006), ii) genetic elements encoding virulence factors, such as adhesins, invasins, iron transport proteins, toxins and secretion systems (Cornelissen *et al*., 2013; Che *et al*., 2014), and iii) tRNA genes, which are hotspots for integration of pathogenicity islands and other genetic

material and are flanked by direct repeats (Gal-Mor and Finlay, 2006; Cornelissen *et al*., 2012). These MGEs lack the necessary genes that are required for horizontal transfer from one bacterium to another and rely on helper phages such as phage 80alpha for excision, replication and packaging into phage-like particles, which allows for transfer (Lindsay, 2010; Melo *et al*., 2014). Mobilisation of SaPIs is specific and can only take place if phage proteins are able to inactivate the SaPI repressor (Dearborn and Dockland, 2012). When repression has been lifted, excision, circularisation and replication of the SaPI is induced (Chen *et al*., 2015). The SaPIs are packaged into a capsid that is encoded by the helper phage (Novick *et al*., 2010). The helper phage's genetic material is subsequently excluded from the capsid (Novick *et al*., 2010). Notable SaPIs include SaPI1, which characteristically carries the toxic shock syndrome toxin (*tst*) genes and is mobilised by phage 80alpha at a high frequency (Plata *et al*., 2009) and SaPbov1, which also carries the *tst* gene and is mobilised by the phages 80alpha, ϕ11 and ϕ147 (Plata *et al*., 2009).

2.4.3 Plasmids harboured by *Staphylococcus aureus*

Plasmids are circular, double-stranded DNA molecules with the ability to replicate independently from the chromosomal DNA due to the presence of an origin of replication (Lodish *et al*., 2000; Pierce, 2012). These MGEs carry a myriad of antimicrobial, biocide, and toxin genes but do not carry genes that are essential for cell growth and replication (McCarthy and Lindsay, 2012; Cornelissen *et al*., 2013). Staphylococcal plasmids have been classified into three classes: i) class I plasmids are small with sizes ranging from 1 kb to 5 kb and usually encode resistance to one or two antibiotics, ii) class II plasmids are of intermediate size (8 kb to 40 kb) and encode resistance to beta-lactams and inorganic ions, and iii) class III plasmids consisting of large conjugative plasmids with sizes ranging from 40 kb to 60 kb, which carry multiple resistance determinants including genes that encode penicillinases, resistance to heavy metals and detergents (Plata *et al*., 2009; Lindsay, 2010; McCarthy and Lindsay, 2012; Schumacher *et al*., 2014). Conjugative plasmids are those that are able to mediate self-transmission by expressing proteins for the mating pore formation system and the replicative DNA transfer system (O'Brien *et al*., 2015). Staphylococcal plasmids replicate either by rolling circle replication as seen with small plasmids or by theta replication as seen with the larger plasmids (Shearer *et al*., 2011). Plasmids in staphylococci may be horizontally transferred through: i) conjugation, a process where two bacteria in close proximity make contact allowing for a plasmid or part of the chromosomal genetic material to be transferred

from the donor cell to the recipient cell, ii) mobilisation, a process in which a non-conjugative plasmid makes use of the mating pore formation system of a conjugative plasmid, and iii) transduction, a type of gene exchange, which involves a bacteriophage carrying genetic material from one bacterium to another (Shearer *et al*., 2011; Pierce, 2012; O'Brien *et al*., 2015).

2.4.4 Staphylococcal Cassette Chromosome *methicillin* in *Staphylococcus aureus*

The staphylococcal cassette chromosome methicillin (SCC*mec*) is a large fragment of DNA, which carries the *mecA* gene encoding resistance to beta-lactams (Amirkhiz *et al.,* 2015; Wang *et al*., 2017). It is believed that the *mecA* gene originated from *Staphylococcus sciuri*, which is considered a commensal of animals, owing to the fact that a homologue that codes for a penicillin-binding protein (PBP) with 88% amino acid sequence homology was found on the chromosome of this bacterium (Deurenberg *et al*., 2007; Nemeghaire *et al*., 2014; Nazari *et al*., 2015). The SCC*mec* element is integrated in the chromosome of *S. aureus* at a site called the *attBscc*, which is located near the *S. aureus* origin of replication (Ibrahem *et al*., 2009). The SCC*mec* element is highly variable and exhibits extensive diversity in different staphylococcal species (Kinnevey *et al*., 2013). This MGE is more prevalent in non-*S. aureus* staphylococci than in MRSA, therefore CONS are considered reservoirs of SCC*mec* elements (Shore *et al*., 2011). The *mec* gene complex is regulated by a sensor inducer called the mecR1 and a repressor known as mecI (Shore *et al*., 2011). In addition to the *mec* complex, these unique fragments of DNA have two essential components namely the cassette chromosome recombinase (*ccr)* complex and the joining regions (J-regions) (Chongtrakool *et al*., 2006). The *ccr* gene complex encodes serine recombinases that are responsible for the mobility, integration and excision of the SCC*mec* element (Milheiriço *et al*., 2007; Shore *et al*., 2011). The integration and excision from the chromosome takes place at a specific site of the 3' end of the open reading frame, referred to as open reading frame X (*orfX*) (Deurenberg *et al*., 2007; Kondo *et al*., 2007; Valsesia *et al*., 2010). The function of the *orfX* was unknown but it has been annotated as a 23S rRNA methyltransferase (Shore *et al*., 2011). To date, five *ccrA* allotypes, seven *ccrB* allotypes and one *ccrC* allotype have been described (Wu *et al*., 2015). The J-regions constitute the non-essential parts of the cassette but these regions have been shown to carry additional antibiotic resistance determinants (Milheiriço *et al*., 2007). The J-1 region is located between the right flanking chromosomal region and the *ccr* complex, J-2 is located between the *ccr* gene complex and the *mec* complex and J-3 is located between *orf*X

and the *mec* complex (Kondo *et* al., 2007; Martínez-Meléndez *et al*., 2015). Variations in the J-regions occur and this allows some SCC*mec* types to further be differentiated into sub-types such as IVa, IVb, IVc, IVd, IVg and IVh (Berglund *et al*., 2009; Monecke *et al*., 2016). Figure 2.2 illustrates the basic structure of a SCC*mec* element (Hiramitsu *et al*., 2013).

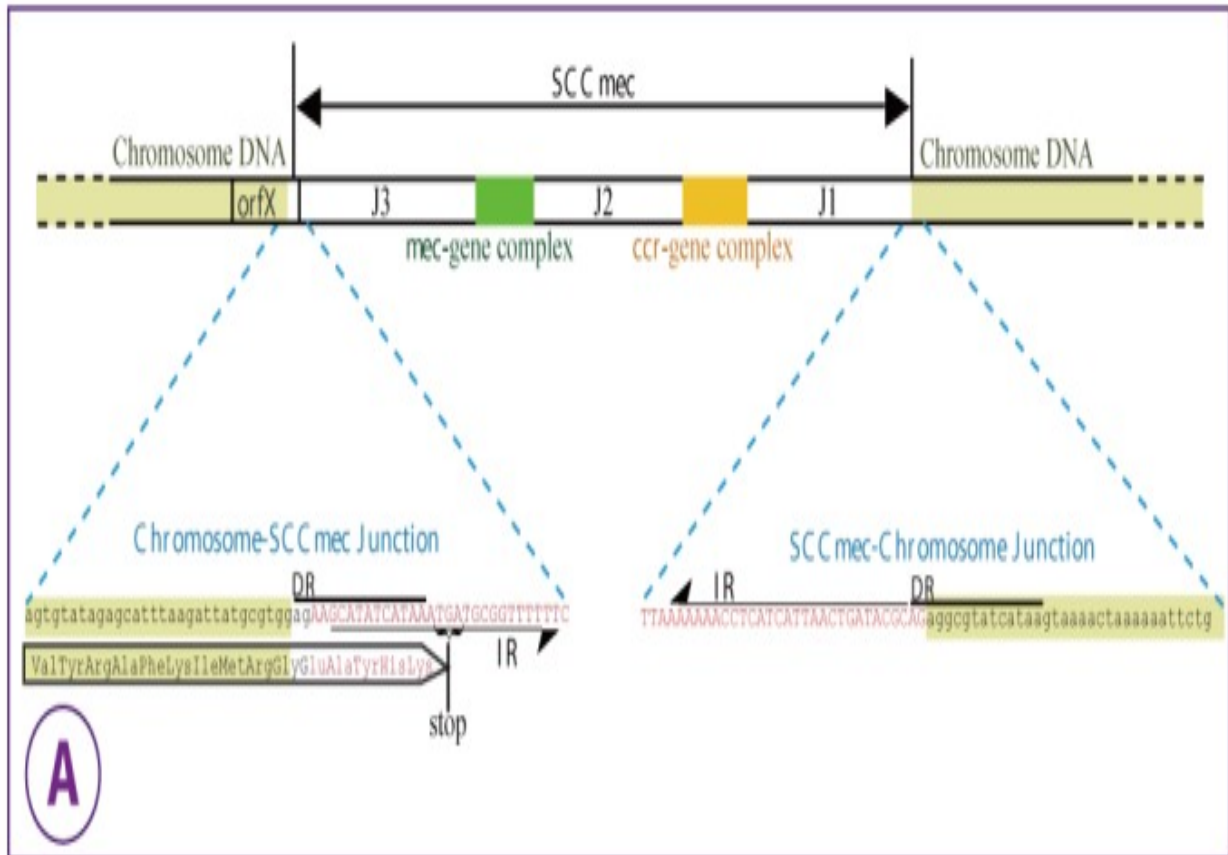

Figure 2.2 **Basic structure of the SCC*mec* element containing the *ccr*-gene complex, J-regions and the *mec*-gene complex (Hiramitsu *et al*., 2013)**

Different types of SCC*mec* elements have been defined based on the different combinations of *ccr* and *mec* complexes (Ghaznavi-Rad *et al*., 2010). To date, there are 13 different types of SCC*mec* elements and 11 subtypes that have been described (Côrtes *et al*., 2018; Yoon *et al*., 2019).

2.4.5 Transposable elements in *Staphylococcus aureus*

Transposable elements are DNA sequences with the ability to move from one location to another in the genome (Munoz-Lopez and Garcia-Perez, 2010). There are various types of transposable elements (Pierce, 2012). Regardless of the variation exhibited by these MGEs, most transposable elements have common features, which include: i) the presence of short flanking repeats on either side of the transposable element (generated during the process of transposition), and ii) the terminal ends of some transposable elements bear terminal inverted repeats, which are recognised by enzymes that catalyse transposition (Pierce, 2012). An insertion sequence (IS) is the smallest transposable elements encoding only transposase, an enzyme required for its transposition (Hegstad *et al*., 2010). Transposons on the other hand are larger than IS elements and contain several other genes in addition to those necessary for their movement (Brooks *et al*., 2013). Composite transposons carry genes for specialised function such as antibiotic resistance and are flanked by IS elements (Malachowa and DeLeo, 2010; Brooks *et al*., 2013). Non-composite transposons carry the transposase gene and have inverted repeats but lack IS elements (Pierce, 2012). Small transposons such as *Tn*552 and *Tn*554 encode resistance to penicillin and erythromycin respectively and can be found integrated in plasmids, SCC elements or the chromosome (Lindsay, 2010). The *S. aureus* genome contains several copies of transposons, many of which are inactive due to mutations and deletions (Hiramitsu *et al*., 2013).

2.5 Colonisation, spread and epidemiology of *Staphylococcus aureus*

Staphylococcus aureus asymptomatically colonises the skin and anterior nasal passage of 20% to 30% of healthy individuals at any given time (Thurlow *et al*., 2012; Foster, 2015). However, colonisation rates may be higher in healthcare workers (HCW) and patients (Ryan *et al*., 2014). Colonisation is higher in HCW due to constant exposure to known and unknown MRSA-positive patients (Sassmannhausen *et al*., 2016). Colonisation serves as a source of infection (Cornelissen *et al*., 2013). Bacteria are shed from the nose onto the skin and clothing of the carrier and the bacteria may be spread to anyone that comes into contact with the carrier or fomites that have been contaminated (Ryan *et al*., 2014). Areas contaminated in the hospital include bedding, clothing, furniture and medical instruments (Grema *et al*., 2015).

Methicillin-resistant *S. aureus* strains that are acquired in the hospital are referred to as hospital–associated MRSA (HA-MRSA) (Green *et al*., 2012). An infection is considered to be HA-MRSA if a positive culture is obtained from a specimen taken at least 48 hours after admission (Nair *et al*., 2018). Risk factors for MRSA infection include a compromised immune system, recent hospitalisation, previous antibiotic usage, dialysis, indwelling catheters and surgery (Taconelli *et al*., 2008; Grema *et al*., 2015). The elderly, infants, chronically ill patients and organ transplant recipients are most at risk of MRSA infections (Green *et al*., 2012).

Methicillin-resistant *S. aureus* was thought to be restricted to hospitals and people in contact with healthcare settings (David *et al*., 2011; Tomada, 2016). However, after the 1990s, there was a global emergence of MRSA in healthy people that lacked the traditional risk factors of MRSA (Edslev *et al*., 2017). This MRSA was referred to as community-associated MRSA (CA-MRSA) (David and Daum, 2010; Green *et al*., 2012). The risk factors for CA-MRSA are referred to as the 5 C's and include crowding, compromised skin, a lack of cleanliness, contaminated items and surfaces and frequent skin-to-skin-contact (Green *et al*., 2012). The epidemiology of MRSA is constantly changing and evolving (Von Dach *et al*., 2016). This changing epidemiology is partly due to the infiltration of CA-MRSA into the hospital setting, where it was initially not encountered (Ramsing *et al*., 2013). The distinction between HA-MRSA and CA-MRSA has become less clear over the past few years (Perovic *et al*., 2017).

2.5.1 Molecular epidemiology of sequence types associated with outbreaks

Methicillin-resistant *S. aureus* can be grouped into sequence types (STs) based on multi-locus sequence typing (MLST), a typing method, which is used to sequence the internal fragments of seven housekeeping genes, which are essential for survival (Bonesso *et al*., 2011; Alkharsah *et al*., 2018). Sequence types can also be determined *in silico* using whole genome sequencing (WGS) data (Page *et al*., 2017). Sequence types that make up a clonal complex (CC) are STs that show single or double-locus variations of the original ST and because single nucleotide polymorphisms will not always result in different allelic numbers, different STs may be placed within the same CC (Suzuki *et al*., 2009). Most of the MRSA isolates responsible for epidemics belong to eight major CCs namely CC1, CC5, CC8, CC22, CC30, CC45, CC59 and CC80 (Boswili and Udo, 2018). Each of the CCs exhibits a distinct geographical distribution, with STs such as ST1 (CC1), ST5 (CC5) and ST8 (CC8) being

commonly found in the United Stated and Japan, whereas CC22 is more prevalent in Europe and Australia (Wang *et al.,* 2016; Alkharsah *et al*., 2018). Clonal complex 80 is widely distributed in Europe but it shows a high prevalence in the Middle East and Northern Africa (Wang *et al*., 2016; Edslev *et al*., 2017). Sequence type 59 (belonging to CC59) is common in China and other Asian countries (Wang *et al*., 2016). Clonal complex 30 has been reported in many parts of the world including the United States, Latin America and the Middle East but is also common in Europe (David and Daum, 2010; Ritchie *et al*., 2014). The CCs that are prevalent in Sub-Saharan Africa include CC5, CC15 and CC30 (Ruffing *et al*., 2017). The most commonly identified STs in South Africa include ST239 (CC8), ST612 (CC8) and ST36 (CC30) (Moodley *et al*., 2010; Perovic *et al*., 2017). The clones ST239-MRSA-III and ST612-MRSA-612 were reported to be a major cause of bacteraemia in South African hospitals by Perovic and colleagues (2017). Clonal complexes 1, and CC80 have been associated with CA-MRSA infections and have been reported in both the community and in hospital settings (Abdulgader *et al*., 2015).

2.6 Pathogenesis and virulence factors of *Staphylococcus aureus*

Staphylococcus aureus is the most significant species of the staphylococci owing to its large arsenal of virulence factors that contribute to its pathogenicity (Stefani and Goglio, 2010; Mahon *et al*., 2015). Many of these virulence factors are encoded on MGEs and are responsible for this pathogen's versatility (Otto, 2014). These virulence factors include cellular components such as protein A, clumping factor and fibronectin binding proteins, cytolytic toxins and exotoxins that play a role in invasion of the host and evasion of the host immune system and mediating adhesion to host cells (Hiramitsu *et al*., 2014; Mahon *et al*., 2015).

2.6.1 Cell wall associated proteins of *Staphylococcus aureus*

Staphylococcus aureus expresses a number of surface proteins that are covalently attached to the peptidoglycan (Foster *et al*., 2015). Cell wall associated proteins are important virulence factors that are involved in iron acquisition, adhesion, biofilm formation, invasion and inflammation (Foster *et al*., 2015). Many *S. aureus* proteins and cell wall components are pro-inflammatory, meaning these proteins have the ability to elicit a cell response in the host that

is similar to the reaction caused by Gram-negative lipopolysaccharide (Powers and Wardenburg, 2014).

2.6.1.1 Adherence of *Staphylococcus aureus*

In order for *S. aureus* to invade host cells, the bacterium needs to attach to the host cell surface and this initial attachment is mediated by cell wall bound adhesins including microbial surface components recognising adhesive matrix molecules (MSCRAMM) and secretable expanded repertoire adhesin molecules (SERAM) (Bur *et al*., 2013). Surface proteins such as clumping factor and fibronectin-binding proteins likely play an important role in early infection (Ryan *et al*., 2014). The MSCRAMMs are involved in the initiation of endovascular infections, bone and joint infections and infections involving prosthetic devices and depending on which MSCRAMMs an *S. aureus* isolate possesses, it may be predisposed to cause certain kinds of infections (Gordon and Lowy, 2008).

Staphylococcus aureus has the ability to attach to indwelling medical devices through direct interaction with the polymer of the device or by interaction with host matrix proteins after they attach onto the device (Perisamy *et al*., 2012). *Staphylococcus aureus* can also attach to damaged host tissue (McCarthy *et al*., 2015). Once the bacterium attaches to the prosthetic device, it is able to grow and form a biofilm (Gordon and Lowy, 2008). A biofilm is defined as an aggregation of surface-bound bacterial cells that are embedded in an extracellular matrix (Lister and Horswill, 2014). This extracellular matrix consists of exopolysaccharides, teichoic acids and DNA (Perisamy *et al*., 2012). Biofilm formation allows bacteria to persist not only by protecting the bacteria from host defences but also by hampering delivery of antibiotics (Neopane *et al*., 2018). In addition, bacteria within a biofilm are not as metabolically active as those that are planktonic, which makes these particular bacteria even more resistant to antimicrobial killing (Lister and Horswill, 2014; Tong *et al*., 2015).

Small colony variants (SCVs) are rare strains of *S. aureus* that are characterised by a small colony size and slow growth (Kahl *et al*., 2016). These strains require carbon dioxide, hemin or menadione for growth (Mahon *et al*., 2015). Another characteristic of SCVs is the down-regulation of virulence genes and the up-regulation of biofilm formation and adhesion genes (Kahl *et al*., 2016). Antibiotics are active against dividing bacteria and the slow growth of SCVs results in tolerance to antibiotics therefore making the SCVs more resistant than the

common colony type (Garcia *et al*., 2013; Vulin *et al*., 2018). *Stapylococcus aureus* SCVs are clinically important because they can result in persistent and recurring infections despite adequate antibiotic therapy owing to their reduced susceptibility to antibiotics (Tubby *et al*., 2013; Proctor *et al*., 2014).

2.6.1.2 Evasion of *Staphylococcus aureus* from host defences

Staphylococcus aureus possesses other virulence factors that are involved in the evasion of the host immune defences during infection (Gordon and Lowy, 2008). A pathogen needs to be able to circumvent host cell responses in order to survive within its host (Rooijakkers *et al*., 2007). These virulence factors include staphylococcal protein A, capsular polysaccharide, chemotaxis inhibitory protein of staphylococci (CHIPS) and staphylococcal complement inhibitors (SCIN) (Foster, 1996; Gordon and Lowy, 2008; Ryan *et al*., 2014; Powers and Wardenburg, 2014).

Staphylococcal protein A is a cell wall anchored protein that is released during staphylococcal growth, belonging to the family of MSCRAMMS (Brooks *et al*., 2013; Keneer *et al*., 2017). This protein has the ability to bind to the F_c and F_{ab} regions of host IgG antibodies and consequently prevents the recognition of staphylococcal antigens (Powers and Wardenburg, 2014; Keneer *et al*., 2017). This evasion of host antibodies prevents opsonisation and clearance (Gordon and Lowy, 2008). Staphylococcal protein A triggers B cell superantigen activity by cross-linking with VH3 type B cell receptors that cause superclonal expansion and apoptosis of activated B cells (Falugi *et al*., 2013).

The majority of *S. aureus* have a polysaccharide capsule, which inhibits phagocytosis (Brooks *et al*., 2013). The capsule allows the bacteria to escape phagocytosis by masking the bacterial surface and surface associated proteins, preventing the bacteria from being recognised by phagocytic cells (Kuipers *et al*., 2016). There are at least 11 serotypes that have been identified but serotypes 5 and 8 have been implicated in most infections, with serotype 8 being the most prevalent (Foster, 1996; Brooks *et al*., 2013; Kuipers *et al*., 2016). In addition to inhibiting phagocytosis, the capsular polysaccharide can also induce abscess formation (Gordon and Lowy, 2008).

Staphylococcus aureus secretes molecules that diminish the recruitment of leukocytes to the site of infection or prevent uptake by phagocytic cells, which results in intracellular killing (Powers and Wardenburg, 2014). These proteins include, but are not limited to, CHIPS and SCIN (Powers and Wardenburg, 2014). Chemotaxis inhibitor protein of *S. aureus* is a 14.1-kDa protein that is found in over 60% of *S. aureus* isolates, which binds to the C5aR and formylated peptide receptors and prevent activation and chemotaxis of leukocytes *via* C5a, a terminal effector of the complement system (Rooijakkers *et al*., 2007; Wright *et al*., 2007; Otto, 2014). Staphylococcal complement inhibitors are 9.8 kDa proteins secreted by *S. aureus* that binds to and blocks C3 convertase activity, which inhibits complement defences due to the deposition of C3b opsonin on the surface of *S. aureus* and decreased neutrophil uptake and killing (Powers and Wardenburg, 2014).

2.6.2 Toxins and enzymes produced by *Staphylococcus aureus*

Toxins and enzymes are secreted by the bacterial cell, unlike cellular components that form part of the bacterial cell (Al-Mabairik *et al*., 2016). These secreted toxins allow the bacterium to invade host tissue and cause an infection in the host (Becker *et al*., 2014). In addition to toxins, *S. aureus* also produces a number of enzymes such as coagulase, elastases, hyaluronidases, proteases and staphylokinases, which play a role in the invasion and penetration of host cells as well as the evasion of host immune defences, allowing the bacteria to be disseminated to other sites (Gordan and Lowy, 2008; Brooks *et al*., 2013; Kong *et al*., 2016).

2.6.2.1 Hemolysins in *Staphylococcus aureus*

Staphylococcus aureus produces four hemolysins namely alpha, beta, gamma and delta hemolysins, with alpha hemolysin being the most important due to its role in the pathogenesis of *S. aureus* infections, especially pneumonia (Mahon *et al*., 2015; Ping *et al*., 2018). Hemolysins are polypeptides with the ability to damage host cell cytoplasmic membrane (Vandernesch *et al*., 2012). This group of toxins disrupt the cytoplasmic membrane by inserting into the lipid bilayer causing perforations and eventually the formation of pores in the cytosplamic membrane (Ryan *et al*., 2014). Consequently, there is a leakage of ions, low molecular weight molecules and water out of the cell and cell lysis (Qiu *et al*., 2012).

Alpha hemolysin, encoded by the alpha hemolysin (*hla*) gene, is a small beta-barrel pore-forming toxin that has long been recognised as an important cause of injury to host cells (Qiu *et al*., 2012; Berube *et al*., 2013). This hemolysin is central to the pathogenesis of pulmonary, intraperitoneal, intramammary and corneal infections (Qiu *et al*., 2012). Numerous host cell types, including red blood cells, epithelial cells and leukocytes, are susceptible to this toxin (Kebaier *et al*., 2012).

Beta-hemolysin is encoded by the beta-hemolysin (*hlb*) gene (Burnside *et al*., 2010). Unlike alpha-hemolysin, beta-hemolysin is not a pore-forming toxin but is a neutral sphingomyelinase with the ability to hydrolyse the plasma membrane lipid called sphingomyelin (Vandenesch *et al*., 2012). In addition to sphingomyelin, beta-hemolysin has the ability to hydrolyse monocytes and at temperatures below 10°C, it lyses erythrocytes (Burnside *et al*., 2010; Kong *et al*., 2016). This hemolysin is also referred to as the hot-cold toxin due to its ability to lyse red blood cells at 4°C and the absence of lysis at 37°C (Moraveji *et al*., 2014).

Delta-hemolysin, a product of the delta-hemolysin (*hld*) gene, is a small amphipathic peptide, meaning it has a hydrophobic and a hydrophilic side (Vandenesch *et al*., 2012). This toxin has the abilty to lyse erythrocytes, membrane bound organelles, spheroplasts and protoplasts (Burnside *et al*., 2010). This heterogenous toxin dissociates into subunits in non-ionic detergents and disrupts biological membranes (Brooks *et al*., 2013). The *S. aureus* delta-toxin has physiochemical and structural properties that are similar to phenol-soluble modulins (PSMs) (Laabei *et al*., 2014).

Gamma-hemolysin is the product of the gamma-hemolysin (*hlg*) genes, namely *hlgA*, *hlgB* and *hlgC* (Havaei *et al*., 2010). This hemolysin is a bi-component leukocidin composed of two proteins designated S and F, with the ability to lyse white blood cells (Brooks *et al*., 2013). Although leukocidins are known to lyse leukocytes, gamma-hemolysin and LukED are the only leukocidins within the family of *S. aureus* leukocidins that have the ability to lyse red blood cells (Yoong and Torres, 2013). This toxin is active against macrophages, monocytes and neutrophils (Vandenesch *et al*., 2012).

2.6.2.2 Enterotoxins produced by *Staphylococcus aureus*

Enterotoxigenic strains of *S. aureus* produce toxins ranging from 26 kDa to 30 kDa known as staphylococcal enterotoxins (DeGrasse, 2012). Staphylococcal enterotoxins belong to a large family of pyrogenic toxin superantigens (Kadariya *et al*., 2014). This superantigenic toxin has the ability to cause immune suppression, non-specific T-cell proliferation and it is also hypothesised that the superantigenic activity of these toxins facilitates transcytosis, which allows the toxin to gain access to the bloodstream where it interacts with antigen presenting cells and T-cells resulting in its superantigen activity (Schelin *et al*., 2011; Kadariya *et al*., 2014). Staphylococcal enterotoxins are highly stable and exhibit heat and environmental resistance in addition to being resistant to low pH and proteolytic enzymes such as pepsin and trypsin, allowing the enterotoxins to survive the gastrointestinal tract after ingestion (Kadariya *et al*., 2014). Staphylococcal enterotoxins can withstand heating at 121°C for 10 minutes and can be produced at temperatures ranging from 10°C to 50°C (optimum at 30°C to 40°C), pH range of 4.8 to 9.0 (optimum at 5.3 to 7.0) and water activity range of 0.87 to 0.99 (optimum at 0.9) (Satomi *et al*., 2013). The genes encoding these toxins are carried on and spread by MGEs such as plasmids, prophages and pathogenicity islands (Schelin *et al*., 2011).

A number of staphylococcal enterotoxins (A to V) have been reported (DeGrasse, 2012; Alfatemi *et al*., 2014). Staphylococcal enterotoxin A (SEA) is frequently involved in staphylococcal food poisoning and SEB has been identified as a possible biological weapon in addition to its ability to cause food poisoning (Wu *et al*., 2016). Staphylococcal enterotoxins are regarded as a threat to food safety and food security (Wu *et al*., 2016).

2.6.2.3 Staphylococcal toxic shock syndrome toxin

Many strains of *S. aureus* are known to carry genes for toxic shock syndrome toxin (TSST), which is the common causative agent of toxic shock syndrome (DeVries *et al*., 2011). This 22-kDa toxin causes toxic shock syndrome (TSS) by stimulating the release of interleukin-1, interleukin-2, tumor necrosis factor α and other cytokines (Otto *et al*., 2014). This toxin is functionally a superantigen that lacks emetic activity and was initially designated staphylococcal enterotoxin F (SEF) (Argudin *et al*., 2010). This toxin is encoded by the toxic shock syndrome toxin (*tst*) gene, which is found in 20% of *S. aureus* strains including MRSA (Brooks *et al*., 2013). The *tst* gene is carried on an *S. aureus* pathogenicity island, which is

mobilised by a bacteriophage (Dearborn and Dockland, 2012). Production of TSST-1 is regulated by the accessory gene regulator (*agr*) system of *S. aureus*, which is a well characterised quorum sensing system that is responsible for the regulation of a wide array of staphylococcal virulence factors (MacPhee *et al*., 2013). Previous studies have shown that elevated oxygen and carbon dioxide and a neutral pH of 6.5 to 7.0 are necessary for the optimal production of TSST-1 (MacPhee *et al*., 2013).

2.6.2.4 Staphylococcal exfoliative toxin

The *S. aureus* exfoliative toxins namely exfoliative toxin A (ETA), ETB and ETD are encoded on different genetic elements; exfoliative toxin A (*eta)* is integrated into the staphylococcal genome by a phage and exfoliative toxin B (*etb*) and exfoliative toxin D (*etd*) are found on a large plasmid named pETB and a pathogenicity island respectively (Kato *et al*., 2011; Shi *et al*., 2011; Grumann *et al*., 2014). Exfoliative toxins are capable of cleaving desmoglein-1, which is a cadherin protein that mediates cell-to-cell adhesion in keratinocytes (Koosha *et al*., 2014). This results in the formation of blisters due to intra-epidermal dissociation of cells and it results in the loss of the superficial skin layer (Kato *et al*., 2011; Cornelissen *et al*., 2013). Exfoliative toxin A and ETB cause staphylococcal scalded skin syndrome (Saida *et al*., 2015) while exfoliative toxin D has been primarily isolated from humans with deep pyoderma (Iyori *et al*., 2010).

2.6.2.5 Panton-Valentine leukocidin in *Staphylococcus aureus*

Panton-Valentine leukocidin, like gamma hemolysin, is a bicomponent beta-barrel toxin that forms pores in the cell (Shore *et al*., 2014). This cytotoxin was first reported by and subsequently named by Sir Phillip Noel Panton and Francis Valentine in 1932 and has since been recognised as an important virulence factor for *S. aureus* (Shrestha *et al*., 2014; Bhatta *et al*., 2016). This leukocidin is cytotoxic to neutrophils and to a lesser extent, to monocytes and macrophages (Spaan *et al*., 2013). This potent toxin is encoded by the *lukS-PV* and *lukF-PV* genes, which are carried by phages such as *φ*Sa2958, *φ*Sa2MW, *φ*PVL, *φ*108PVL and *φ*SLT of the family *Siphoviridae* (Marriem *et al*., 2013; Saeed *et al*., 2018). The assembly of one class F subunit and one class S subunit is integral to form a functional hetero-oligomeric pore (Jayasinghe and Baylet, 2005). There are at least six class F proteins (LukF-PV, LukF-R, LukD, LukF'-PV, HlgB and LukF-I) and seven class S proteins (LukS-PV, LukS-R, LukE,

LukM, HlgA, HlgC and LukS-I) (Jayasinghe and Baylet, 2005). The two components are secreted by *S. aureus* before assembling into a heptamer on neutrophil membranes resulting in neutrophil lysis (Shallcross *et al*., 2013). In addition to PVL and gamma-hemolysin AB and CB, the genome of human *S. aureus* strains encodes other leukocidins namely leukocidin ED (LukED) and leukocidin AB/GH (LukAB/GH) (Spaan *et al*., 2013; Powers and Wardenburg, 2014). The LukAB/GH protein is reported to be both a secreted protein and one of the predominant surface proteins of *S. aureus* at the late exponential phase of growth, which may be involved in targeting immune cells when they are in contact with the bacterium (Vandernesch *et al*., 2012). In contrast to the other bi-component pore-forming toxins that show a high sequence identity (70% to 80%), LukA and LukB share a low sequence identity (30% to 40%) with the other leukocidins (Kailasan *et al*., 2019). Due to its ability to lyse monocytes and lymphocytes, LukED plays a role in bloodstream infections and it has the ability to adversely affect phagocytosis and therefore promotes dissemination of the bacteria (Powers and Wardenburg, 2014). Panton-Valentine leukocidin producing MRSA is usually responsible for mild skin and soft tissue infections and sepsis and is also associated with necrotising pneumonia and bone and joint infections (Muttaoyah *et al*., 2010; Bhatta *et al*., 2016). Although PVL has been epidemiologically linked to CA-MRSA, some HA-MRSA strains have been described carrying PVL genes (Yamuna *et al*., 2017). The PVL has been reported in MSSA and it has been shown that PVL-positive MSSA is likely a reservoir for the development of PVL-positive MRSA (Al-Hassnawi *et al*., 2013; Saeed *et al*., 2018).

2.7 *Staphylococcus* aureus in burn patients and neonates in the healthcare setting

Outbreaks of MRSA are usually nosocomial infections and are frequently observed in intensive care units and burn units (Agca *et al*., 2014). Methicillin-resistant *S. aureus* is a significant pathogen in these patient populations, who are uniquely susceptible to colonisation and infection (Barbut *et al*., 2013). Outbreaks in these units are common and can continue for many years (Barbut *et al*., 2013).

2.7.1 Burn wound patients

The intact human skin surface is an important component for the preservation of body fluid homeostasis, thermoregulation and protection against infection (Church *et al*., 2006). Thermal injury results in a breach in the skin's surface (Church *et al*., 2006). In addition to destruction

of the skin barrier, the local and systemic host cellular and humoral immune responses are depressed and this is one of the major factors that contribute to infection and complication in patients with severe burn wounds (Church *et al*., 2006). Underlying vasculature of the skin is damaged during thermal injury and this makes it difficult for various components of the immune system to reach the affected site to help fight infection (Saaiq *et al*., 2015). Infection in burn wound patients is still considered one of the most important causes of disability and mortality in all ages in both developed and developing countries (Saaiq *et al*., 2015). Burn wound patients are at a high risk of infection for a variety of reasons such as an exposed body surface, the immunocompromising effects of burns, invasive diagnostic and therapeutic procedures and a protracted hospital stay (Church *et al*., 2006; Saaiq *et al*., 2015).

Although the surface of the skin is sterile immediately after thermal injury, the wounds eventually become colonised with microorganisms (Rode *et al*., 2008). A study conducted by Saaiq and colleagues (2015) identified *S. aureus* as the third most frequently isolated bacteria from burn wound patients following *Pseudomonas aeruginosa* and *Klebsiella* spp. but it is the leading cause of burn related morbidity and mortality (Tejiram *et al*., 2017). Bacteria such as *S. aureus* can be found deep within sweat glands and hair follicles and are therefore able to survive heat and colonise the wound surface within 48 hours of thermal injury if topical antimicrobials are not used (Church *et al*., 2006). Burn wound patients colonised with MRSA represents a reservoir for the spread of the pathogen to patients in other wards and to HCWs (Barbut *et al*., 2013). Destruction of the skin integrity provides a protein-rich, avascular and necrotic environment that favours colonisation and proliferation of microorganisms (Tejiram *et al*., 2017). Negative effects of colonisation include delayed wound healing, an increased need for surgical intervention and prolonged stay at the burn centre (Jaspers *et al*., 2014).

2.7.2 Neonatal patients

Methicillin-resistant *S. aureus* has become a frequent source of infections that affect premature and critically ill neonates (Nelson and Gallagher, 2012). Despite administration of the appropriate antibiotic therapy, neonatal infections can have long-term sequelae such as poor neurodevelopment and growth outcomes (Popoola *et* al., 2014). Host factors that make neonates particularly vulnerable to MRSA infection include an immature immune system, the use of broad spectrum antibiotics, prolonged hospitalisation, overcrowded and understaffed neonatal intensive care units (NICU), frequent contact with HCWs and incomplete bacterial

microbiome development (Nelson *et al*., 2015; Heinrich *et al*., 2011; Pierce *et al*., 2017). Invasive devices are frequently used in NICU patients, which further increases the risk of acquiring invasive MRSA infections (Geraci *et al*., 2014). Due to an increase in the prevalence of MRSA in the healthcare setting and in the community, the eradication of MRSA in the NICU has become increasingly challenging (Milstone *et al*., 2010).

Colonised neonates are the main reservoir of MRSA in the NICU and relatives and HCWs play a role in the transmission of this pathogen to other patients (Geraci *et al*., 2015). Transmission of MRSA through breast milk and the birth canal has also been described (Zervou *et al*., 2013). In a phenomenon known as colonisation pressure, the risk of healthcare-associated transmission is shown to increase as the density of colonised patients increases (Pierce *et* al., 2017). Community reservoirs have been implicated in the introduction of MRSA into NICUs by increasing the colonisation prevalence among patients and visitors (Azarian *et al*., 2015). A NICU is a very complex hospital ward regarding infection control practices because HCWs and parents are closely involved in the care of the neonates and despite being instructed, parents are not trained to follow good infection control procedures (Ramsing *et al*., 2013). Current strategies to prevent the transmission of MRSA in NICUs and burn units include identifying colonised patients and placing these patients on contact precautions, cohorting, hand hygiene of HCWs and in some cases decolonisation of patients and healthcare workers (Barbut *et al*., 2013; Popoola *et al*., 2014).

2.8 Infections in burn patients and neonates caused by *Staphylococcus aureus* and treatment

Infection occurs as a consequence of *S. aureus* gaining access into host cells through a break in the skin or an open wound (Liu, 2009). The severity of infection is also affected by host factors, which include co-morbidities such as diabetes mellitus and acquired immunodeficiency syndrome (AIDS) (Ki and Rotstein, 2008).

Intravascular catheters may be required in neonates and burn patients for various reasons such as the administration of medication and nutritional support, making these patients susceptible to bloodstream infections (Shah *et al*., 2013). Bloodstream infections are diseases defined by the presence of viable bacteria in the blood stream (Viscoli *et al*, 2016). Bloodstream infections caused by *S. aureus* are associated with high morbidity and mortality and can result

in long-term disability (Corey, 2009; Rodio *et* al., 2017). Bloodstream infections are mostly associated with intravascular catheters or devices, surgical site infections and in some cases pneumonia caused by *S. aureus* (Que and Moreillon, 2015). The presence of viable bacteria in the blood can often result in metastatic infections such as infective endocarditis (Corey, 2009). The bacteria can attach to cardiac valves and form a vegetation made up of fibrin and bacterial cells (Tong *et al*., 2015). In order to treat a bloodstream infection, it is imperative to remove the original focus if infection is due to infected intravascular or prosthetic devices (Que and Moreillon, 2015). Empircal antibiotic treatment must take into account MRSA as a cause of the infection (Que and Moreillon, 2015).

Pneumonia is a microbial infection that is followed by inflammation of the lungs, leading to an accumulation of inflammatory cells and fluid in the bronchioles and alveoli (Dahal and Schwan, 2018). Pneumonia can be classified as community-acquired, healthcare-acquired or ventilator associated pneumonia (VAP). Ventilator-associated pneumonia is defined as pneumonia that occurs 48 hours to 72 hours following endotracheal intubation (Kalanuria *et al*., 2014). Ventilator-associated pneumonia is a common nosocomial infection in NICU and is associated with increases in morbidity and mortality, hospital stay and costs (Augustyn, 2007; Foglia *et al*., 2007). Intubation is indicated in burn patients with inhalation injury if upper airway patency is in jeopardy (Onishi *et al*., 2017). Although intubation or mechanical ventilation may save lives, it is associated with an increased risk of infections such as VAP (Al-Omari *et al*., 2015). The rate of VAP is high in burn patients and it increases mortality (Al Ashry *et al*., 2016). Based on the time of onset, VAP can be divided into two types: i) early onset VAP, which occurs within the first four days of mechanical ventilation and is usually caused by antibiotic susceptible bacteria, and ii) late onset VAP, which develops five or more days after mechanical ventilation and is usually caused by antibiotic resistant bacteria (Charles *et al*., 2014). Linezolid is used as treatment for VAP due to vancomycin's poor cure rate (Que and Moreillon, 2015). Telavancin is another alternative that can be used for VAP caused by MRSA and it has been found to be effective as treatment of VAP caused by MSSA due to its activity against several Gram-positive pathogens (Dahal and Schwan, 2018). Nebulised antibiotics for VAP are an area of research with the potential to be used in staphylococcal pneumonia (Burnham and Kollef, 2017).

Sepsis is described as life-threatening organ dysfunction caused by a disregulated host response to infection (Greenhalgh, 2017). It has been reported that sepsis is currently the most

common manifestation of invasive MRSA infections, with one-third of cases presenting as MRSA sepsis (Dong *et al*., 2018). Sepsis is a leading cause of death following serious burn injury and sepsis-related failure of multiple organs (Jones *et al*., 2014; Tridente, 2018). Patients that have sustained severe burn injuries present with hypermetabolic and hyperinflammatory responses that lead to physiological changes and demonstrate signs of systemic inflammatory response syndrome (SIRS) (Ren *et al*., 2015). Since similar manifestations and test results are seen in patients with sepsis and those with non-infectious inflammation such as burn patients, diagnosis of sepsis based on clinical findings alone is difficult (Madenci *et al*., 2014). Delay in diagnosis and treatment of sepsis results in a rapid progression to circulatory collapse, multiple organ failure and eventually death (Ren *et al*., 2015). Optimal treatment of sepsis depends on early diagnosis and includes administration of antibiotics and management of hemodynamic changes and organ dysfunction (Nunez Lopez, 2017). Vancomycin is the empiric treatment for patients presenting with sepsis, especially in regions where the prevalence of MRSA is high (Dong *et al*., 2018). Cloxacillin can be used to treat sepsis caused by *S. aureus* isolates that are susceptible (Paulsen *et al*., 2015).

Staphylococcal scalded skin syndrome (SSSS) is a blistering disease of the skin that is caused by strains of *S. aureus* that produce exfoliative toxins (Ruocco *et al*., 2017). The severity of SSSS ranges from a couple of blisters on the skin to severe exfoliation, which is observed over the entire surface of the body (Mishra *et al*., 2016). Neonates and children are more susceptible to SSSS due to their immature immune system, which is unable to produce antibodies to neutralise exfoliative toxins (Meshram *et al*., 2018). Additionally, neonates and children do not possess the renal capacity to excrete these toxins (Mishra *et al*., 2016; Meshram *et al*., 2018). Staphylococcal scalded skin syndrome due to infection of burn wounds has been described (Tsujimoto *et al*., 2018). In addition to the administration of antibiotics such as cefazolin and vancomycin, treatment also includes maintaining fluid and electrolyte balance, temperature regulation, skin care and nutritional management (Kapoor *et al*., 2008; Kouakou *et al*., 2015; Tsujimoto *et al*., 2018).

2.9 Antibiotic resistance in *Staphylococcus aureus*

Antibiotics work by targeting bacteria using various means that start with the physical interaction of an antibiotic and its bacterial target, which causes a change in the physiology of the bacteria and leads to cell death or inhibition of growth (Garcia *et al*., 2017). Strains of

S. aureus have developed resistance to many of the commonly used antibiotics (Deyno *et al*., 2017). Misuse of antibiotics by the public, extensive use of antimicrobials as growth enhancers in animal feed and movement of resistant bacteria across geographical barriers due to an increase in international travel all contribute to the emergence and spread of antibiotic resistance (Al-Mebairik *et al*., 2016; Deyno *et al*., 2017). *Staphylococcus aureus* is an ESKAPE pathogen, which is a group of bacteria (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.) that are virulent and resistant to multiple antibiotics (Mulani *et al*., 2019). The ESKAPE pathogens have been implicated in hospital-acquired infections among critically ill individuals and present a challenge due to their numerous antibiotic resistance mechanisms (Ramsamy *et al*., 2018). Resistance determinants may be found on the chromosome or on MGEs such as plasmids and transposons (Santajit and Indrawattana, 2016). Due to their genetic plasticity, bacteria have developed mechanisms that allow them to thrive in the presence of antibiotics (Munita and Arias, 2016). These mechanisms include inactivation/alteration of the antibiotic, modification of binding sites, changes in permeability of the cell leading to reduced intracellular concentrations of the said antibiotic, multidrug efflux pumps and biofilm formation (Tang *et al*., 2014; Munita and Arias, 2016; Santajit and Indrawattana, 2016). Spontaneous mutations in chromosomal genes that involve single nucleotide polymorphisms can also result in resistance to antibiotics (Tang *et al*., 2014). The rate of spontaneous mutations that can generate antibiotic resistance is low compared to acquisition of resistance genes (Coculescu, 2009).

2.9.1 Penicillin

Beta-lactam antibiotics such as penicillins work by acylation of the transpeptidase domain of the active site of penicillin-binding proteins (PBPs), blocking access to their substrate and preventing cross-linking of peptidoglycan strands during cell wall synthesis (Ba *et al*., 2014). Prior to the introduction of penicillin, the mortality rate of patients with staphylococcal infections was up to 80% (Deurenberg *et al*., 2007). Staphylococcal infections were primarily treated with penicillin, which was introduced in the late 1940s (Bonesso *et al*., 2011). However, two years following the introduction of penicillin, strains that were resistant to this beta-lactam antibiotic arose in hospital settings (Bonesso *et al*., 2011). By 1948, resistance to penicillin was reported in over 50% of staphylococcal isolates recovered from patients in a hospital in the United Kingdom (Grema *et al*., 2015). This observed resistance was due to the acquisition of a plasmid carrying a gene encoding a penicillinase (Que and Moreillin, 2015).

Penicillinase is a penicillin-hydrolysing enzyme with the ability to cleave the beta-lactam ring of penicillin and other penicillinase-susceptible compounds into inactive penicillinoic acid (Que and Moreillin, 2015). This renders the antibiotic inactive (Turlej *et al*., 2011; Fernandes *et al*., 2013; Que and Moreillin, 2015). The penicillinase enzyme is a product of the beta-lactamase Z (*blaZ*) gene and is under the regulatory control of two genes namely beta-lactamase I (*blaI*) and beta-lactamase RI (*blaRI*), which encode the repressor, BlaI and the anti-repressor BlaRI respectively (Lowy, 2003; Pantosti *et al*., 2007). In the presence of a beta-lactam antibiotic such as penicillin, BlaRI, which is a sensor transducer, undergoes autolytic cleavage and serves as a protease for the cleavage of BlaI, thereby lifting repression of the *blaZ* gene and allowing the transcription to continue (Tang *et al*., 2014; Al-Mebairik *et al*., 2016). *Staphylococcus aureus* resistant to beta-lactams is usually resistant to other antibiotics such as streptomycin, erythromycin and tetracycline, which poses a threat to the treatment of staphylococcal infections (Llarrull *et al*., 2009; Stryjweski and Corey, 2014).

2.9.2 Methicillin

Methicillin, which is a semi-synthetic derivative of penicillin that is not degraded by penicillinases was introduced in the 1960s to circumvent the rising resistance to penicillin (Chongtrakool *et al*., 2006; Ventola, 2015). However, methicillin-resistant *S. aureus* (MRSA) was reported a few years later in 1962 (Stryjewski and Corey, 2014; Ventola, 2015). The mechanism of resistance to methicillin was described in 1981 and it was found that resistance was not due to the production of a penicillinase but is based on the expression of the *mecA* gene, which encodes a penicillin-binding protein (PBP) 2A (Valsesia *et al*., 2010; Stryjewski and Corey, 2014). This altered PBP, a 74 kDA protein, has an active site that has reduced affinity for beta-lactam antibiotics therefore allowing cell wall synthesis and growth to continue in the presence of beta-lactam antibiotics (Llarrull *et al*., 2009). The *mec* operon, which is also referred to as the *mec* complex, contains the regulatory genes *mecI* and *mecRI* that respond to beta-lactam antibiotics in a manner similar to the *blaI* and *blaRI* genes involved in the regulation of the *blaZ* gene (Lowy, 2003). The product of the *mecRI*, a sensor transducer like *blaRI*, also undergoes autolytic cleavage, which leads to the metallo-protease domain of the protein becoming active and therefore permitting MecRI to cleave MecI that is bound to the operator region of *mecA*, allowing transcription of the *mecA* gene (Deurenberg *et al*., 2007). The *mecI* gene is usually deleted or rendered inactive by mutations (Hiramitsu *et al*., 2014). Deletion of the *mecI* gene, which encodes the repressor protein MecRI, results in

constitutive expression of *mecA* and therefore constant production of PBP2A (Llarrull *et al*., 2009). However, it has been found that the DNA sequences bound by BlaI and MecI in order to repress gene expression are identical (Lowy, 2003). Consequently, BlaI has the ability to repress the *mecA* gene in addition to the *blaZ* gene (Hiramitsu *et al*., 2014). This is most likely a protective mechanism that prevents over-production of PBP2a (Lowy, 2003).

A novel PBP gene designated *mecB*, was discovered in *S. aureus* isolated from China, Japan and Thailand that showed resistance to beta-lactams but lacked the *mecA* gene (Hiramitsu *et al*., 2013). The gene encoding the novel PBP shares 61.7% homology with *mecA* and is adjoined to transposase genes that form transposon *Tn6045* (Hiramitsu *et* al., 2013). This transposon is found on a plasmid or the chromosome of *Macrococcus caseolyticus*, which colonises the skin of animals (Becker *et al*., 2018). A study on bovine mastitis paved the way for the discovery of an isolate carrying a novel *mecA* homologue that shared approximately 59% identity to *mecA* at DNA level and about 63% at amino acid level (Paterson *et al*., 2014). This novel *mecA* gene, which was initially designated $mecA_{LGA251}$, was later renamed *mecC* (Ballhausen *et al*., 2014). The *mecC* gene encodes PBP2C, which also confers resistance to beta-lactam antibiotics but it is slightly different from PBP2A that is encoded by *mecA* (Harrison *et al*., 2013; Chan *et al*., 2016). The product of the *mecC* gene exhibits a binding affinity for oxacillin that is four times higher than the binding affinity shown by *mecA* (Ballhausen *et al*., 2014). Additionally, PBP2C has been shown to be thermosensitive, with decreased activity at 37°C (Harrison *et al*., 2013; Ballhausen *et al*., 2014). After its initial discovery in Denmark, the Republic of Ireland and the United Kingdom, *mecC* was identified in other countries such as France, Germany and Norway (Kumurya, 2015). Methicillin-resistant *S. aureus* isolates harbouring any of the alternative *mec* genes pose a potential threat to public health owing to the fact that differences in nucleotides will give rise to negative PCR results and the differences in the products of these genes will result in negative latex agglutination results that can affect diagnosis and treatment of patients (Kumarya, 2015).

2.9.3 Vancomycin

The glycopeptide vancomycin, was first released in 1958 (Howden *et al*., 2010). It is a cationic antibiotic derived from the organism *Amycolatopsis orientalis* (Hu *et al*., 2016). Glycopeptides are similar to beta-lactams in that they kill bacteria by inhibiting cell wall synthesis (Munita and Arias, 2016). Inhibition is achieved by vancomycin binding to the

terminal D-Ala-D-Ala of the peptidoglycan precursors and blocks the incorporation of precursors into the nascent peptidoglycan, thereby preventing cross-linking (Pantosti *et* al., 2007; Munita and Arias, 2016).

In 1997, the first MRSA isolate showing decreased susceptibility to vancomycin was reported in Japan (Gardete and Tomasz, 2014). The isolate's minimum inhibitory concentration (MIC) value for vancomycin was slightly increased, falling within the range of 4 to 8 µg/mL and became known as vancomycin intermediate *Staphylococcus aureus* (VISA) (Gardete and Tomasz, 2014; Garba *et al*., 2018). This mechanism of resistance is non-transferrable and is not maintained in the absence of vancomycin (Walters *et al*., 2015). The VISA phenotype leads to a number of common phenotypic and physiological changes including a thickened cell wall, excess production of peptidoglycan, reduced autolytic activity and reduced biofilm formation (Alam *et al*., 2014). The VISA phenotype seems to have a negative impact on the fitness of *S. aureus* and exhibits attenuated virulence (Alam *et al*., 2014; Walters *et al*., 2015). It is also thought that the thickened cell wall in VISA strains prevents the diffusion of vancomycin to its target, further rendering the VISA strains less susceptible to vancomycin (Di Gregorio *et al*., 2017).

Heteroresistant VISA (hVISA) refers to vancomycin susceptible *S. aureus* strains, which contain subpopulations of VISA at a rate of one bacterium per 10^5 to 10^6 organims (Stryjewski and Corey, 2014; Garba *et al*., 2018). The low frequency at which the hVISA strains occur makes it difficult to detect this phenotype using Clinical Laboratory Standards Institute (CLSI) methods (Howden *et al*., 2010). Heteroresistant VISA strains have been related to persistent bacteraemia, higher rates of complications and vancomycin treatment failure (Di Gregorio *et al*., 2015).

Staphylococcus aureus strains with an MIC greater than or equal to 16 µg/mL are defined as vancomycin resistant *Staphylococcus aureus* (VRSA) (Garba *et al*., 2018). The first VRSA strain was recovered in the USA from a foot wound of a diabetic patient that had previously been treated with vancomycin and had an MIC of 128 µg/mL (Hu *et al*., 2016; McGuinness *et al*., 2017). A vancomycin resistant *Enterococcus* (VRE) isolate was also recovered from the same patient (Hu *et al*., 2016). Resistance to vancomycin is a result of the acquisition of the vancomycin A (*vanA*) gene from VRE, which is carried on a conjugative transposon, *Tn1546* (Walters *et al*., 2015; Santajit and Indrawattana, 2016). The *vanA* gene mediates high-level

resistance to vancomycin as well as to teicoplanin (Perichon and Courvalin, 2009). The *vanA* operon contains a number of genes responsible for different aspects of the resistance to vancomycin (Pantosti *et al*., 2007). The *vanH* gene encodes a dehydrogenase, which reduces pyruvate to D-Ala-D-Lac and *vanA* encodes a ligase, which is involved in the formation of an ester bond between D-Ala-D-Ala and D-Ala-D-Lac, which replaces D-Ala-D-Ala in the peptidoglycan synthesis process lowering vancomycin's binding affinity (Courvalin, 2006; Pantosti *et al*., 2007). The *vanA* operon is an inducible operon under the regulatory control of *vanS* and *vanR* and is only active in the presence of vancomycin (Pantosti *et al*., 2007). Although there are other genes associated with vancomycin resistance (*vanB*, *vanC*, *vanC2*/*C3*, *vanD*, *vanE*, *vanG*, *vanL*, *vanM* and *vanN*), *vanA* and *vanB* are the most important (Praharaj *et al*., 2013; Moosavian *et al*., 2018). The *vanA* and *vanB* genes are the most common genotypes and the most clinically relevant because possession of the *vanA* gene confers high-level resistance to vancomycin and teicoplanin and the *vanB* gene confers high-level resistance to vancomycin and susceptibility to teicoplanin (Moosavian *et al*., 2018). Additionally, *vanA* and *vanB* gene clusters are located on MGEs such as plasmids and transposons, which means high level glycopeptide resistance can disseminate through the acquisition of these MGEs (Eshaghi *et al*., 2015).

2.10 Diagnostic techniques for the identification and characterisation of *Staphylococcus aureus*

The rapid and accurate identification of infection-causing bacteria such as MRSA and susceptibility testing are cardinal in selecting the appropriate treatment as well as to prevent further dissemination of MRSA (Kriegskorte *et al*., 2018). A number of phenotypic and genotypic methods are employed for this purpose (Ryan *et al*., 2014).

2.10.1 Phenotypic methods for the detection of *Staphylococcus aureus*

Staphylococcus aureus is isolated by culturing a clinical specimen, e.g. blood, pus and tissue onto solid media such as blood agar or trypticase soy agar (Foster, 1996; Mahon *et al*., 2015). Culturing on growth media is followed by Gram staining, which can provide information that is helpful in the early diagnosis and treatment of infection; however, it cannot be used to identify the bacteria to species level (Mahon *et al*., 2015).

Agglutination tests where protein A reacts with latex particles coated with immunoglobulin G can be used for the rapid detection of *S. aureus* (McPherson and Pincus, 2011). One such agglutination test is the Pastorex™ Staph Plus (Bio-Rad, USA), which has a high sensitivity and specificity and can therefore be used to rapidly identify *S. aurues* (Das *et al*., 2018). Pastorex™ Staph Plus (Bio-Rad, USA) is designed to simultaneously detect the *S. aureus* clumping factor, protein A and capsular polysaccharide (Argemi *et al*., 2017). These results can be confirmed using the Vitek® 2 automated system (bioMèrieux, France).

Staphylococcus aureus can be distinguished from other staphylococci based on the production of coagulase (Cheng *et al*., 2010). Two forms of coagulase exist, one bound to the cell wall (clumping factor), which is detected using the slide coagulase test where the test organism is suspended in water or saline to which a drop of plasma is added on a glass slide. If agglutination is observed, the isolate is considered coagulase positive and can be identified as *S. aureus* (McPherson and Pincus, 2011; Mahon *et al*., 2015). The other form of coagulase, which is also referred to as staphylocoagulase, is not attached to the cell wall and is detected with the tube coagulase test where the bacterium in question is suspended in plasma and incubated for four hours after which clot formation will be observed if the isolate is coagulase positive (Kateete *et al*., 2010).

The API Staph identification kit is a biochemical phenotypic test that uses metabolic activity and morphological features to identify *S. aureus* (Sheraba *et al*., 2010). The API Staph identification kit (bioMèrieux, France) contains up to 20 miniaturised biochemical tests (www.bioMèrieux-usa.com). A bacterial suspension is added to the wells of the strip; after incubation for 24 hours and the addition of the reagents accompanying the kit, reactions in each well are interpreted and the organism is identified (Cunha *et al*., 2004).

2.10.1.1 Automated identification and antibiotic susceptibility testing of *Staphylococcus aureus*

A number of automated systems are available that have the ability to positively identify microorganisms and to determine their antimicrobial susceptibility profiles (Ligozzi *et al*., 2002; Mahon *et al*., 2015). These automated systems make use of phenotypic tests such as microdilution and biochemical tests to identify microbes from a pure culture (Rodio *et al*., 2017). The MicroScan (Siemens Healthcare Diagnostics, Germany) makes use of a 96-well

microtitre tray containing 32 reagents for the identification of bacteria (Mahon *et al*., 2015). It has the ability to perform MIC testing and read the results using a spectrophotometer (McPherson and Pincus, 2011). The Vitek® 2 automated system (bioMèrieux, France) makes use of a card, which contains 64 wells that contain different antibiotics of varying concentrations (Mahon *et al*., 2015). This automated system makes use of photometers or fluorometers that monitor the reactions in each of the wells and reports changes within three hours to 24 hours (Jorgensen and Ferraro, 2009; Schumacher *et al*., 2018). The software linked to this system can integrate information from different reactions to comment on the mechanisms of resistance (Schumacher *et al*., 2018). Some advantages of the Vitek® 2 automated system (bioMèrieux, France) is that it reduces workload and can provide results in a short space of time (Koçaglu *et al*., 2019). The disadvantages are that it requires a large inoculum size, it has a limited organism spectrum and equipment and reagents have a high cost (van Belkum and Dunne, 2013).

Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) (Bruker Deltonic, Germany) is an automated system used in clinical laboratories for the identification of bacteria or yeast (Schuster *et al*., 2018). Bacteria from a single colony are directly smeared onto a MALDI target plate and 1 µL of α-cyano-4-hydroxycinnamic acid matric solution (prepared with 50% acetonitrile and 2.5% trifluoroacetic acid in water) is dropped onto the smear and allowed to air dry at ambient temperature (Hou *et al*., 2019). The target plate is then inserted into the mass spectrophotometer and the sample is exposed to a laser, which creates gas phase ions that are pulsed into a flight tube (Carbonelle *et al*., 2011). The mass/charge ratio, which can be determined by the time of flight of ions with the use of a detector, is used for the identification of the bacterial species in question using a reference database (Carbonelle *et al*., 2011; Hou *et al*., 2019). This technique offers the advantage of requiring minimal sample preparation, being less labour intensive, rapid and accurate (Croxatto *et al*., 2012; Singhal *et al*., 2015; Sauget *et al*., 2017; Schuster *et al*., 2018). The disadvantages of this system include the high initial cost of the MALDI-TOF equipment, it requires trained personnel and data acquisition and analysis are time-consuming (Singhal *et al*., 2015). Automated systems have a quicker turn-around time than non-automated, culture-based tests (Machen *et al*., 2014; Rodio *et al*., 2017). The implication of this is that test results are available sooner and patients can be treated timeously thus decreasing the morbidity and mortality of patients (Machen *et al*., 2014).

2.10.1.2 Antimicrobial susceptibility testing of *Staphylococcus aureus*

Antimicrobial susceptibility testing is performed to confirm MRSA by detecting oxacillin resistance and it is done using standard guidelines that are provided by organisations such as the CLSI and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Mahon *et al*., 2015; Kassim *et al*., 2016). The breakpoints that are set out by these organisations play a role in clinical decision making by deciding whether an organism will be reported as susceptible or not (Kassim *et al*., 2016). Previously, oxacillin was used for the detection of methicillin resistance but the CLSI recommends that cefoxitin should be used instead (Brown *et al*., 2005). This is because cefoxitin is a strong inducer of the *mecA* gene (Aruna and Rajesh, 2017). Additionally, cefoxitin provides results that are more accurate and that are reproducible [Centers for Disease Control and Prevention (CDC), 2013].

Broth microdilution and disk diffusion are methods employed for susceptibility testing of MRSA isolates (Brooks *et al*., 2013). Broth microdilution makes use of cation-adjusted Mueller-Hinton broth supplemented with 2% NaCl (CLSI, 2018). According to CLSI recommendation, isolates with an MIC value that is ≤ 4 µg/L should be reported as sensitive and those with an MIC value ≥ 8 µg/L should be reported as resistant (CLSI, 2018). The EUCAST guidelines recommend that a *S. aureus* isolate having an MIC value > 4 mg/L should be reported as resistant (EUCAST, 2019).

Mueller-Hinton agar is used for disk diffusion testing (CLSI, 2018; EUCAST, 2019). The CLSI (2018) recommends that isolates that show a zone that is ≥ 22 mm should be reported as susceptible and those that exhibit a zone ≤ 21 mm should be reported as resistant (McPherson and Pincus, 2011; CDC, 2013; CLSI, 2018). The EUCAST guidelines also recommend that an isolate showing a zone ≥ 22 mm should be reported as susceptible and those showing a zone < 22 mm should be reported as resistant (EUCAST, 2019). Expression of resistance is affected by growth conditions such as temperature, osmolarity of the medium and concentration of NaCl and incorrect results may be due to failure to detect resistance (Sakoulas *et al*., 2001; Kassim *et al*, 2016).

The Epsilometer test (Etest) is a commercial test that uses a thin plastic strip that is coated with varying concentrations of an antibiotic and the upper surface is marked with the concentration of the antibiotic (Jorgensen and Ferraro, 2009; Khan *et al*., 2019). The strip is placed on pre-inoculated agar and incubated for 24 hours (Schumacher *et al*., 2018). The

concentration at which the zone of inhibition intersects with the test strip is the MIC (Jergensen and Ferraro, 2009). The E-test offers the advantage of being simple to use, accurate and reliable (Khan *et al*., 2019). Another advantage is that the gradient is stable for up to 20 hours and can therefore be used for fast growing bacteria as well as the slow growing fastidious bacteria (Schumacher *et al*., 2018). A disadvantage of this method is that it requires manual measurement of the zone of inhibition, which may make its use impractical in some laboratories (Varela *et al*., 2008).

2.10.2 Genotypic methods for the identification of *Staphylococcus aureus*

Techniques such as conventional and real-time polymerase chain reaction (PCR) assays are routinely used by laboratories for the detection of staphylococci and other species of bacteria (Brown *et al*., 2005; Turlej *et al*., 2011). Molecular identification of *S. aureus* is based on the detection of the 16S rRNA gene that is specific for the *Staphylococcus* genus and the *nuc* gene, which encodes a thermostable nuclease specific for *S. aureus*. (Ghaznavi-Rad *et al*., 2010; Bonesso *et al*., 2011; Al-Talib *et al*., 2014). Polymerase chain reaction assays can also be used to detect antibiotic resistance genes and virulence factors such as the *pvl* genes (Mkize *et al*., 2017). The *mecA* gene, which encodes PBP2A, is highly conserved among *Staphylococcus* spp., making it a suitable candidate for the detection of MRSA (Al-Talib *et al*., 2014; Salem-Bekhit, 2014; Mahon *et al*., 2015). In fact, detection of the *mecA* gene is considered the gold standard for the detection of MRSA (Brown *et al*., 2005). Multiplex PCR assays have the ability to detect numerous genes in one PCR assay (Okolie *et al*., 2015). The advantage of a M-PCR assay is that it provides a tool for the rapid identification and characterisation of staphylococci, which is important in clinical settings (Pichon *et al*., 2012). Real-time PCR assays have been used as an alternative to the traditional culture based methods as well as conventional PCR based methods because it decreases the amount of time needed to obtain results and is less laborious (Turlej *et al*., 2011; Velasco *et al*., 2014).

Huletsky and colleagues (2005) described a real-time PCR assay for the identification of MRSA. This assay detected the *orfX* gene that is unique to *S. aureus* and the SCC*mec* element carrying *mecA*, thereby linking methicillin resistance specifically to *S. aureus* (Tenover and Tickler, 2015). There were reports of this assay giving false positive results due to the assay detecting the presence of the SCC*mec* element even though the *mecA* gene was absent (Donnio *et al*., 2007). To overcome this problem, other assays were developed that could

simultaneously detect the 16S rRNA genes, *mecA* gene and the SCC*mec-orfX* junction (Kim *et al*., 2013). The BD Max StaphSR system (BD Diagnostics, USA) is an automated multiplex real-time PCR assay that is used for the molecular detection of MRSA (Lee *et al*., 2017). This assay amplifies the SCC*mec* right extremity junction (MREJ), the *nuc* and *mecA*/*C* to positively identify MRSA (Dalpke *et al*., 2015). Another real-time PCR assay targeting DNA sequences within the *orfX*–SCC*mec* junction is the Xpert MRSA assay (Cepheid, USA) and it uses the GeneXpert real-time PCR platform (Rossney *et al*., 2008). This automated system integrates all the steps involved in detection of SCC*mec* in two hours (Oh *et al*., 2013). This assay performs PCR using a cartridge containing all the necessary reagents (Oh *et al*., 2013). A newer Xpert MRSA NxG assay has received FDA approval and unlike the previous Xpert MRSA assay, it includes primers and probes that can detect more SCC*mec* types and can detect both *mecA* and *mecC* (Yarbrough *et al*., 2018). These assays offer the advantage of being rapid methods of detecting MRSA (Tenover and Tickler, 2015). Some of the disadvantages are that they are costly and require specialised equipment and reagents (Turlej *et al*., 2011; Tenover and Tickler, 2015).

2.11 *Staphylococcus aureus* typing methods

Various discriminative methods can be employed to type MRSA isolates and to determine their molecular epidemiology (Sit *et al*., 2017). Typing of MRSA isolates is important for investigations pertaining to the spread and transmission of MRSA within and among hospitals and is an essential component of a good surveillance system and infection control strategies (Nübel *et al*., 2013; Rebic *et al*., 2016). A good typing method is inexpensive, rapid, easy to interpret, highly reproducible and must have a high enough discriminatory power to differentiate isolates that are epidemiologically unrelated (Al-Obaidid *et al*., 2018). Typing methods used for the characterisation of bacterial isolates include SCC*mec* typing, pulsed-field gel electrophoresis (PFGE), multi-locus sequence typing (MLST), spa typing, AGR typing and whole genome sequencing (Enright *et al*., 2000; Schwalm *et al*., 2011). These typing methods vary in their discriminatory power, reproducibility, cost and labour (Mehndiratta and Bhalla, 2012). No single method is ideal for typing MRSA; a combination of different typing methods will differentiate isolates better because using different typing methods provide more data about the bacterial populations than a single method (Clark *et al*., 2012; Bilung *et* al., 2018).

2.11.1 Staphylococcal cassette chromosome *methicillin* typing

This is a PCR-based typing method that can be used for the assignment of SCC*mec* types, allowing for the identification of HA-MRSA and CA-MRSA (Sit *et al*., 2017). This typing method targets specific sites of the SCC*mec* element that are characteristic to each SCC*mec* type making the assignment of SCC*mec* types possible (Zhang *et al*., 2005; Kondo *et al*., 2007; Milheiriço *et al*., 2007). The advantage of this PCR-typing method is that it is quick and cost-effective (Turlej *et al*., 2011; Bonesso *et al*., 2011). One of the disadvantages of this method is that there is not a single PCR method available that can correctly identify all the *SCCmec* types and all the subtypes (Lakhundi and Zhang, 2018). Many different methods have been described for the typing of SCC*mec* elements; however, there is no concordance between the typing methods and they generate inconsistent results (Turlej *et al*., 2011). Some of the typing schemes are complex and difficult to interpret and some are unable to subtype the *SCCmec* elements (Zhang *et al*., 2005). Additionally, most of these described methods require multiple PCR reactions, which make these methods laborious and time-consuming Ghaznavi-Rad *et al*., 2010). The SCC*mec* typing schemes that are available do not take into account the plasticity of SCC*mec* elements, which results in an increase in the number of nontypeable SCC*mec* elements (Kaya *et al*., 2018).

2.11.2 Pulsed-field gel electrophoresis

This typing method has long been regarded the 'gold standard' method for the genotyping of MRSA and has been widely used for local outbreak investigation, long term surveillance of MRSA infections at regional and national levels and for comparison at an international level (Rebic *et al*., 2016). Pulsed-field gel electrophoresis uses enzymes such as *Sma*I, a rare cutting endonuclease, to digest chromosomal DNA into large fragments (Thurlow *et al*., 2012). The large DNA fragments are separated using electrophoresis on an agarose gel, with periodic changes in the orientation of the electric field across the gel (He *et al*., 2014). Following separation of the DNA fragments, a DNA fingerprint is created and similarities in banding patterns are a reflection of genetic relatedness between isolates (Thurlow *et al*., 2012; Golding *et al*., 2015).

The PFGE results are presented in the form of a dendrogram and patterns with ≥80% similarity (less than six bands difference) are considered to be part of the same pulsotype with

closely related isolates (Tenover *et al*., 1995; Calvez *et al*., 2015). In contrast to MLST that indexes changes that accumulate over a long period of time, PFGE indexes changes that accumulate rapidly, thus making it appropriate for outbreak studies or short-term epidemiological studies (Frickmann *et al*., 2012; Uhlemann *et al*, 2014). Advantages of PFGE are that it is highly discriminatory and because MLST is sequence-based, it remains cheaper than MLST (Thomas and Pettigrew, 2009). The standardisation of protocols has improved the reproducibility of PFGE between laboratories (Golding *et al*., 2015). Another advantage of PFGE is that it can be used to type most bacterial strains even though there are a few that are referred to as PFGE non-typeable; these cannot be typed using PFGE due to methylation at the restriction sites, which prevents the endonuclease from cutting the chromosomal DNA (Bosch *et al*., 2010). Some of the pitfalls of PFGE is that it takes a long time for results to become available, which can be a problem in cases where real-time surveillance is required (i.e an on-going outbreak) (Frickmann *et al*., 2012). Additionally, it is laborious and can only be performed in a well-equipped laboratory due to the fact that PFGE requires the use of specialised equipment (Ross *et al*., 2005; Mongkolrattanothai, 2013).

2.11.3 Multi-locus sequence typing

This typing method sequences an internal fragment from the seven housekeeping genes, which are responsible for the normal functioning of the bacterial cell (Bonesso *et al*., 2011). The seven housekeeping genes are carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glpF*), guanilate kinase (*gmk*), phosphate acetyltransferase (*pta*), triosephosphate isomerase (*tpi*) and acetyl-co-enzyme A acetyltransferase (*yqiL*) (Deurenberg *et al*., 2007; Budimir, 2012). Different sequences of each of the housekeeping genes are assigned specific allele numbers and each isolate is defined by these allele numbers, resulting in an allelic profile or sequence type (Deurenberg *et al*., 2007). Due to the fact that many alleles exist at each of the seven loci, it is very unlikely for two isolates to have the same allelic profile by chance and therefore isolates with the same allelic profile can be assigned to the same clonal complex using eBURST analysis (Enright *et al*., 2000; Suzuki *et al*., 2009). This typing method indexes changes that accumulate slowly over time and can thus be used to measure evolution of isolates over time (Uhlemann *et al*., 2014). Advantages of MLST are that it exhibits a high resolution power, an internet database is available, which allows for easy exchange of results and the portability of sequence data (Petersson *et al*., 2010; Unemo and Dillon, 2014). The disadvantage of this typing method is that it is time consuming and is

currently more expensive than WGS (Larsen *et al*., 2012; Mongkolrattanothai, 2013; Chen *et al*., 2015).

2.11.4 Staphylococcal protein A typing

Staphylococcal protein A (spa) typing involves the sequencing of a polymorphic 24-bp variable number tandem repeat within the 3' coding region of protein A (Koreen *et al*., 2004). Differences in the types and number of repeats, result in different spa types (Alkharsar *et al*., 2018). The differences between the repeats are a result of deletions, mutations and duplications of nucleotides (Budimir, 2012). This typing method is useful for investigating local and global epidemiology of *S. aureus* (Mazi *et al*., 2015). Staphylococcal protein A typing is popular due to the fact that it is based on the sequencing of a single gene, making it less expensive and time consuming than other methods (Alkharsar *et al*., 2018). It has a higher discriminatory power than MLST and shows a high degree of concordance with other typing methods (Strommenger *et al*., 2006; Goudarzi *et al*., 2016). Another added advantage of this typing method is the fact that an online database is available where electronic submissions can be made and spa types can be assigned (Mehndiratta and Bhalla, 2012). The fact that this typing method is based on the sequencing of a single gene can also be a disadvantage because misclassifications can occur as a result of recombination or sequencing errors (Satta *et al*., 2013). Another disadvantage is that it has high running costs, which have limited its use to laboratories that can afford it (Mazi *et al*., 2015).

2.11.5 Whole genome sequencing

Whole genome sequencing has vastly improved our knowledge of MRSA and has shed light on areas such as evolution, outbreak investigations as well as the resistome, virulome and mobilome of MRSA strains (Wang *et al*., 2016). Additionally, knowledge of the epidemiology as well as the spread of specific clones assists in efficient infection control (Sabat *et al*., 2017). Improvements in the sequencing technology and the tools used for analysis have increased the output and the speed at which data can be analysed as well as the cost of WGS (Quainoo *et al*., 2017). With whole genome sequencing becoming increasingly cheaper, it has the potential to be used routinely in diagnostics and surveillance (Jeoensen *et al*., 2014). One of the advantages of WGS is that it has the ability to provide information about an isolate such as the species, strain type, antibiotic resistance and virulence (Besser *et al*., 2018). Whole genome sequencing allows for genome wide comparisons of clinical

isolates and has a high discriminatory power (Lee *et al*, 2015). Another advantage of WGS is that when the sequence of the entire genome is available, molecular typing of bacteria can be performed *in silico* using various bioinformatic tools such as MLST 2.0, *spa*Typer 1.0, eBURST v3 and PlasmidFinder v1.3 (Amoako *et al*., 2019). A disadvantage of WGS is that the genetic information of an organism does not necessarily translate into knowledge of gene expression and transcription; in other words, the presence of a particular gene does not equate to expression of the gene (Kwong *et al*., 2015). Consequently, most published evidence does not currently support the replacement of routine phenotypic antibiotic susceptibility testing with WGS-inferred susceptibility (Ellington *et al*., 2017). Although the cost of WGS has decreased significantly and more laboratories are able to use it, it still remains expensive specifically for resource limited laboratories in developing countries (Lee *et al*, 2015). Furthermore, the analysis of the data generated needs to be performed by a bioinformaticist (Lee *et al*, 2015). Despite the expenses associated with WGS, it still remains relevant during outbreak investigations because it provides the ultimate resolution power and it has the ability to correctly exclude epidemiologically unrelated isolates (Sabat *et al*., 2017).

2.12 Infection control in the healthcare setting

Infection control is an aggregate of all the methods that are employed to prevent nosocomial infections (Ryan *et al*., 2014). Since the 1970s, infection prevention programmes have been recognised as an integral part of infection control in hospitals (Harris *et al*., 2017). Healthcare associated infections (HAIs), especially those caused by antibiotic resistant bacteria, are associated with high morbidity and mortality (Harris *et al*., 2017). Patients that are infected pose a threat to other patients in the hospital as they can transmit pathogens to other patients directly or through contact with a staff member (Ryan *et al*., 2014). Standard precautions are the minimum infection prevention practices needed for safe care of patients in all healthcare settings and include hand hygiene, the use of personal protective equipment, safe injection practices, respiratory hygiene or cough etiquette and safe handling of equipment or surfaces that may be contaminated (Ryan *et al*., 2014; Kim and Oh, 2015; Olson *et al*., 2016). These standard precautions need to be adhered to at all times irrespective of the diagnosis of infectious status of patients because blood and other bodily fluids of patients could potentially be infectious (Kim and Oh, 2015; Rajbhandari *et al*., 2018).

Transmission-based precautions are used in conjunction with standard precautions and these additional precautions are employed in cases of suspected or proven infection (Siegel *et al*., 2007; Ryan *et al*., 2014). Contact precautions, which fall under the category of transmission-based categories, are recommended to control the spread of multi-drug resistant organisms such as MRSA and include the use of gloves and gowns and isolation of patients in private rooms (Kullar *et al*., 2016). Optimal hand hygiene is considered to be the cornerstone of healthcare associated infection prevention (Allegranzi and Pittet, 2009; Agca *et al*., 2014). Hand hygiene in conjunction with the cleaning of surfaces and inanimate objects within the healthcare setting, is another essential measure to prevent the spread of pathogens (Allegranzi and Pittet, 2009). Excessive usage of antiseptic agents can lead to decreased sensitivity to antiseptics or it can lead to resistance to the antiseptics (Shamsudin *et al*., 2012). One such antiseptic is chlorhexidine, which is a bactericidal bisguanide that is widely used in hospitals (Prag *et al*., 2014). Genes such as quartenary ammonium compound A (*qacA*) and *qacB* are found in some *S. aureus* strains and encode multidrug efflux pumps associated with a higher minimum bactericidal concentration (MBC) for chlorhexidine (McNeil *et al*., 2016). The efficacy of chlorhexidine may be negatively affected by biological material or biofilm (Prag *et al*., 2014). Multiple strains of MRSA can be found circulating in the hospital during a period of no outbreaks, which indicates that the transmission of MRSA persists despite infection control interventions (Popoola *et al*., 2014). A study conducted by Barbut and colleagues (2013) showed that the use of hydrogen peroxide vapour to decontaminate the environment proved to be an effective way to eliminate pathogens from the environment. Controlling the spread of resistant bacteria is a major challenge in the healthcare setting and often involves labour intensive and protracted efforts (Derde *et al*., 2014). Infection prevention and control involves activities aimed at preventing and reducing the spread of infections in broad and varied settings (Mahon *et al*., 2015). Curbing healthcare associated infections prevents patients and healthcare workers from acquiring healthcare associated infections that can be avoided, it prevents avoidable death, saves money, promotes high quality healthcare and reduces the spread of antimicrobial resistance, which is a global problem (WHO, 2019).

2.13 Summary

Staphylococcus aureus is a significant pathogen that has caused an increase in the morbidity and mortality of patients across the globe (Green *et al*., 2012). This pathogen has the ability to

cause a wide array of infections and owes its success to its possession of a plethora of virulence factors that assist in causing infection, evading host defences and persisting within the host (Foster *et al*, 2015). The genome of *S. aureus* is known to contain a number of MGEs, which contribute to its plasticity and adaptability (Malachowa and DeLeo, 2010). *Staphylococcus aureus* has the ability to survive on inanimate objects and therefore spread due to its hardy nature and its ability to withstand high heat and dessication (Cornelissen *et al*., 2013). *Staphylococcus aureus* is also known to be resistant to a number of antimicrobials that are commonly used for treatment, further making it a threat to public health (Li and Webster, 2018).

Methicillin resistant *S. aureus* is one of the leading causes of nosocomial infections (Green *et al*., 2012). Outbreaks of MRSA are prevalent in burn wound and neonatal ward patients (Agca *et al*., 2014). There are a number of factors that make these patient groups particularly vulnerable to MRSA infections and these include: i) an immature or suppressed immune system, ii) protracted hospital stay, iii) use of antimicrobials, iv) breach in the skin surface leading to an exposed body surface, v) the use of invasive devices such as catheters, and vi) overcrowding (Church *et al*., 2006; Milstone *et al*., 2010; Saaiq *et al*., 2015).

In order to investigate outbreaks and to track the source of outbreaks and the transmission of pathogens, various typing methods are employed (Price *et al*., 2013). These typing methods include MLST, PFGE, SCC*mec* typing, spa typing and WGS (Enright *et al*., 2000; Schwalm *et al*., 2011). Each of these typing methods differs in their discriminatory power, reproducibility, cost, labour and the time needed to obtain results (Mehndiratta and Bhalla, 2012). Whole genome sequencing has the highest discriminatory power of all the typing methods mentioned above (Sabat *et al*., 2017). Whole genome sequencing has the ability to detect single nucleotide differences between strains that would not be detected using the other typing methods, thereby giving WGS the ultimate resolution power (Sabat *et al*., 2017). In addition, WGS provides information that the other typing methods do not such as information regarding the resistome, the virulome and the mobilome (responsible for the dissemination of resistance determinants and virulence factors) (Price *et al*., 2013). In this study, PFGE and WGS were used to characterise MRSA isolates that were responsible for outbreaks at a number of healthcare centres in Gauteng. The study also proposed to establish whether the MRSA isolates from the different healthcare centres are genetically related.

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CHAPTER 3

MOLECULAR CHARACTERISATION OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* OBTAINED FROM BURN AND NEONATAL WARDS FOLLOWING AN OUTBREAK

The editorial style of the Journal of Medical Microbiology was followed in this chapter

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3.1 ABSTRACT

Introduction: Methicillin-resistant *Staphylococcus aureus* (MRSA) is a common cause of outbreaks in healthcare settings and is linked to a high morbidity and mortality in hospitalised patients. Outbreaks are commonly reported in neonates and burn patients, who are susceptible to healthcare associated infections due to having a compromised immune system, undergoing surgical procedures, the use of indwelling catheters and antibiotic therapy.

Aim: This study was conducted to molecularly characterise MRSA isolates responsible for outbreaks in burn and neonatal patients.

Methodology: Eighty-five MRSA isolates obtained from burn and neonatal ward patients from four different hospitals in Gauteng were included in the study. These isolates were collected between 2015 and 2019 and were randomly selected. A multiplex PCR (M-PCR) assay was performed to confirm the identity of the MRSA isolates and to screen for the methicillin A (*mecA*) and Panton-Valentine leukocidin (*pvl*) genes. The MRSA isolates were genotyped using pulsed-field gel electrophoresis (PFGE). Five representative isolates from major pulsotypes were selected for whole genome sequencing (WGS).

Results: All 85 isolates were confirmed to be *S. aureus* and all the isolates were MRSA due to the presence of the *mecA* gene. None of the isolates harboured the *pvl* gene. Forty-two of the 85 (49%) isolates clustered together into three major pulsotypes following PFGE. Pulsotype A consisted of 32 isolates from all four hospitals and included isolates from burn and neonatal wards. Pulsotypes B and C consisted of five isolates each collected from neonatal wards from three of the four hospitals (hospitals I, III and IV for Pulsotype B and hospitals I, II and III for pulsotype C). Whole genome sequencing results showed that the isolates were assigned to three sequence types (STs), namely ST239, ST612 and ST5 and carried type III, IVa and I SCC*mec* elements respectively. The isolates were assigned spa

types t037, t1257 and t045. The clone MRSA-III-t037 was identified in different hospitals. The virulence factors identified in the isolates included proteases, enterotoxins, gamma-hemolysin, leukocidin ED and the staphylococcal complement inhibitor.

Conclusion: The clustering of the MRSA isolates indicates high genetic relatedness among MRSA isolates that were circulating within the four hospitals. The pandemic clone ST239-MRSA-III-t037 has been reported in many countries around the world. This clone has also been found to be one of the most prevalent clone in hospitals in South Africa. In this study, ST239-MRSA-III-t037 was detected in three different hospitals and the spread of this clone between hospitals is most likely due to the transfer of patients from one hospital to another. Other clones identified in this study that have been reported in South Africa and countries around the world include ST5-MRSA-I and ST612-MRSA-IV. Strict infection control and prevention measures, surveillance and monitoring need to be implemented to limit the spread of MRSA within the hospital and between different hospitals.

Keywords: MRSA, burn patients, neonatal patients, outbreak, genetic relatedness

3.2 INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a commensal bacterium that forms part of the normal flora of humans [1]. However, it is an opportunistic pathogen with the ability to cause severe disease in individuals with an immature or compromised immune-system [2]. *Staphylococcus aureus* possesses a battery of virulence factors that assist in invading the host, evading the host immune defences and ultimately causing disease in the host [3,4]. These virulence factors include, but are not limited to, enterotoxins, exfoliative toxins, hemolysins, toxic shock syndrome toxin (TSST) and Panton-Valentine leukocidin (PVL) [5]. The spectrum of diseases caused by *S. aureus* range from superficial skin infections to deep-seated infections associated with a high mortality such as bacteraemia and infective endocarditis [5,6]. *Staphylococcus aureus* becomes MRSA after acquiring a large mobile genetic element (MGE) called the staphylococcal cassette chromosome (SCC), which carries the *mecA* gene that is responsible for resistance to beta-lactams [7]. Infections caused by MRSA have become difficult to treat due to antibiotic resistance [8]. Resistance to antibiotics used for the treatment of MRSA infections can arise through the acquisition of resistance determinants carried on MGEs such as plasmids, transposons and the SCC*mec* element or as a result of mutations in the chromosomal genes [9].

Bacteria have been implicated as the most common cause of healthcare associated infections (HAIs) [10]. Methicillin-resistant *Staphylococcus aureus* is one of the leading causes of HAIs, which contribute to the increased morbidity and mortality of patients worldwide [11]. Burn patients, infants in the neonatal wards as well as other hospitalised patients are at risk of acquiring HAIs owing to the fact that they are often immunocompromised or have an immature immune system, undergoing antibiotic treatment, have indwelling devices and protracted hospitalisation [12,13]. In addition to a disrupted skin barrier and a supressed immune system, burn patients also have necrotic and avascular tissue, which favours the growth of microbes [12,14]. In neonates, an immature immune system, prematurity and surgery are some of the factors that compound the risk of acquiring HAIs [15].

The emergence of MRSA within the hospital has encouraged surveillance and strict infection control programmes [16]. Outbreaks of MRSA are investigated using typing methods that can promptly and accurately determine the genetic relatedness of outbreak isolates [13]. These typing methods include multiplex PCR (M-PCR) assays, pulsed-field gel electrophoresis (PFGE) and whole genome sequencing (WGS) [11]. Although PFGE is considered the gold standard for the typing of *S. aureus* in surveillance studies, WGS has been shown to have a higher discriminatory power [11,17]. Whole genome sequencing has the ability to exclude isolates that are epidemiologically unrelated and it provides information pertaining to the resistome, virulome and mobilome of isolates therefore making it superior to the other typing methods [18,19].

There has been an increase in the number of reports of MRSA from burn and neonatal ward patients between 2015 and 2018 at four tertiary academic hospitals in Gauteng, South Africa. The aim of this study was to determine the genetic relatedness of MRSA isolates at these healthcare centres with the use of PFGE and WGS on selected representative strains and to determine the antibiotic and virulence gene profiles associated with these outbreak strains.

3.3 MATERIALS AND METHODS

3.3.1 Study design and collection of bacterial isolates

This was a descriptive laboratory based study conducted at the Department of Medical Microbiology, University of Pretoria. Ethical approval for this study was obtained from the

Research Ethics Committee, University of Pretoria (Ethics number 326/2018). A total of 85 non-repeat clinical MRSA isolates that were stored following routine diagnostic analysis were randomly selected from the culture bank of the Department of Medical Microbiology, University of Pretoria/National Health Laboratory Service (NHLS) diagnostic laboratory, Tshwane Academic Division (TAD). The collection period was from 2015 to 2019. The isolates were collected from burn and neonatal ICU wards from hospitals I, II, III and IV. These hospitals are teaching hospitals in the Gauteng province, South Africa. Hospital I, II and IV are regional hospitals and hospital III is a reference tertiary hospital. There is movement of patients between the hospitals, with up referral of patients from the regional hospitals to the reference tertiary hospital or down referral of patients from the reference tertiary hospital to the regional hospitals.

3.3.2 Culture and storage of bacterial isolates

The MRSA isolates were sub-cultured on blood agar plates using the streak plate method to obtain single colonies. The plates were incubated (Vacutec, UK) at 37°C for 18 hours to 24 hours. Following incubation, a single colony was picked and used to inoculate 5 mL of sterile 3.7% brain heart infusion (BHI) broth (LabM Limited, UK), which was incubated (Vacutec, UK) at 37 °C for 18 hours to 24 hours. A volume of 900 µL of the turbid broth was mixed with an equal volume of sterile 50% glycerol (Merck, Germany). Each of the isolates were stored in triplicate. One cryotube (Nest biotechnology, China) was placed in the -80°C freezer (New Brunswick, USA) for long-term storage and the remaining cryotubes (Nest Biotechnology, China) were stored in the -20°C freezer (Samsung, South Korea) until needed for further processing.

3.3.3 Antibiotic susceptibility testing of methicillin-resistant *Staphylococcus aureus*

Susceptibility testing using the Vitek® 2 automated system (bioMèrieux, France) was performed on isolates as part of routine diagnostic analysis by the NHLS/ TAD laboratory. Susceptibility testing was performed using antibiotics from the following classes: aminoglycosides, antimycobacterials, beta-lactams, fluoroquinolones, fusidanes, glycopeptides, glycyclines, lincomycins, macrolides, oxazolidinones, quinolones and sulphonamides. Some of the isolates were not tested for the full panel of antibiotics but were

tested for resistance to penicillin (beta-lactam), erythromycin (macrolide) and vancomycin (glycopeptide).

3.3.4 Total genomic DNA extraction and identification of methicillin-resistant *Staphylococcus aureus* isolates

Two millilitre of overnight broth inoculated with a pure colony of MRSA was used for DNA extraction (Appendix). The genomic DNA was extracted using the *ZR Fungal/Bacterial DNA Miniprep*™ commercial kit (Zymo Research USA) as per manufacturer recommendations. Five hundred microlitre of beta-mercaptoethanol (Merck, Germany) was added to the binding buffer in the extraction kit (Zymo Research, USA) prior to use. The extracted DNA was transferred into a sterile 2 mL microcentrifuge tube (Eppendorf, Germany) and stored at 20°C until required.

An identification multiplex polymerase chain reaction (ID M-PCR) assay was used to confirm the identity of clinical MRSA isolates. The primers were synthesised by Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa. This ID M-PCR assay used primers that targeted the following genes: i) 16S rRNA gene (597 bp) [20] specific for the genus *Staphylococcus*, ii) nuclease (*nuc*) gene (359 bp) [21] specific for *S. aureus*, iii) *sep* gene (124 bp) [22] specific for *Staphylococcus epidermidis*, iv) *mvaA* gene (271 bp) [22] specific for *Staphylococcus haemolyticus*, v) *hom* gene (177 bp) [21] specific for *Staphylococcus hominis*, vi) *cap* gene (525 bp) [21] specific for *Staphylococcus capitis*, vii) *mecA* gene (162 bp) [23] which indicates resistance to beta-lactam antibiotics and viii) *pvl* gene (433 bp) [24] for the detection of the Panton-Valentine leukocidin. The primer sequences and amplicon sizes are shown in Table 3.1. A M-PCR assay reaction mastermix was prepared by mixing the following: 12.5 µL 2X Bioline® Mastermix (Bioline®, UK), 2.5 µL of a 10X primer mix, 2 µL template (<1 µg DNA/25 µL) and 8 µL nuclease free water (Qiagen, Germany), bringing the final reaction volume to 25 µL. Primers with a concentration of 2 µM were used to prepare a 10x primer mix. The positive control used in this study was an in-house PVL-positive MRSA strain identified in previous studies. Nuclease-free water (Qiagen, Germany) was used as a negative control. The amplification of DNA was performed in a G-storm thermocycler (Vacutec, UK) and the cycling conditions that were used for the amplification can be found in Table 3.2. The resulting amplicons were subjected to gel electrophoresis (see section 3.3.4).

3.3.5 Gel electrophoresis for the detection and analysis of M-PCR assay amplicons

Amplicons produced during amplification were separated using a 1.5% (w/v) SeaKem® LE agarose gel (Lonza, Switzerland), stained with 5 µL of ethidium bromide [(10 µg/mL) Promega, USA)]. Gel electrophoresis was conducted in 1X Tris-Borate EDTA (TBE) buffer (pH 8.0) at 90V for 90 minutes. A 50 bp molecular weight marker (Bioline, UK) was used as a size reference for the amplicons. After gel electrophoresis, the amplicons were visualised under UV light using the TFM-26 Ultra Transilluminator (UVP, USA). The images were captured and saved for analysis.

3.3.6 Molecular typing of methicillin-resistant *Staphylococcus aureus* isolates using pulsed-field gel electrophoresis

The genetic relatedness of 85 clinical MRSA isolates from four different healthcare centres in Gauteng was determined with the use of PFGE. The protocol described by McDougal *et al* [25] was used together with guidelines provided by the manufacturer of the Rotaphor Type VI (Biometra, Germany). *Staphylococcus aureus* chromosomal DNA was restricted using *Sma*I (New England Biolabs, USA) and separated on a 1.2% (w/v) SeaKem® LE agarose gel (Lonza, Switzerland). The banding patterns were analysed using the GelCompar II (Applied Maths, Belgium) software program. A dendrogram was reconstructed from the distance matrix (constructed using Dice coefficient) using the unweighted pair group method with arithmetic mean (UPGMA). A similarity value of ≥80% was used to assign major and minor pulsotypes to the MRSA isolates. A major pulsotype was defined as a pulsotype with five or more isolates, whereas a minor pulsotype consists of less than five isolates [26].

3.3.7 Whole genome sequencing of methicillin-resistant *Staphylococcus aureus* isolates

Five representative MRSA isolates from the major pulsotypes were selected for WGS. Isolates from the burn and neonatal wards were selected from the different pulsotypes and also according to their antibiotic resistance patterns. The genomic DNA of representative MRSA isolates was sent to the National Institute for Communicable Diseases (NICD) for sequencing and analysis. Sequencing of the isolates was performed using the MiSeq (Illumina, USA). The sequence data was analysed using CLC Genomics (CLC Bio, USA). Online databases were used for the analysis of the resistome and virulome of the MRSA

isolates. ResFinder 3.2 was used to identify antimicrobial resistance genes, VirulenceFinder 2.0 was used for the identification of virulence genes and MLST 2.0 was used to determine the sequence types of the MRSA isolates.

3.4 RESULTS

All 85 isolates were positively identified as MRSA using an ID M-PCR assay targeting the genus specific 16S rRNA gene, *S. aureus* specific *nuc* gene and the *mecA* gene, which confirms methicillin-resistant *S. aureus*. None of the isolates in this study were positive for the *pvl* gene, which encodes the Panton-Valentine leukocidin. The MRSA isolates in this study were cultured from a number of specimens, including blood cultures, intravenous catheters, nasal swabs, pus, superficial swabs, tissue and tracheal aspirates.

The collection period was between 2015 and 2019. Twelve MRSA isolates [all from neonatal intensive care wards, (NICU)] were obtained in 2015; six isolates (50%) were from Hospital I, five (42%) were from Hospital II and one (8%) was from Hospital III. Eight isolates were obtained in 2016 and all the isolates were from Hospital I; seven (88%) of the isolates were from burn patients and one (12%) was from NICU. Forty-six isolates were obtained in 2017; 27 (59%) from Hospital I, 14 (30%) from hospital II and five (11%) from Hospital III. These isolates originated from the NICU (36/46; 78%) and the burn ward (10/46; 22%). Six isolates were obtained in 2018 and all were originated from Hospital I; four (67%) from the NICU and two (33%) from the burn ward. Thirteen isolates were obtained in 2019 and all of the isolates were from the NICU of Hospital IV. The number of isolates obtained from the different hospitals between 2015 and 2019 are shown in Figure 3.1. Overall, 66 of the 85 (78%) MRSA isolates were from the NICUs, whereas only 19 (22%) originated from the burn ward. The age for neonates ranged between 0 days and 74 days (average 13.74 days) and the age for the burn patients ranged between 23 days and 55 years (average 21.98 years). The gender of a few patients (3/85; 4%) was not available. More than 50% of the neonatal patients were female (37/66; 56%). The gender of burn patients was equally distributed with 10 (53%) being female and nine (47%) being male. There were more adult burn patients (18 years and older) than patients under the age of 18 (11/19; 58% and 7/19; 37% respectively). One burn patient's age was unknown (1/19; 5%).

Phenotypic identification was performed on the isolates using the Vitek® 2 automated system (bioMèrieux, France). The Vitek® 2 automated system (bioMèrieux, France) results were confirmed using an ID M-PCR assay and both results were 100% concordant. The phenotypic identification results of 14 patients (16%) showed that more than one bacterial species was identified in a single patient. Four of the 14 (29%) patients were neonates and 10 (71%) were burn patients. The species identified in the neonates were *Acinetobacter baumannii, Enterococcus faecalis*, *Klebsiella pneumoniae* and *Serratia marcescens*. The species identified in burn patients were *Escherichia coli, Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Pseudomonas putida*.

Susceptibility results showed that all the isolates were resistant to penicillin/ampicillin and cloxacillin; 98% (66/67) of isolates were resistant to gentamycin; 97% (68/70) were resistant to clindamycin; 97% (68/70) were resistant to erythromycin/azithromycin; 91% (63/69) were resistant to ciprofloxacin; 84% (56/67) of isolates were resistant to tetracycline; 83% (58/70) were resistant to trimethoprim-sulfamethoxazole; 63% (42/67) were resistant to moxifloxacin. All of the isolates were sensitive to fusidic acid, vancomycin, teichoplanin, linezolid and tigecycline. One isolate (1.49%) was found to be resistant to rifampicin. All these results are summarised in Figure 3.2.

A dendrogram was constructed after PFGE, which showed three major pulsotypes, seven minor pulsotypes and 17 singletons at a similarity value of ≥80%. The dendrogram of the MRSA isolates is shown in Figure 3.3. Pulsotype A contained 32 isolates from all four hospitals. Seventeen of the 32 isolates in pulsotype A were from Hospital I, eight were from Hospital II, two were from Hospital III and five were from Hospital IV. Four of the 32 isolates in pulsotype A were from the burn ward and 28 were from the neonatal wards. The largest number of isolates in pulsotype A (20/32) was from 2017 followed by five from 2019, five from 2015 and two from 2018.

Pulsotype B consisted of five isolates from Hospitals I, III and IV. Three of the five isolates were from Hospital III and single isolates each from Hospital I and Hospital IV. All the isolates originated from the NICU. The isolates were from 2017 (3), 2018 (1) and 2019 (1).

Pulsotype C consisted of five isolates from Hospitals I, II and III. Equal numbers (two each) of the isolates were obtained from Hospital II and III and one isolate was from Hospital I. All isolates were from the NICU. All the isolates [5/5 (100%)] were from 2017.

Five representative isolates were selected for WGS based on antibiotic resistance profiles and their clustering on the dendrogram. The STs that were identified *in silico* in the representative isolates were ST239, ST5 and ST612. Three of the five isolates belonged to ST239 and had an allelic profile of 2-3-1-1-4-4-3. These three isolates harboured a type III SCC*mec* element and had the spa type t037. The clone ST239-MRSA-III was identified in three different hospitals (I, II and III). One of the five isolates belonged to ST5 and had an allelic profile of 1-4-1-4-12-1-10. This isolate harboured a type I SCC*mec* element and had the spa type t1257. The remaining isolate belonged to ST612 and had an allelic profile of 2-3-1-1-4-4-3. This ST612 isolate carried a type IVa SCC*mec* element and had the spa type t1257.

The WGS data showed that all the representative isolates carried the *aur* and *spl* genes, which encode the proteases aureolysin and serine protease-like (Spl) proteins respectively. Although the *pvl* gene was not detected in the isolates, genes that encode other bi-component leukocidins were detected. These genes were *hlg*A, *hlgB* and hlg*C*, which encode gamma-hemolysin and *lukE* and *lukD*, which encode Leukocidin ED. The representative isolates all carried genes that code for staphylococcal enterotoxins (SE). These genes included *sea*, *seb*, *sek*, *seg*, *sen*, *seo*, and *seq*. Staphylococcal enterotoxins are gastrointestinal toxins but these toxins also display potent superantigenic acivity and cause disruptions in the adaptive immunity [27,28]. Resistance genes such as *blaZ*, *tetM*, *ermA*, *aac*(6')-*aph*(2''), *aph*(3'-lla) which confer resistance to penicillins, tetracyclines, macrolides and aminoglycosides respectively, were detected. These results showed concordance with susceptibility testing results that were obtained using the Vitek® 2 automated system (bioMèrieux, France). The *scn* and *sak* genes, which encode the staphylococcal inhibitor protein and staphylokinase, respectively, were identified in the MRSA isolates. No *chp* and *sep* genes were identified in any of the isolates.

3.5 DISCUSSION

The M-PCR assay results showed the presence of the 16S rRNA, *nuc* and *mec*A genes, therefore confirming MRSA. Concordance between the ID M-PCR assay results and the

identification results from the Vitek® 2 automated system (bioMèrieux, France) indicates that PCR can be employed to rapidly and reliably identify bacteria. Furthermore, PCR is more cost-effective compared to automated systems. None of the MRSA isolates carried the *pvl* gene, which encodes the Panton-Valentine leukocidin. This is in agreement with a study conducted by Ramsing and colleagues [29], which found no *pvl*-positive MRSA isolates during an outbreak investigation using molecular methods in neonates at a Danish NICU. This was also observed by Geraci and colleagues [30] who reported no *pvl*-positive MRSA isolates from neonates in an Italian NICU.

Outbreaks in NICUs caused by MRSA have been reported in several countries including Germany, Israel, Japan and Taiwan [29]. Neonates in the hospital are susceptible to colonisation with MRSA due to close contact with healthcare workers, whose MRSA carriage rate is two to three times higher than that of the general population [31]. In addition to MRSA, *Acinetobacter baumannii, Enterococcus faecalis*, *Klebsiella pneumoniae* and *Serratia marcescens* were identified in blood cultures of four neonates using the Vitek® 2 automated system (bioMèrieux, France). These species are included in the list of ESKAPE (*Enterococcus faecalis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterbacter* spp.) pathogens, which is a group of pathogens that commonly cause life-threatening infections in critically ill patients and are resistant to commonly used antibiotics [32,33]. A systematic review by Birt and colleagues [34] showed that MRSA was one of the most common causes of outbreaks in neonates along with *Klebsiella pnuemoniae* and *Serratia marcescens*. The frequency of outbreaks in neonatal wards in low- and middle-income countries is poorly reported but is likely to be higher than in high-income countries due to overcrowding, lack of resources and understaffing [35]. Additionally, the majority of hospitals in low-income countries do not always have adequate infection control infrastructure and most do not always have nationwide surveillance systems [36].

In addition to MRSA, Gram-negative bacteria such as *Escherichia coli, Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Pseudomonas putida* were identified in blood cultures, swabs and an intravenous catheter tip in 10 burn patients using the Vitek® 2 automated system (bioMèrieux, France). A study conducted on burn patients at a burn centre in Iran by Namvar and colleagues [37] also identified *K. pneumonia*, and *P. aeruginosa* in burn patients. A study conducted by Bahemia and colleagues [38] at a burn

unit in Johannesburg, South Africa reported MRSA as the third most cultured bacterium from burn patients following *A. baumannii* and *P. aeruginosa*. In contrast, a study conducted by Forson and colleagues [39] at a tertiary teaching hospital in Ghana predominantly isolated *Pseudomonas* spp. and *Acinetobacter* spp., whereas *Proteus mirabilis* and *S. aureus* were less frequently isolated. Burn units are a major reservoir for MRSA and outbreaks are commonly reported [40]. Patients in the burn unit are susceptible to local and systemic infection due to a disrupted skin barrier, a compromised immune system and a protracted hospital stay.

Antibiotic susceptibility testing using the Vitek® 2 automated system (bioMèrieux, France) showed that all of the MRSA isolates in this study were multi-drug resistant (MDR) as they showed resistance to more than three classes of antibiotics. High levels of resistance to penicillin, cloxacillin, gentamycin, clindamycin, erythromycin, ciprofloxacin, tetracycline and trimethoprim-sulfamethaxozole were observed in this study. A study conducted by Chen and colleagues [42] in China also reported MRSA isolates showing high levels of resistance to penicillin, erythromycin, clindamycin and tetracycline. However, Chen and colleagues [42] reported higher resistance to rifampicin (49%) and much lower resistance rates to trimethoprim-sulfamethaxozole (19%). One patient in this study showed resistance to rifampicin, which is an antibiotic mainly reserved for use in the treatment of tuberculosis (TB) in South Africa [43]. South Africa has a high TB burden and therefore rifampicin is widely used and this could be driving resistance to rifampicin in MRSA [43]. Sixty-three percent of the isolates in this study showed resistance to moxifloxacin, which is another antibiotic that is mainly used for the treatment of TB in South Africa [44]. As all the isolates were susceptible to vancomycin and linezolid these antibiotics are still feasible treatment options for patients with MRSA infections. A study conducted by Hassan and colleagues in 2016 at a burn unit in Bangladesh found that 28% of MRSA isolates were resistant to vancomycin [45]. Co-colonization or co-infection of *Enterococcus faecium* and *S. aureus*, as observed in one of the neonates in this study, is of concern if the *E. faecium* carries the *van*A/B genes because these genes can be transferred to the *S. aureus*, giving rise to vancomycin-resistant *S. aureus* (VRSA) [46]. However, the *Enterococcus faecium* isolate in the neonate in this study was sensitive to vancomycin and therefore did not harbour the *van*A/B genes. Although reports of VRSA are still rare, only a few cases of VRSA have been reported around the world since 2002, interventions need to be put in place that will keep the spread of VRSA to a minimum [47].

Methicillin-resistant *S. aureus* is well-known for its ability to persist in hospital environments combined with its resistance to antibiotics and its high potential to cause outbreaks [48]. Pulsed-field gel electrophoresis can be employed for the surveillance and genotyping of MRSA [49]. Pulsotype A consisted of MRSA isolates that were recovered from patients in 2015, 2017, 2018 and 2019, pulsotype B was made up of isolates from 2017, 2018 and 2019 and pulsotype C consisted of isolates collected only in 2017. These results highlight the ability of MRSA to persist in the hospital environment, allowing this pathogen to spread and continuously cause outbreaks. None of the MRSA isolates that were collected in 2016 formed part of the three major pulsotypes. This may be because the MRSA isolates that were circulating in the hospitals in 2016 were genetically distinct.

Pulsotype A was made up of isolates from the burn unit and NICU, which highlights the spread and establishment of pulsotype A between and within wards and hospitals, whereas pulsotypes B and C isolates were circulating and spreading within and between NICUs. Furthermore, all the pulsotypes are made up of isolates recovered from different hospitals. These results highlight the intra- and inter-hospital spread of MRSA. This is most likely due to the movement of patients and healthcare workers between wards and hospitals.

The clone ST239-MRSA-III is a pandemic clone that has been circulating in numerous countries all over the world since the 1970s [50]. This clone has many names but is commonly known as the Brazillian/Hungarian clone and is associated with hospital-associated infections [51]. A study conducted by Moodley and colleagues on MRSA isolates collected from public and private laboratories in the nine provinces in South Africa found that ST239-MRSA-III was the second most prevalent clone among MRSA isolates after ST36-MRSA-II [52]. This clone was also the most dominant in the study done by Liu and colleagues in 2012 across seven cities focusing on children in China [53].

The ST5-MRSA-I is an epidemic clone that has been reported worldwide in countries such as Argentina, Denmark and Poland [54]. A study conducted in 2011 by Jansen van Rensburg and colleagues on MRSA isolates collected from five hospitals in Cape Town showed that ST5-MRSA-I accounted for 37% of the isolates and was the second most prevalent clone among the hospitals after ST612-MRSA-IV [55]. The ST5-MRSA-I clone in the study conducted by Jansen van Rensburg and colleagues [55] and the clone identified in this present study were both associated with spa type t045. In contrast, a study conducted by Shittu and

colleagues on MRSA isolates collected from 13 healthcare centres in South Africa found that the MRSA isolates that belonged to ST5 had a spa type of t045 and carried a type II SCC*mec* element [56]. This might be attributed to the acquisition of a different SCC*mec* element by this clone [52]. In a study using *S. aureus* isolates from 22 countries in Africa, Asia, Australia, Europe, North America and South America, Nübel and colleagues showed that MRSA that belong to ST5 have had numerous SCC*mec* acquisition events and that this lineage has been associated with a minimum of six different SCC*mec* types [57].

The ST612-MRSA-IV is an MRSA clone that has been reported to be prevalent in South African hospitals and is sporadically isolated from horses and veterinarians that work with horses in Australia [16]. This clone was found to be among the most prevalent clones identified in a study conducted by Perovic and colleagues on isolates obtained from five hospitals in Gauteng and the Western Cape [58]. The ST612-MRSA-IV isolate in this present study was associated with spa type t1257, whereas in the study conducted by Perovic and colleagues [58], it was associated with spa types t064 and t1257. Isolate 21, which is the only isolate in this study that showed resistance to rifampicin was identified as a ST612-MRSA-IV clone. This is in agreement with another study conducted by Jansen van Rensburg and colleagues [43] on previously characterised MRSA isolates obtained from hospitals in Cape Town that found that the rifampicin resistant MRSA isolates were all ST612-MRSA-IV with the exception of one isolate that corresponded to ST5-MRSA-I. All the ST612-MRSA-IV isolates carried an uncommon double amino acid substitution in RpoB ($H_{481}N$ and $I_{527}M$). The same double amino acid substitution was observed in isolate 21 of this current study.

The ST239 and ST5 isolates harboured type III and type I SCC*mec* elements respectively. These isolates can therefore be classified as HA-MRSA. The ST5 isolate carrying a type I SCC*mec* is sensitive to all non-beta-lactam antibiotics (with the exception of erythromycin and clindamycin). The same results were observed for ST5-MRSA-I isolates in the study done by Jansen van Rensburg and colleagues [55]. This may be due to the fact that SCC*mec* type I (34.3 kb) is smaller than type II (53.0 kb) and type III (66.9 kb) and therefore has the capacity to carry fewer resistance genes than type II and III, which have integrated plasmids and transposons, resulting in resistance to multiple classes of antibiotics [54].

The ST612 isolate carried a type IV SCC*mec* element and was classified as CA-MRSA. Due to the changing epidemiology and increasing prevalence of CA-MRSA in the hospital setting,

the distinction between CA-MRSA and HA-MRSA has become less clear [59]. Community-associated MRSA is known to carry SCC*mec* types that are smaller than those carried by HA-MRSA and it is believed that these smaller *SCCmec* types are more mobile [60]. Consequently, these SCC*mec* elements can be transferred more efficiently to other staphylococci, making the presence of CA-MRSA in the hospital a cause of concern [61].

Staphylococcus aureus produces a variety of secreted proteases that degrade host tissue, interfere with the host immune system and are involved in nutrient acquisition [62,63]. Aureolysin and Serine protease-like (Spl) proteases were identified in the representative isolates. Aureolysin, a well-characterised metaloprotease, is known to hamper phagocytosis of *S. aureus* by cleaving complement factor 3 [62]. Unlike aureolysin, very little is known about Spls and their function has been hard to elucidate [64]. However, evidence suggests that Spls affect host immune responses [65]. All the representative isolates harboured the bi-component pore-forming genes *hlgA, hlgB*, *hlgC*, *lukE* and *lukD* genes. Leukocidin ED (LukED), which is found in about 85% of *S. aureus* strains, has reduced cytotoxic effects compared to PVL and this normally leads to a local inflammatory response among infected patients [66]. Although the LukED leukocidin is less cytotoxic than PVL, it still remains an important virulence factor for bloodstream infections caused by *S. aureus* due to its ability to cause damage to monocytes and lymphocytes thereby diminishing phagocytic uptake and facilitating dissemination [3,67]. Additionally, both gamma hemolysin and leukocidin ED have the ability to lyse erythrocytes. The immune evasion complex (IEC) genes allow MRSA to colonise, adapt to and evade the host defences [68]. The IEC genes identified in this study were *scn*, *sea*, and *sak*. A study conducted by Ariarad and colleagues [69] in 2015 on MRSA isolates collected from patients admitted to hospitals affiliated with the Ahvaz Jundishapur University of Medical Sciences identified the same combination of genes in 12.4% of isolates. One isolate in this study belonging to ST5 only carried the *scn* gene. Ariarad and colleagues found that 6.9% of isolates only harboured the *scn* gene [69]. The IEC genes are encoded by a phage and different phages carry different combinations of these IEC genes. Bacteria can either lose these phages or be infected by different phages, resulting in an assortment of IEC genes that are harboured by a single isolate [70,69]. The combined effects of the ability of *S. aureus* to evade the host immune defences and spread due to virulence factors and infections that are hard to treat owing to antibiotic resistance, increase morbidity and mortality in affected patients.

This study highlights the spread and establishment of epidemic and pandemic MRSA clones across hospitals and wards. This study is limited by a small sample size as only 85 isolates were randomly selected to characterise. Another limitation of the study is that not all isolates were subjected to WGS. Rapid and cost-effective ways of screening patients and HCWs prior to their transfer to different hospitals should be implemented to prevent the spread of antibiotic resistant bacteria between hospitals. Whole genome sequencing has proved to be a powerful tool in outbreak investigations and more efforts should be put into finding ways to integrate WGS into routine diagnostics for surveillance and outbreak investigations.

3.6 CONCLUSION

Most of the outbreaks caused were due to a few successful and virulent clones established in the hospitals and wards. This study highlights the need for the implementation of improved screening, decolonisation and educational measures for personnel and patients moving between or entering hospitals. The study also emphasised the importance of surveillance and adhering to strict infection prevention and control strategies to prevent the spread of pathogens, especially those that are multidrug resistant, between patients and between hospitals.

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bacteriophages in MRSA ST5 isolates from swine, swine facilities, humans with swine contact and humans with no swine contact. *PLoS ONE*

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Table 3.1 Sequences of primers used for the identification of methicillin-resistant *Staphylococcus aureus* and the expected band sizes of resultant amplicons

Target gene	Oligonucleotide sequence (5'→3')	Size (bp)	Reference
16S rRNA gene for *Staphylococcus* spp.	F- GCAAGCGTTATCCGGATTT R- CTTAATGATGGCAACTAAGC	597	[20]
S. aureus specific gene (*nuc*)	F- GCGATTGATGGTGATACGGTT R- AGCCAAGCCTTGACGAACTAAAGC	280	[21]
S. epidermidis specific gene (*sep*)	F-ATCAAAAAGTTGGCGAACCTTTTC R-CAAAAGAGCGTGGAGAAAAGTATC	124	[22]
S. haemolyticus specific gene (*mvaA*)	F-GGTCGCTTAGTCGGAACAAT R-GTTTCTGGTGTATCAACACC	271	[22]
S. hominis specific gene (*hom*)	F-TACAGGGCCATTTAAAGACG R-GTTTCTGGTGTATCAACACC	177	[21]
S. capitis specific gene (*cap*)	F-ACTACGCCTATGATTATTGC R-GAYGCTTCTTTACCATAGGG	525	[21]
mecA gene	F- TCCAGATTACAACTTCACCAGG R-CCACTTCATATCTTGTAACG	162	[23]
PVL gene	F-ACACACTATGGCAATAGTTATTT R- AAAGCAATGCAATTGAT GTA	433	[24]

Table 3.2 Cycling conditions of multiplex PCR assay for the identification of methicillin-resistant *Staphylococcus aureus*

Step	Temperature (^{o}C)	Duration	Number of cycles
Initial denaturation	95	15 minutes	1
Denaturation	94	30 seconds	30
Primer annealing	58	3 minutes	
Initial extension	72	1.5 minutes	
Final extension	68	15 minutes	1

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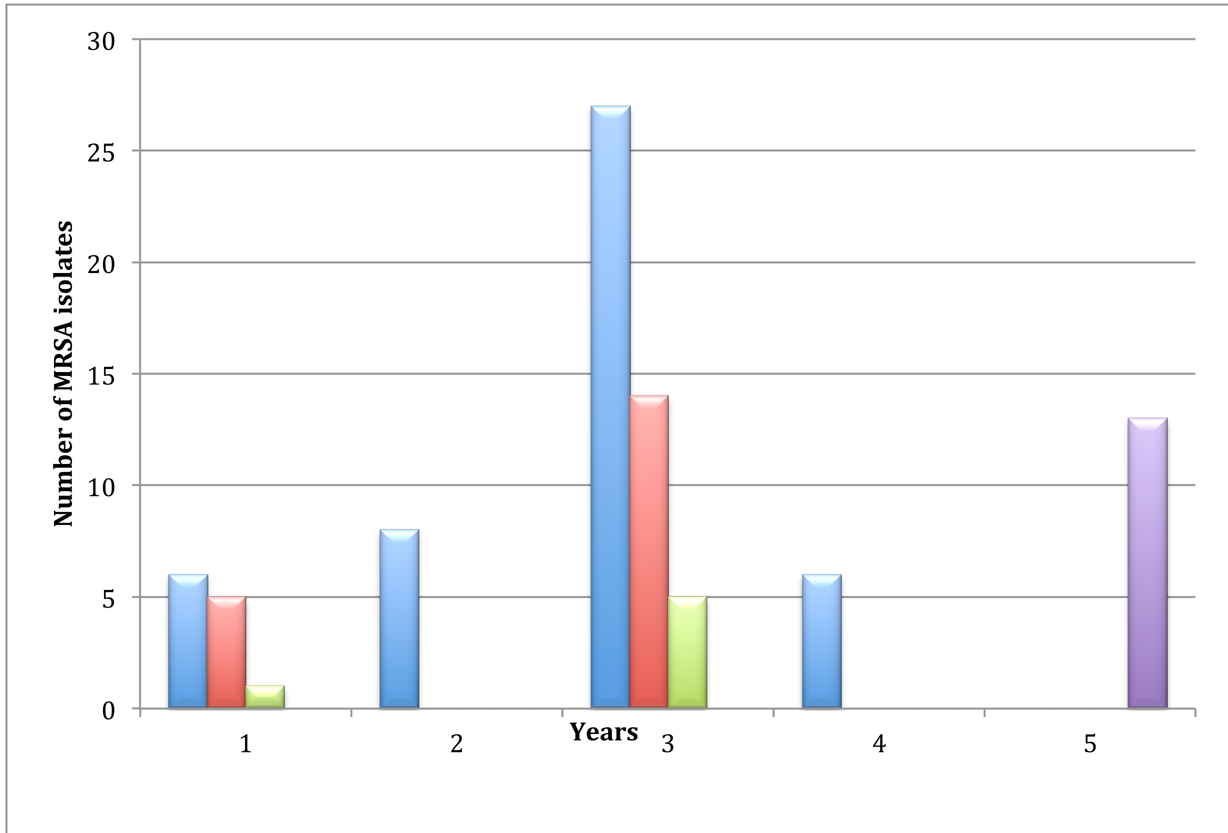

Figure 3.1: Number of isolates from the different hospitals (Hospital I=blue; Hospital II= red; Hospital III=green and Hospital IV=purple) between 2015 (1), 2016 (2), 2017 (3), 2018 (4) and 2019 (5). Continuous outbreaks are highlighted by the isolation of MRSA over a period of five years.

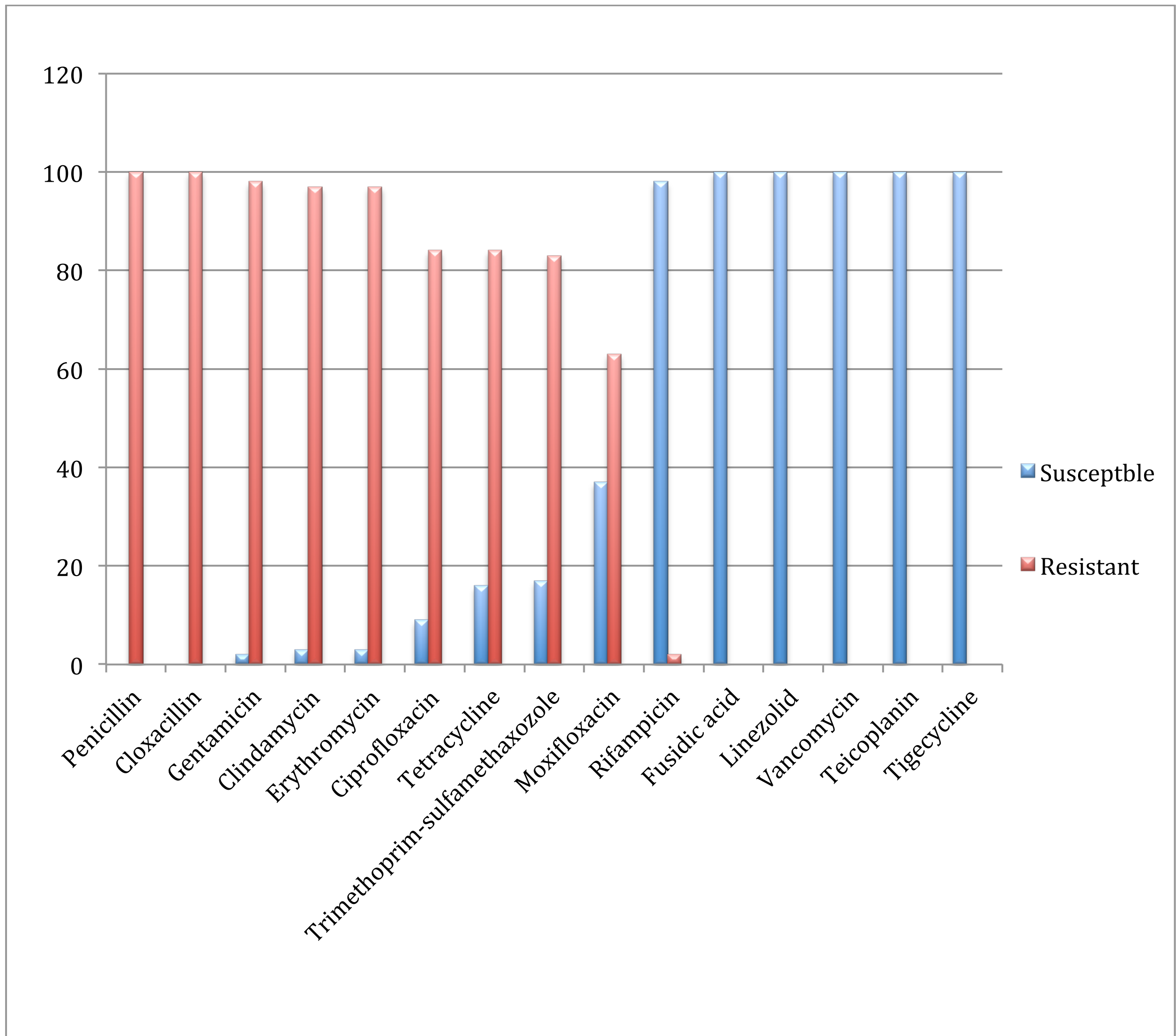

Figure 3.2 **Antibiotic resistance patterns of clinical methicillin-resistant *Staphylococcus aureus* isolates obtained from burn and neonatal patients from four different hospitals in Gauteng. The isolates show resistance to different classes of antibiotics and are therefore multi-drug resistant.**

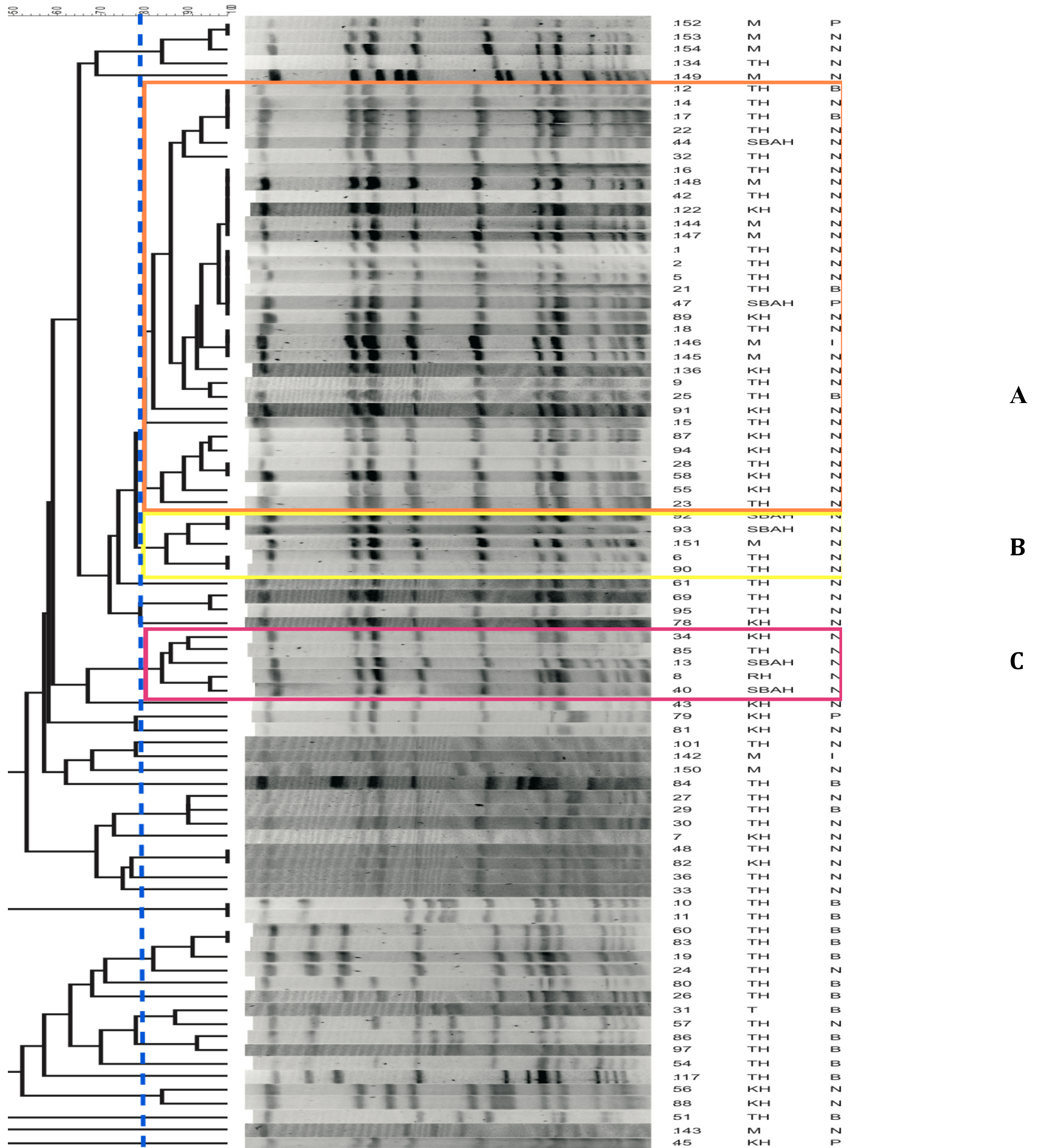

Figure 3.3 **Dendrogram constructed after pulsed-field gel electrophoresis of MRSA isolates obtained from burn and neonatal wards from 2015 to 2019 showing the major pulsotypes that formed at a similarityvalue of ≥80%. The major pulsotypes are made up of MRSA isolates from different wards and hospitals, indicating intra –and inter-hospital spread of MRSA**

CHAPTER 4

CONCLUSIONS

4.1 Concluding remarks

Staphylococcus aureus (*S. aureus*) is an important cause of healthcare-associated infections (HAIs) and it has been shown that nosocomial infections caused by *S. aureus* are a significant burden on the healthcare system (Kim *et al*., 2014). Before becoming established as a healthcare-associated pathogen, reports of methicillin-resistant *S. aureus* (MRSA) among *S. aureus* isolates causing infections within the healthcare setting were infrequent (Chandrakar *et al*., 2016). The epidemiology of MRSA is constantly changing and evolving, since its first appearance in the healthcare setting and the community (Chandrakar *et al*., 2016). The rise and dissemination of antibiotic resistant bacteria is one of the most formidable threats to the public health system (Wang and Ruan, 2016). A considerable proportion of HAIs are caused by highly antibiotic resistant bacteria such as MRSA and vancomycin resistant enterococci (VRE); MRSA in particular has been associated with substantial morbidity and mortality among patients (Cosgrove *et al*., 2005). Methicillin-resistant *S. aureus* is linked with a increase in the length of hospitalisation and hospital costs (Cosgrove *et al*., 2005). The increased use of antibiotics (or those that may be more expensive) and the need for additional laboratory and diagnostic tests all play a role in increasing the cost of healthcare (WHO, 2002). Increased length of hospitalisation may incur indirect costs by negatively affecting the patient due to the possibility of loss of income and the ability to provide for dependents (Nair *et al*., 2018). Populations in the hospital that are most vulnerable to nosocomial infections are patients in the intensive care unit (ICU), burn units, those undergoing organ transplants and neonates (Khan *et al*., 2017). This study focused only on neonates and burn patients.

Since their discovery, antibiotics have been key in treating common infections. However, the misuse of antibiotics has played a role in the rise of antibiotic resistance worldwide (Aslam *et al*., 2018). Multi-drug resistant bacteria have resulted in difficult to treat or even untreatable infections using conventional antibiotics (Frieri *et al*., 2017). The MRSA isolates in this study were multi-drug resistant and showed resistance to antibiotics such as penicillin, cloxacillin, erythromycin, gentamycin, clindamycin, tetracycline, ciprofloxacin and trimethoprim-sulfamethoxazole. The presence of the genes conferring resistance to these antibiotics was

confirmed using whole genome sequencing (WGS) data. The concordance of phenotypic susceptibility testing results and the resistance genes detected using WGS show that WGS could potentially be a promising method of predicting antimicrobial resistance (Kong *et al*., 2016). However, phenotypic testing cannot be replaced by WGS because although the presence of a gene can be detected with WGS, expression of that gene cannot be predicted using WGS data. Expression of resistance gene can be detected with phenotypic susceptibility testing. Antibiotic stewardship, which is defined as "coordinated interventions designed to improve and measure the appropriate use of antibiotic agents by promoting the selection of the optimal drug regimen including dosing, duration of therapy and route of administration", needs to be practiced in order to curb the development of drug resisance (Barlam *et al*., 2016).

A multiplex polymerase chain reaction (M-PCR) assay targeting the *Staphylococcus* 16S rRNA gene, the *nuc* gene specific for *S. aureus* and the *mec*A gene indicating MRSA was used in this study to confirm that the 85 selected isolates were MRSA. The isolates were also screened for the Panton-Valentine leukocidin (*pvl*) gene. None of the isolates carried the *pvl* genes and this may be due to its low prevalence in this setting. The M-PCR assay results and the identification results obtained using the Vitek® 2 automated system (bioMèrieux, France) were 100% concordant. This is an indication that PCR is a reliable way to identify bacteria isolated from patients. In addition, M-PCR assays can also be used to characterise isolates by targeting virulence and antibiotic resistance genes as well as mobile genetic elements that are harboured by isolates. It is rapid, reproducible and has a high sensitivity and specificity (Staggemeier *et al*., 2015).

Pulsed-field gel electrophoresis is a valuable technique that can be employed for the characterisation of bacteria responsible for outbreaks (Moghadam *et al*., 2017). It is a discriminative approach for typing *S. aureus* based on the fragmentation of the bacterial chromosome with the use of an endonuclease such as *Sma*I. Although the MRSA isolates displayed genetic diversity, three major pulsotypes consisting of five or more isolates with a minimum similarity value ≥80% were observed following electrophoresis. Each of the three pulsotypes were made up of isolates from different hospitals, which indicates genetic relatedness of MRSA isolates. Despite the fact that this technique is labour intensive and time-consuming, it was successful in genotyping all of the MRSA isolates, therefore making it an appropriate method.

Whole genome sequencing data identified three clones namely ST239-MRSA-III, ST5-MRSA-I and ST612-MRSA-IV. These clones were associated with spa types t037, t045 and t1257 respectively. The ST239-MRSA-III and ST5-MRSA-I clones have been reported worldwide. Although ST612-MRSA-IV was previously reported as an infrequent clone, it was found to be the dominant clone in hospitals in the Western Cape, South Africa by Janse van Rensburg and colleagues (2011); it has also been reported in Australia (Murphey *et al*., 2019). In the present study, ST239-MRSA-III was identified in neonates from hospitals I, II and III collected in 2017 and 2018. Isolation of the same clone in different hospitals over a period of two years is an indication of this pathogen's ability to become established and to persist in the hospitals and to spread between patients and between hospitals.

The pathogenicity of *S. aureus* can be attributed to the carriage of virulence factors that assist in the colonisation, invasion and evasion of the host defences (Wang *et al*., 2016). Genes that form part of the immune evasion complex (IEC) were identified in the five representative isolates from WGS data. The presence of these genes allow the *S. aureus* to colonise susceptible individuals and escape host defences that are already compromised due to a supressed and immature immune system in burn patients and neonates respectively. Antibiotic resistance in these isolates further complicates treatment of infections caused by these isolates. With the rise of antibiotic resistance and the dwindling treatment options, it is important to study and identify virulence factors because they serve as potential targets for the treatment of *S. aureus* infections (Ahmadrajabi *et al*., 2017).

4.2 Future research

Surveillance of HAIs in South Africa is overlooked and is poorly resourced (Lowman, 2016). As a result, the true burden of HAIs is unestablished but it is widely acknowledged that the burden is higher in the public sector than in the private sector (Lowman, 2016; Mahomed *et al*., 2017). Most public healthcare facilities in Sub-Saharan Africa, including South Africa, are unable to successfully carry out HAI surveillance due to lack of resources, trained infection control staff and infrastructure that facilitate surveillance (Dramowski *et al*., 2017). Surveillence of HAIs allows for the determination of the burden of disease and can also be used to keep track of the effectiveness of infection control strategies (Olivier *et al*., 2018). The results in this study showed the ability of MRSA to persist in the hospital environment for years and to continuously cause outbreaks, which further highlighted the importance of

infection control and surveillance in order to identify outbreaks early and to contain the spread of the pathogens responsible.

In order to provide the best healthcare to patients, it may be necessary for patients to be transferred between wards or even between hospitals (Kulshrestha and Singh, 2016). Patients may need to be transferred from one hospital to another because one hospital may have the equipment or expertise needed to provide care that may be unavailable at the other hospital. The transfer of patients colonised with multi-drug resistant (MDR) bacteria from one facility to another may result in the introduction and and subsequent spread of MDR bacteria in the new facility admitting the patient. Ideally, patients and staff need to be screened prior to transfer to other hospitals. However, screening patients is expensive and may not be feasible in settings with limited resources. Therefore future research needs to focus on developing rapid and cost effective ways of screening patients so as to prevent the intra- and inter-hospital spread of antibiotic resistant bacteria and to prevent outbreaks.

The combination of the decreasing cost and increasing speed of WGS makes it an attractive tool for routine diagnostics and public health microbiology (Leekitcharoenphon *et al*., 2014). Despite enthusiasm in the community and the many advantages WGS has over standard microbiological methods, WGS has not been widely adopted in routine diagnostics and clinical microbiology (Balloux *et al*., 2018; Mintzer *et al*., 2019). To facilitate the use of WGS in diagnostics, typing and surveillance, changes in laboratory workflow will be required and ways to convert the data generated from WGS to clinically relevant information that can be easily interpreted by clinicians and public health professionals with limited bioinformatics capabilities need to be developed (Joensen *et al*., 2014; Rossen *et al*., 2018).

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APPENDIX A

REAGENTS AND BUFFERS USED IN THE EXPERIMENTAL PROCEDURES

1. Agarose gel (1.2%)(100 mL

SeaKem LE agarose powder (Lonza, USA)	1.2 g
1X TBE Buffer (pH 8.0)	100 mL
Ethidium bromide [10mg/mL (Sigma-Aldrich, USA)]	5 µL

Add 1 g of SeaKem LE agarose powder to 100 mL of a 1X TBE buffer solution. Heat the solution until the agarose powder is completely dissolved. Allow to cool and add 5 µL of ethidium bromide. Swirl to mix then pour into a casting tray with the comb and allow to set for 1 hour

2. Brain Heart Infusion (BHI) broth (500 mL)

Brain Heart Infusion broth (LabM Limited, UK) 1	8.5 g
Ultrapure water (Purite Select HP, Purite Ltd., UK)	500 mL

Dissolve 18.5 g of BHI broth powder in 400 mL of ultrapure water. Once dissolved, bring volume to 500 mL. Autoclave at 121°C for 15 mins

3. Ethidium bromide (EtBr) solution

Ethidium bromide (10 mg/mL stock solution, Sigma-Aldrich, USA)	250 µL
Ultrapure water (Purite Select HP, Purite Ltd., UK)	1 L

Add 250 µL of EtBr in 1 L of ultrapure water, mix well and cover with foil and store in dark cupboard to prevent exposure to light

4. Ethylene diamine tetra-acetate (EDTA) (0.5 M; pH 8.0) (1 L)

EDTA (Sigma-Aldrich, USA)	93.5 g
Ultrapure water (Purite Select HP, Purite Ltd., UK)	500 mL

Dissolve 93.5 g of EDTA in 400 mL of ultrapure water. Adjust the pH to 8.0 using sodium hydroxide (NaoH) pellets (Merck, Germany) and bring the final volume to 500 mL. Autoclave at 121°C for 15 min

5. Glycerol (50%) (500 mL)

Glycerol (Merck millipore, USA)	250 mL
Utrapure water (Purite Select HP, Purite Ltd., UK)	250 mL

Add 250 mL of glycerol to 250 mL of ultrapure water. Mix thoroughly by inverting and autoclave at 121°C for 15 min

6. Gram-positive lysis buffer (pH 8.0) (500 mL)

1 M Tris-HCl solution (pH 8.0) (See 14)	25 mL
0.5 M EDTA (pH 8.0) (Prepared in 4)	50 mL
10% Sarcosyl (Sigma-Aldrich, USA)	50 mL
Ultapure water (Purite Select HP, Purite Ltd., UK)	500 mL

Add 25 mL of 1 M Tris-HCl solution, 50 mL of 0.5 M EDTA solution and 50 sarcosyl solution to 400 mL of ultrapure water. Mix thoroughly by inverting and bring volume to 500 mL

7. Lysostaphin (50 mg/mL) (5 mL)

Lysostaphin (Sigma-Aldrich, USA)	5 mg
20 mM Sodium acetate (pH 4.5) (Merck, Germany)	5 mL

Dissolve 5 mg of lysostaphin in 5 mL of sodium acetate, mix thoroughly and store in aliquots of 1 mL at -20°C (Defy, South Africa)

8. Lysozyme (20 mg/mL) (5 mL)

Lysozyme (Sigma-Aldrich, USA)	100 mg
Tris-EDTA (TE) buffer (Prepared in 13)	5 mL

Dissolve 100 mg of lysozyme powder in 5 mL of TE buffer and mix well and store at -20°C (Defy, South Africa)

9. Proteinase K (20 mg/mL) (12.5 mL)

Proteinase K (Roche, Switzerland)	250 mg
Nuclease-free water (Qiagen, Germany)	12.5 mL

Dissolve 250 mg of Proteinase K in 12.5 mL of nuclease-free water (on ice). Mix thoroughly by swirling and aliquot the final solution into volumes of 1 mL each and store at -20°C (Defy, South Africa)

10. Sodium acetate (20 mM; pH 4.5) (10 mL)

Sodium acetate (Calbiochem, USA)	164.01 mg
Ultrapure water (Purite Select HP, Purite Ltd., UK)	10 mL

Dissolve 164.01 mg of sodium acetate powder in 10 mL of ultrapure water and mix thoroughly. Adjust the pH to 4.5 using glacial acetic acid (MerckMillipore, USA) and autoclave at 121°C for 15 min

11. Tris-boric EDTA (TBE) buffer (5X; pH 8.0) (500 mL)

Tris-base (Sigma-Aldrich, USA)	54 g
Boric acid (Sigma-Aldrich, USA)	27.5 g
0.5 M EDTA (pH 8.0) (Prepared in 4)	20 mL
Ultrapure water (Purite Select HP, Purite Ltd., UK)	1000 mL

Dissolve 54 g of Tris-base, 27.5 g of boric acid and 20 mL of 0.5 M EDTA in 800 mL ultrapure water. Once dissolved, adjust the pH to 8.0 and bring volume to 1 000 L. Autoclave

at 121°C for 15 min

12. Tris-EDTA (TE) buffer (pH 8.0) (1 L)

1 M Tris-HCl (pH 8.0)	10 mL
0.5 M EDTA (pH 8.0)	2 mL
Ultrapure water (Purite Select HP, Purite Ltd., UK)	1000 mL

Add 10 mL of Tris-HCl and 2 mL of 0.5 M EDTA to 800 mL of ultrapure water and mix thoroughly by inverting. Bring the volume to 1 000 mL and autoclave at 121°C for 15 min

13. Tris-Hydrochloric acid (HCl) (1 M; pH 8.0) (500 mL)

Tris-base (Sigma-Aldrich, USA)	60.55 g
Hydrochloric acid (HCl) (Merckmillipore, USA)	21 mL
Ultrapure water (Purite Select HP, Purite Ltd., UK)	500 mL

Dissolve 60.55 g of Tris-base in 400 mL of ultrapure water, add 21 mL of HCl and mix thoroughly by swirling. Bring the volume to 500 mL and autoclave at 121°C for 15 min

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APPENDIX B

EXPERIMENTAL PROCEDURES USED IN THIS STUDY

DNA extraction protocol according to the ZR Fungal/Bacterial DNA Miniprep™ commercial kit (Zymogen Fermentas, USA) (Modified)

1. Pipette 2 000 µL of overnight BHI Broth inoculated with a single colony in a 2 mL Eppendorf tube. Centrifuge the broth for 5 min at 5 000 g in a centrifuge (Eppendorf, Germany). Discard the supernatant and resuspend the pellet in 200 µL of phosphate buffered saline (PBS).
2. Add 600 µL of the Lysis solution into the tube containing the resuspended pellet and transfer the entire volume into a ZR BashingBeadTM Lysis Tube.
3. Secure the Lysis Tube in a bead beater or vortex (Vortex Genie, USA) fitted with a 2 mL tube holder assembly and process at maximum speed (900 g) for 5 min.
4. Centrifuge the ZR BashingBeadTM Lysis Tube in a centrifuge (Eppendorf, Germany) for 1 min at 10 000 g.
5. Transfer 400 µL of the supernatant from the BashingBeadTM Lysis Tube to a Zymo- SpinTM IV Spin Filter (orange top) in a Collection Tube and centrifuge (Eppendorf, Germany) for 1 min at 7 000 g (snap off the base of the Zymo-Spin IVTM Spin Filter prior to use).
6. Add 500 µL of Beta-Mercaptoethanol to the Fungal/Bacterial DNA Binding Buffer prior to use. Add 1 200 µL of Fungal/Bacterial DNA Binding Buffer to the filtrate in the Collection Tube from step 5.
7. Transfer 800 µL of the mixture from step 6 to a Zymo-SpinTM IIC Column in a Collection Tube and centrifuge (Eppendorf, Germany) for 1 min at 10 000 g.
8. Discard the flow through from the Collection Tube and repeat step 7.
9. Add 200 µL of the DNA Pre-Wash Buffer to the Zymo-SpinTM IIC Column in a new Collection Tube and centrifuge (Eppendorf, Germany) for 1 min at 10 000 g.
10. Add 500 µL of the Fungal/Bacterial DNA Wash Buffer to the Zymo-SpinTM IIC Column and centrifuge (Eppendorf, Germany) for 1 min at 10 000 g.
11. Transfer the Zymo-SpinTM IIC Column to a clean 1.5 ml Eppendorf Tube and add 100 µL of the DNA Elution Buffer into the column matrix. Centrifuge (Eppendorf, Germany) the Zymo-SpinTM IIC Column with Eppendorf Tube for 30 sec at 10 000 g to elute the DNA.

12. Store DNA at -20°C until required.

Pulsed-field gel electrophoresis of *Staphylococcus aureus* isolates (Mcdougal *et al*., 2003)

1. Inoculate blood agar plates with bacterial isolates and *Staphylococcus aureus* ATCC 12600, which will serve as the molecular size standard. Incubate (Vacutek, UK) at 37°C for 16 hours to 18 hours.
2. Using a swab, suspend bacterial cells into 1000 µL of TE buffer [0.1 M EDTA (Sigma-Aldrich, USA), 10 mM Tris-HCL (Sigma-Aldrich, USA), pH 8.0] and adjust the optical density of the suspension so that it is between 1.2 and 1.8 at 630 nm (Elx800, Biotek Instruments Inc., USA).
3. Add 400 µL of the adjusted cell suspension to a sterile microcentrifuge tube (Eppendorf, Germany) then add 20 µL of lysozyme [20 mg/mL stock solution (Sigma-Aldrich, USA)] and incubate (AccuBlock^{TM} Digital Dry Bath, Labnet International Inc., USA) at 56 °C for 20 minutes.
4. After incubation, add 20 µL of Proteinase K [20 mg/mL stock solution (Roche, Switzerland)] and 5 µL of lysostaphin [1 mg/mL (Sigma-Aldrich, USA)] to the mixture.
5. Prepare 1.2% agarose by adding 0.3 g of SeaKem agarose powder (Lonza, USA) to 25 mL of TE buffer [0.1 M EDTA (Sigma-Aldrich, USA), 10 mM Tris-HCL (Sigma-Aldrich, USA), pH 8.0]. Heat until the agarose dissolves completely.
6. Add 400 µL of the molten agarose to the cell suspension prepared in step 4, mix well and dispense the mixture into a plug mould (Rotaphor® Biometra, Germany) and allow to solidify at 4°C (Samsung, South Korea) for 10 minutes.
7. Prepare Gram-positive/Proteinase K mixture [50 mM Tris (Sigma-Aldrich, USA); 50 mM EDTA (Sigma-Aldrich, USA), pH 8.0; 1% N-Lauroylsarcosine (Sigma-Aldrich, USA); 0.1 mg/mL Proteinase K (Roche, Switzerland)] and add 5 mL of the mixture into 15 mL Falcon tubes (Nest Biotechnology, China).
8. Once solidified, add the plugs into the Falcon tubes (Nest Biotechnology, China) containing the Gram-positive/Proteinase K mixture [50 mM Tris (Sigma-Aldrich, USA); 50 mM EDTA (Sigma-Aldrich, USA), pH 8.0; 1% N-Lauroylsarcosine (Sigma-Aldrich, USA); 0.1 mg/mL Proteinase K (Roche, Switzerland)] and incubate (Stuart Shaking Incubator Si500, Bibby Scientific Group, UK) overnight at 54°C with constant shaking at 170 rpm.

9. After incubation, remove plugs from the Gram-positive/Proteinase K mixture [50 mM Tris (Sigma-Aldrich, USA); 50 mM EDTA (Sigma-Aldrich, USA), pH 8.0; 1% N-Lauroylsarcosine (Sigma-Aldrich, USA); 0.1 mg/mL Proteinase K (Roche, Switzerland)] and add to a new 15 mL Falcon tube (Nest Biotechnology, China) containing sterile ultrapure water (Purite Select HP, Purite Ltd, UK) and incubate (Stuart Shaking Incubator Si500, Bibby Scientific Group, UK) at 51°C with constant shaking at 170 rpm for 15 minutes.
10. Decant the ultrapure water (Purite Select HP, Purite Ltd, UK) without losing the plug and add 10 mL of sterile ultrapure water (Purite Select HP, Purite Ltd, UK). Incubate (Stuart Shaking Incubator Si500, Bibby Scientific Group, UK) at 51°C with constant shaking at 170 rpm for 15 minutes.
11. Pour out the ultrapure water (Purite Select HP, Purite Ltd, UK) and add 10 mL of TE buffer [0.1 M EDTA (Sigma-Aldrich, USA), 10 mM Tris-HCL (Sigma-Aldrich, USA), pH 8.0] and repeat step 10 four times using sterile TE buffer [0.1 M EDTA (Sigma-Aldrich, USA), 10 mM Tris-HCL (Sigma-Aldrich, USA), pH 8.0] for each wash step.
12. After the last wash step, remove the plugs from the TE buffer [0.1 M EDTA (Sigma-Aldrich, USA), 10 mM Tris-HCL (Sigma-Aldrich, USA), pH 8.0] and transfer into a sterile microcentrifuge tube (Eppendorf, Germany) containing 1 500 µL of sterile TE buffer [0.1 M EDTA (Sigma-Aldrich, USA), 10 mM Tris-HCL (Sigma-Aldrich, USA), pH 8.0] and store at 4°C (Samsung, South Korea) until required for restriction.
13. In preparation for restriction digestion, remove plugs from TE buffer [0.1 M EDTA (Sigma-Aldrich, USA), 10 mM Tris-HCL (Sigma-Aldrich, USA), pH 8.0] and cut the plugs into 2 mm thick slices using a scalpel.
14. Add each plug slice into a microcentrifuge tube (Eppendorf, Germany) containing 200 µL of a pre-restriction buffer [20 µL 10X CutSmart buffer (New England Biolabs, USA); 180 µL nuclease free water (Qiagen, Germany)] and incubate (AccuBlock™ Digital Dry Bath, Labnet International Inc., USA) at 37°C for 10 minutes.
15. Decant the pre-restriction enzyme mixture and replace it with 200 µL of restriction enzyme [173 µL nuclease free water (Qiagen, Germany); 22 µL 10X CutSmart buffer (New England Biolabs, USA); 5 µL *Sma*I (20 U/µL) restriction enzyme (New England Biolabs, USA)] and incubate (AccuBlock™ Digital Dry Bath, Labnet International Inc., USA) at 37°C for 2.5 hours.

16. During incubation, prepare a 1.2% agarose gel by adding 3.9 g of SeaKem agarose powder (Lonza, USA) to 325 mL of 0.25X TBE buffer (Sigma-Aldrich, USA), heat until completely dissolved. Pour agarose into an assembled gel tray (Rotaphor® Biometra, Germany) and allow the gel to set at room temperature (25° C ± 5°C) for at least 45 minutes. Leave 25 mL of agarose behind to seal wells.
17. Add 2 700 mL of ultrapure water (Purite Select HP, Purite Ltd, UK) to the cooling tank (Rotaphor® Biometra, Germany) of the Rotaphor® System 6.0 (Rotaphor® Biometra, Germany) and add 2 400 mL of 0.25X TBE buffer (Sigma-Aldrich) to the electrophoresis chamber (Rotaphor® Biometra, Germany). Set the temperature of the cooling tank to 5°C and the electrophoresis chamber to 13°C and switch on the cooling tank and pump of the electrophoresis chamber to allow the ultrapure water (Purite Select HP, Purite Ltd, UK) and the 0.25X TBE buffer (Sigma-Aldrich) to cool down to the desired temperatures.
18. When restriction is complete, remove plug slices from the restriction buffer and replace it with 200 µL of 0.25X TBE buffer to stop restriction and allow to stand for 5 minutes at room temperature (25°C ± 5°C).
19. Remove plug slices and place them into the wells of the gel, reserving lanes 1, 9 and 18 for the molecular size standard. Seal wells with the remaining 1.2% agarose (SeaKem LE agarose, Lonza, USA) from step 16.
20. Once sealed, cut off excess agarose on the well using a scalpel to make the gel level.
21. Cut the gel from the casting tray (Rotaphor® Biometra, Germany) and remove the frame and loose agarose. Put the corner insulators into place and put the tray (Rotaphor® Biometra, Germany) into the electrophoresis chamber.
22. Set the running parameters for electrophoresis on the Rotaphor® system (Rotaphor® Biometra, Germany) as follows: 22 hours at 13°C, constant linear angle of 120°, a voltage of 200 V to 220 V, interval of 5 s linear to 25 s.
23. After electrophoresis is complete, remove the gel from the electrophoresis chamber (Rotaphor® Biometra, Germany) and stain with 1 L of ethidium bromide solution [250 µL of ethidium bromide (10 mg/mL stock solution)(Sigma-Aldrich, USA)] for 30 minutes in the dark. De-stain the gel in 1 L of ultrapure (Purite Select HP, Purite Ltd, UK) water for 15 minutes in the dark.
24. View the gel under ultraviolet (UV) light using the UVP-Doc 1 system (Transilluminator, UVP Products Inc., USA) and capture the image. Analyse the gel images using the GelCompar II (Applied Maths, Belgium) software and generate

dendrograms and calculate the percentage of relatedness of the isolates using the Dice coefficient method and the unweighted pair group method with arithmetic mean (UPGMA) method.

25. Using a similarity value of ≥80% as a cut-off value assign pulsotypes to the isolates, with isolates showing a similarity of 80% and more being regarded as members of the same pulsotype.

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APPENDIX C

DETAILED RESULTS OF THIS STUDY

Table C1: Patient demographics and specimen types

Lab ID	Year	Ward	Specimen	Gender	Age	MRSA	PVL	Hospital
1	2017	NICU	BC	F	11d	+	-	I
2	2017	NICU	BC	M	7d	+	-	I
5	2017	NICU	BC	F	6d	+	-	I
6	2018	NICU	BC	M	21d	+	-	I
7	2017	NICU	BC	F	16d	+	-	II
8	2017	NICU	BC	F	18d	+	-	II
9	2017	NICU	BC	F	15d	+	-	I
10	2018	B	P	M	5y	+	-	I
11	2017	B	BC	M	2y	+	-	I
12	2017	B	P	F	26y	+	-	I
13	2017	NICU	BC	F	8d	+	-	III
14	2018	NICU	BC	F	5d	+	-	I
15	2017	NICU	BC	F	5d	+	-	I
16	2017	NICU	BC	F	0d	+	-	I
17	2017	B	BC	F	23d	+	-	I
18	2017	NICU	BC	F	3d	+	-	I
19	2016	B	P	M	43y	+	-	I
21	2017	B	P	F	30y	+	-	I
22	2017	NICU	BC	F	6d	+	-	I
23	2017	NICU	BC	F	6d	+	-	I
24	2017	NICU	BC	NA	NA	+	-	I
25	2017	B	BC	F	23y	+	-	I
26	2017	B	P	F	22y	+	-	I
27	2017	NICU	BC	F	13d	+	-	I
28	2017	NICU	BC	F	21d	+	-	I
29	2017	B	S	F	NA	+	-	I
30	2015	NICU	BC	F	2m4d	+	-	I
31	2018	B	P	F	2y	+	-	I
32	2018	NICU	BC	M	8d	+	-	I
33	2018	NICU	BC	F	3d	+	-	I
34	2017	NICU	BC	F	28d	+	-	II
36	2015	NICU	BC	M	10d	+	-	I
40	2017	NICU	TA	F	6d	+	-	III
42	2017	NICU	BC	F	14d	+	-	I
43	2017	NICU	BC	F	7d	+	-	II
44	2017	NICU	BC	F	11d	+	-	III
45	2017	PICU	BC	F	19d	+	-	II
47	2015	PICU	BC	F	1m4d	+	-	III
48	2017	PICU	BC	F	4d	+	-	I
51	2016	B	P	M	1y	+	-	I
54	2016	B	P	M	55y	+	-	I
55	2015	NICU	BC	M	9d	+	-	II
56	2015	NICU	P	M	12d	+	-	II
57	2015	NICU	BC	M	7d	+	-	I
58	2015	NICU	BC	F	11d	+	-	II
60	2016	B	IC	M	31y	+	-	I
61	2016	NICU	BC	M	5d	+	-	I
69	2015	NICU	BC	M	5d	+	-	I
78	2017	NICU	BC	M	3d	+	-	III
79	2017	PICU	S	M	7d	+	-	II

+: positive; **-**: negative; **B**: adult and paediatric burn unit; **N**: **BC**: blood culture neonatal ward; **IC**: intravenous catheter; **ICU**: intensive care unit; **NICU**: neonatal intensive care unit; **NS**: nasal swab; **P**: pus; **PICU**: paediatric intensive care unit; **S**: swab; **TA**: tracheal aspirate; **T**: tissue

Table C1: Patient demographics and specimen types (continued)

Lab ID	Year	Ward	Specimen	Gender	Age	MRSA	PVL	Hospital
80	2017	B	S	F	43y	+	-	I
81	2017	NICU	BC	F	4d	+	-	II
82	2017	NICU	S	F	4d	+	-	II
83	2017	B	P	F	29y	+	-	I
84	2017	B	P	M	31y	+	-	I
85	2017	NICU	S	F	32y	+	-	I
86	2017	B	P	M	19m1d	+	-	I
87	2017	NICU	BC	F	1m	+	-	II
88	2017	NICU	NS	NA	2m14d	+	-	II
89	2017	NICU	T	M	13d	+	-	II
90	2017	NICU	BC	M	4d	+	-	I
91	2017	NICU	BC	M	12d	+	-	II
92	2017	NICU	BC	F	27d	+	-	III
93	2017	NICU	BC	F	8d	+	-	III
94	2017	NICU	BC	F	6d	+	-	II
95	2015	NICU	BC	M	9d	+	-	I
97	2016	B	P	F	2y	+	-	I
101	2015	NICU	BC	F	6d	+	-	I
117	2016	B	S	M	49y	+	-	I
122	2015	NICU	BC	M	6d	+	-	II
134	2016	NICU	BC	F	9d	+	-	I
136	2015	NICU	BC	F	19d	+	-	II
142	2019	ICU	BC	F	5d	+	-	IV
143	2019	N	S	M	1m12d	+	-	IV
144	2019	N	S	NA	N/A	+	-	IV
145	2019	N	BC	M	9d	+	-	IV
146	2019	ICU	BC	M	5d	+	-	IV
147	2019	N	S	F	14d	+	-	IV
148	2019	N	BC	F	11d	+	-	IV
149	2019	N	S	F	9d	+	-	IV
150	2019	N	S	M	2m13d	+	-	IV
151	2019	N	NS	M	NA	+	-	IV
152	2019	P	NS	F	5d	+	-	IV
153	2019	N	BC	M	8d	+	-	IV
154	2019	N	BC	F	10d	+	-	IV

Key: +: positive; **-**: negative **B**: adult and paediatric burn unit; **N**: **BC**: blood culture neonatal ward; **IC**: intravenous catheter; **ICU**: intensive care unit; **NICU**: neonatal intensive care unit; **NS**: nasal swab; **P**: pus; **PICU**: paediatric intensive care unit; **S**: swab; **TA**: tracheal aspirate; **T**: tissue

Table C2: Antibiotic susceptibility patterns of MRSA isolates obtained from the burn and neonatal wards

Isolate ID	Pen/amp	Clox	Ery/Azi	Clin	Fus	Tmp	Van	Tei	Lin	Cip	Moxi	Tet	Gen	Tig	Rif
1	R	R	R	R	S	R	S	S	S	R	I	R	R	S	S
2	R	R	R	R	S	R	S	S	S	R	I	R	R	S	S
5	R	R	R	R	S	R	S	S	S	R	R	R	R	S	S
6	R	R	R	R	S	R	S	S	S	R	R	R	R	S	S
7	R	R	R	R	S	R	S	S	S	R	I	R	R	S	S
8	R	R	R	R	S	R	S	S	S	R	I	R	R	S	S
9	R	R	R	R	S	R	S	S	S	R	I	R	R	S	S
10	R	R	R	R	S	S	S	S	S	I	S	S	R	S	S
11	R	R	R	R	S	S	S	S	S	I	S	S	R	S	S
12	R	R	R	R	S	R	S	S	S	R	R	R	R	S	S
13	R	R	-	-	-	-	S	-	-	-	-	-	-	-	-
14	R	R	R	R	S	R	S	S	S	R	I	R	R	S	S
15	R	R	R	R	S	R	S	S	S	R	R	R	R	S	S
16	R	R	R	R	S	R	S	S	S	R	R	R	R	S	S
17	R	R	R	R	S	R	S	S	S	R	R	R	R	S	S
18	R	R	R	R	S	R	S	S	S	R	R	R	R	S	S
19	R	R	R	R	S	S	S	S	S	S	S	S	R	S	S
21	R	R	S	S	S	R	S	S	S	R	R	R	R	S	R
22	R	R	R	R	S	R	S	S	S	R	R	R	R	S	S
23	R	R	R	R	S	R	S	S	S	R	I	R	R	S	S
24	R	R	R	R	S	R	S	S	S	R	R	R	R	S	S
25	R	R	R	R	S	R	S	S	S	R	I	R	R	S	S
26	R	R	R	R	S	S	S	S	S	S	S	R	R	S	S
27	R	R	R	R	S	R	S	S	S	R	I	R	R	S	S
28	R	R	R	R	S	R	S	S	S	R	R	R	R	S	S
29	R	R	R	R	S	R	S	S	S	R	R	R	R	S	S
30	R	R	R	R	S	R	S	S	S	R	S	R	R	S	S
31	R	R	R	R	S	S	S	S	S	I	S	S	R	S	S
32	R	R	R	R	S	R	S	S	S	R	R	R	R	S	S
33	R	R	R	R	S	R	S	S	S	R	R	R	R	S	S
34	R	R	R	R	S	R	S	S	S	R	I	R	R	S	S
36	R	R	R	R	S	R	S	S	S	R	S	R	R	S	S
40	R	R	R	R	S	R	S	S	S	R	S	R	R	S	S
42	R	R	R	R	S	R	S	S	S	R	R	R	R	S	S
43	R	R	R	R	S	R	S	S	S	R	S	R	R	S	S
44	R	R	R	R	S	R	S	S	S	R	S	R	R	S	S
45	R	R	R	R	S	R	S	S	S	R	R	R	R	S	S
47	R	R	R	R	S	R	S	S	S	R	S	R	R	S	S
48	R	R	R	R	S	R	S	S	S	R	S	R	R	S	S
51	R	R	S	S	S	S	S	S	S	R	R	S	S	S	S
54	R	R	R	R	S	S	S	S	S	S	S	S	R	S	S
55	R	R	R	R	S	R	S	S	S	R	S	R	R	S	S
56	R	R	R	R	S	R	S	S	S	R	R	R	R	S	S
57	R	R	R	R	S	R	S	S	S	R	S	R	R	S	S
58	R	R	R	R	S	R	S	S	S	R	S	R	R	S	S
60	R	R	R	R	S	S	S	S	S	S	S	S	R	S	S
61	R	R	R	R	S	R	S	S	S	R	S	R	R	S	S
69	R	R	-	R	-	R	S	-	-	-	-	-	-	-	-
78	R	R	-	-	-	-	S	-	-	R	-	-	-	-	-
79	R	R	R	R	-	R	S	-	-	R	-	-	-	-	-

Key: **pen/amp**: penicillin/ampicillin; **ery/azi**: erythromycin/azithromycin; **Clin**: clindamycin; **Fus**: fusidic acid; **Tmp**: trimethoprim-sulfamethoxazole; **Van**: vancomycin; **Tei**: teicoplanin; **Lin**: linezolid; **Cip**: ciprofloxacin; **Moxi**: moxifloxacin; **Tet**: tetracycline; **Gen**: gentamicin; **Tig**: tigecycline; **Rif**: rifampicin

Table C2: Antibiotic susceptibility patterns of MRSA isolates obtained from the burn and neonatal wards (continued)

Isolate ID	Pen/amp	Clox	Ery/Azi	Clin	Fus	Tmp	Van	Tei	Lin	Cip	Moxi	Tet	Gen	Tig	Rif
80	R	R	R	R	-	-	S	-	-	-	-	-	-	-	-
81	R	R	-	-	-	-	S	-	-	-	-	-	-	-	-
82	R	R	R	R	-	R	S	-	-	-	-	-	-	-	-
83	R	R	R	R	S	S	S	S	S	S	S	S	R	S	S
84	R	R	R	R	S	S	S	S	S	S	S	S	R	S	S
85	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
86	R	R	R	R	S	S	S	S	S	I	S	S	R	S	S
87	R	R	R	R	S	R	S	S	S	R	R	R	R	S	S
88	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
89	R	R	R	R	S	R	S	S	S	R	R	R	R	S	S
90	R	R	R	R	S	R	S	S	S	R	R	R	R	S	S
91	R	R	R	R	S	R	S	S	S	R	R	R	R	S	S
92	R	R	R	R	S	R	S	S	S	R	R	R	R	S	S
93	R	R	R	R	S	R	S	S	S	R	R	R	R	S	S
94	R	R	R	R	S	R	S	S	S	R	R	R	R	S	S
95	R	R	R	R	S	R	S	S	S	R	S	R	R	S	S
97	R	R	R	R	S	S	S	S	S	I	S	S	R	S	S
101	R	R	R	R	S	R	S	S	S	R	R	R	R	S	S
117	R	R	-	-	-	-	S	-	-	-	-	-	-	-	-
122	R	R	R	R	S	R	S	S	S	R	S	R	R	S	S
134	R	R	R	R	S	R	S	S	S	R	S	R	R	S	S
136	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
142	R	R	R	R	S	R	S	S	S	R	R	R	R	S	S
143	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
144	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
145	R	R	R	R	S	R	S	S	S	R	R	R	R	S	S
146	R	R	R	R	S	R	S	S	S	R	R	R	R	S	S
147	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
148	R	R	R	R	S	R	S	S	S	R	R	R	R	S	S
149	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
150	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
151	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
152	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
153	R	R	R	R	S	R	S	S	S	R	R	R	R	S	S
154	R	R	R	R	S	R	S	S	S	R	R	R	R	S	S

Key: **pen/amp**: penicillin/ampicillin; **ery/azi**: erythromycin/azithromycin; **Clin**: clindamycin; **Fus**: fusidic acid; **Tmp**: trimethoprim-sulfamethoxazole; **Van**: vancomycin; **Tei**: teicoplanin; **Lin**: linezolid; **Cip**: ciprofloxacin; **Moxi**: moxifloxacin; **Tet**: tetracycline; **Gen**: gentamicin; **Tig**: tigecycline; **Rif**: rifampicin

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APPENDIX D

RESEARCH ETHICS APPROVAL LETTER

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020.

9 October 2019

Approval Certificate
Annual Renewal

Ethics Reference No.: 326/2018
Title: Molecular characterisation of methicillin-resistant Staphylococcus aureus isolates associated with outbreaks in burn wound and neonatal ward patients at healthcare centres in Gauteng, South Africa

Dear Miss KB Gama

The **Annual Renewal** as supported by documents received between 2019-09-03 and 2019-10-09 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 2019-10-09.

Please note the following about your ethics approval:

- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2020-10-09.
- Please remember to use your protocol number (326/2018) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers
MBChB MMed (Int) MPharmMed PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)