Genetic diversity of methicillin resistant *Staphylococcus aureus* strains in the Pretoria region in South Africa

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by

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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 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_____2014

Spending time with GOD is the key to our strength and success in all areas of life. Be sure that you never try to work GOD into your schedule, but always work your schedule around HIM

Joyce Meyer

Dedication

To my dear husband* (*Babatunde Rotimi*)*: Thank you for your support, love and understanding

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LIST OF SYMBOLS AND ABBREVIATIONS

SYMBOLS

α	Alpha
β	Beta
^{o}C	Degree Celsius
γ	Gamma
%	Percentage

ABBREVIATIONS

ACME	Arginine catabolic mobile element
ADI	Active detection and isolation
agr	Accessory gene regulator
attBscc	bacterial chromosomal attachment site
AIP	Auto-inducing peptide
AST	Antibiotic susceptibility testing
arc	Carbamate kinase
*aro*E	Shikimate dehydrogenase
BC	Blood culture
BECC	Brazilian epidemic clonal complex
BHI	Brain-heart infusion broth
BORSA	Borderline oxacillin resistant *Staphylococcus aureus*
BURST	Based upon repeated sequence types
bp	Base pair
CA-MRSA	Community-associated methicillin resistant *Staphylococcus aureus*
CC	Clonal complex
ccr	Cassette chromosome recombinase
CDC	Centers for Disease Control and Prevention
CDSs	Coding sequences
CHIPS	Chemotaxis inhibitory protein of staphylococci
CLSI	Clinical Laboratory Standards Institute
coa	Coagulase typing
CoNS	Coagulase negative staphylococci
Cont	Contaminated
CV	Core variable
CVP tip	Central venous pressure tip
D	Days
*dna*J	Heat shock protein 40

DNA	Deoxyribonucleic acid
Eap	Extracellular adherence protein
EARSS	European Antimicrobial Resistance Surveillance System
ET	Exfoliative toxin
EMRSA	Epidemic methicillin resistant *Staphylococcus aureus*
Fc	Fibronectin
FDA	Food and Drug Administration
FRET	Fluorescence resonance energy transfer
*glp*F	glycerol kinase
gmk	guanylate kinase
h	Hour
hVISA	heteroresistant vancomycin-intermediate *Staphylococcus aureus*
HA-MRSA	Healthcare-associated methicillin resistant *Staphylococcus aureus*
HIV	Human immunodeficiency virus
HVR	Hypervariable region
ICU	Intensive care unit
ID	Intravascular device
IE	Infective endocarditis
IgG	Immunoglobulin G
IM	Internal medicine
IS	Insertion sequence
kDa	kilodalton
KZN	KwaZulu-Natal
LA-MRSA	Livestock-associated MRSA
Luki	Endotracheal aspirate
M	Molar
m	Months
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time of flight mass spectrometry
Mbp	Megabase pair
mg	Milligram
MgCℓ	Magnesium chloride
MGEs	Mobile genetic elements
MIC	Minimum inhibitory concentration
min	Minute
ml	Millilitre
µg	Microgram
µl	Microlitre
µM	Micromolar
MLST	Multilocus sequence typing
MP	Medical pulmonology
M-PCR	Multiplex polymerase chain reaction

MRSA	Methicillin resistant *Staphylococcus aureus*
MSCRAMM	Microbial surface components recognising the adhesive matrix molecules
MSSA	Methicillin sensitive *Staphylococcus aureus*
MW	Molecular weight
NaCl	Sodium chloride
NAG	N-acetylglucosamine
NAM	N-acetylmuramic acid
ND	Not detected
NHLS	National Health Laboratory Service
NP	Not provided
NT	Not typeable
nm	Nanometer
ORF	Open reading frame
OP	Orthopaedic
PBP2a	Penicillin binding protein 2
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
pH	Hydrogen ion activity
PS	Paediatric surgery
PSM	Phenol-soluble modulin
pta	phosphate acetyltransferase
PVL	Panton-Valentine leukocidin
*rpo*B	β-subunit of RNA polymerase
RE	Restriction enzyme
s	Second
SaPIs	*Staphylococcus aureus* pathogenicity islands
SBAH	Steve Biko Academic Hospital
SCC	Staphylococcal cassette chromosome
SEs	Staphylococcal enterotoxins
SEI	Staphylococcal enterotoxin-like
SFP	Staphylococcal food poisoning
STX	Staphyloxanthin
SOPs	Standard operating procedures
spa	Staphylococcal protein A
SSSS	Staphylococcal scalded skin syndrome
SSTI	Skin and soft tissue infection
ST	Sequence type
*Spe*G	gene spermidine acetlytransferase
TAE	Tris-acetate ethylene diamine tetraacetate
TB	Tuberculosis
TBE	Tris-borate ethylene diamine tetraacetate

tuf	elongation factor Tu
Tn	Transposon
tpi	triosephosphate isomerase
TSS	Toxic shock syndrome
TSST-1	Toxic shock syndrome toxin-1
UK	United Kingdom
UP	University of Pretoria
UPMGA	Unweighted pair group method with arithmetic mean
USA	United States of America
UV	Ultraviolet
VISA	Vancomycin intermediate-resistant *Staphylococcus aureus*
VRSA	Vancomycin-resistant *Staphylococcus aureus*
y	year
*yqi*L	acetyl coenzyme A acetyltransferase

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PUBLICATIONS

1. **Salawu AM, Kock MM, Maphanga TG and Ehlers MM** (2013) Genetic diversity of methicillin resistant *Staphylococcus aureus* strains in the Pretoria region in South Africa. To be submitted for publication to: the *Journal of Clinical Microbiology and Infection*

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SUMMARY

Staphylococcus aureus (*S. aureus*) is one of the leading causes of human diseases in hospital and community settings. Methicillin resistant *Staphylococcus aureus* (MRSA) has emerged as a significant problem worldwide, resisting all known β-lactam antibiotics, including penicillin and cephalosporins. Increased morbidity and mortality caused by MRSA have increased financial burden on healthcare systems worldwide. Information regarding the genetic diversity of MRSA in the Pretoria region of South Africa is limited.

The relatedness of MRSA isolates has been reported in a previous departmental study, using staphylococcal cassette chromosome *mec* (SCC*mec*) and pulsed-field gel electrophoresis (PFGE). Staphylococcal protein A (*spa*) types, accessory gene regulatory (*agr*) groups and multilocus sequence types (MLST) were not reported. A total of 194 MRSA isolates were collected from the Steve Biko Academic Hospital in the Gauteng province (South Africa) from April 2010 to August 2011. This study analysed the isolates using *spa, agr* and MLST typing.

The results for *spa* typing included 187 isolates as one of the isolate was contaminated (isolate 4) and six isolates were untypeable using *spa* typing. Twelve distinct clusters (A to

L) were obtained. Cluster A had five subclusters (A1 to A5), cluster B had four subclusters (B1 to B4), cluster C and D had two subclusters each (C1 and C2 and D1 and D2, respectively), cluster E had four subclusters (E1 to E4), clusters G and K both had two subclusters (G1 and G2 and K1 and K2, respectively). Clusters F, H, I, J and L had no subclusters. The *agr* typing showed that majority of isolates belonged to *agr* group I [84.5% (163/193)] followed by *agr* group III [7.3% (14/193)], *agr* group II [4.7% (9/193)] and *agr* group I and III [3.6% (7/193)]. No *agr* group IV was detected. Ten representative isolates were selected for *spa* sequencing and MLST typing based on the PFGE results. The sequence types (ST) corresponded to three pandemic clonal complexes (CC), which have been reported worldwide *spa* type t012-ST36/CC30, *spa* type t037-ST239/CC8, *spa* type t891-ST22/CC22 and *spa* type t1257-ST612/CC8. The dominant pulsotype from PFGE typing (57%, 110/191) corresponded to pulsotype A and was represented by *spa* type t037-ST239. One Panton-Valentine leukocidin (PVL) positive isolate was detected and was identified as *spa* type t891-ST22/CC22.

Combining different molecular techniques showed that typing assays, such as *spa*, *agr* and MLST typing can accurately be used to determine the genetic diversity of MRSA. Compared to PFGE typing, the three Polymerase chain reaction (PCR) based techniques were rapid and less labour intensive but MLST typing was more expensive. The *spa* types and sequence types identified in the study represent major clones that are disseminated in South Africa and worldwide. The detection of an epidemic clone [t891-ST22/CC22 (EMRSA-15)] harbouring the PVL gene as well as one of the most common pandemic clones [(spa type t037-ST239/CC8)] in this clinical setting is alarming. Results from this study emphasise the need for strict infection control policies to prevent possible outbreaks of these epidemic and pandemic clones that are already circulating in this clinical setting.

Keywords: MRSA, PVL, *spa, agr,* MLST

CHAPTER 1

1.1 INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a commensal bacterium and an important cause of skin and soft tissue infections, pneumonia, osteomyelitis, septic arthritis, endovascular infections and foreign body infections (David and Daum, 2010). *Staphylococcus aureus* has an adaptive nature and is able to survive in the environment, making this bacterium one of the most successful known to man (De Lencastre *et al.,* 2007).

Penicillin was the first antibiotic of choice for the treatment of *S. aureus* infections in the 1940s (Deurenberg and Stobberingh, 2008). However, by the 1950s many strains of *S. aureus* that produced penicillinases were able to inactivate penicillin (Ahmed, 2011). Methicillin, a penicillinase resistant penicillin, was introduced in the late 1950s and used as the antibiotic of choice for the treatment of penicillin resistant staphylococcal infections (Deurenberg and Stobberingh, 2008). However, the first methicillin resistant *S. aureus* strains were observed within a year after the introduction of methicillin (Palavecino, 2007). Resistance to methicillin was due to the acquisition of the *mec*A gene (Palavecino, 2007).

The *mec*A gene is located on a mobile staphylococcal cassette chromosome *mec* complex (SCC*mec*), which also mediates resistance to antibiotics, such as the β-lactams (penicillins, cephalosporins and carbapenems) (Deurenberg and Stobberingh, 2008). The origin of the SCC*mec* is unknown; the cassette is thought to have originated from staphylococci other than *S. aureus*, such as coagulase negative staphylococci (CoNS) (Deurenberg *et al.,* 2007, Tulinski *et al.,* 2012). Healthcare-associated MRSA (HA-MRSA) infections are caused by multi-drug resistant strains harbouring SCC*mec* types I, II, III and VI (Valsesia *et al.,* 2010). Community-associated MRSA (CA-MRSA), SCC*mec* types IV, V, VII, VIII, IX, X and XI are susceptible to the majority of non β-lactam antibiotics, such as gentamycin and ciprofloxacin (Kennedy and DeLeo, 2009; Valsesia *et al.,* 2010). It was recently documented that a new divergent *mec*A homolog called the *mec*C/*mec*A_{LGA251} was identified in human and bovine populations in the UK, Denmark and France (Laurent *et al.,* 2012; Stefani *et al.,* 2012). The *mec*C/*mec*A_{LGA251} gene is located on a novel SCC*mec* called SCC*mec* type XI (Laurent *et al.,* 2012).

Panton-Valentine leukocidin (PVL) is a staphylococcal pore forming exotoxin, which lyses leukocytes and causes tissue destruction (Zanger, 2010). Panton-Valentine leukocidin toxin is encoded by the *luk*S-PV and *luk*F-PV, two contiguous and co-transcribed genes that produce proteins of 32 and 38 kDa, respectively (Duerenberg and Stobberingh, 2008). It is assumed that PVL encoding genes have spread by means of bacteriophages among different *Staphylococcus* strains of healthcare or community origin (Monecke *et al.,* 2007; Otter *et al.,* 2010). Infections caused by PVL positive MRSA strains, such as furunculosis and severe necrotising pneumonia are often associated with CA-MRSA (Lo and Wang, 2011). The role of PVL in the pathogenesis of CA-MRSA is still controversially debated among different researchers and increasingly more studies are reporting the presence of the PVL gene in methicillin sensitive *Staphylococcus aureus* (MSSA) and coagulase negative staphylococci (CoNS) (Wardenburg *et al.,* 2008; Villaruz *et al.,* 2009; Salaam-Dreyer, 2010; Lo and Wang, 2011).

Infections associated with MRSA are usually associated with high morbidity and mortality (Graveland *et al.,* 2009). Understanding the molecular epidemiology and evolution of MRSA provide useful information for controlling transmission in healthcare and community settings (Chen *et al.,* 2009). It is important to develop effective strategies to prevent and control the spread of MRSA (Deurenberg and Stobberingh, 2008). Molecular typing techniques used to investigate outbreaks of MRSA include pulsed-field gel electrophoresis (PFGE), SCC*mec* typing, staphylococcal protein A typing (*spa* typing), accessory gene regulator typing (*agr* typing) and multilocus sequence typing (MLST) (Deurenberg and Stobberingh, 2008). The PFGE typing is described as a highly discriminatory method for monitoring an outbreak of MRSA and is referred to as the gold standard for typing MRSA strains (Vainio *et al.,* 2011). However, the analysis of PFGE typing has become more difficult and labour intensive as the number of profiles in the MRSA database continues to grow (Vainio *et al.,* 2011). In cases of MRSA outbreaks, rapid molecular typing techniques are required to cluster epidemiologically linked cases and separate them from sporadic cases (Strandèn *et al.,* 2003).

The development of sequence-based molecular typing techniques, such as *spa* and MLST typing, represents improvements over the PFGE due to the reproducibility, ease of use and interlaboratory comparison of results (Strommenger *et al.,* 2008). The use of software algorithms for the *spa* and MLST typing enables the classification of MRSA strains into specific lineages (Strommenger *et al.,* 2008). Staphylococcal protein A typing is based on

sequence polymorphisms in the variable X region of the *spa* gene for *S. aureus* surface protein A and has the advantage of being rapid, with a high throughput and interlaboratory portability (Makgotlho *et al.,* 2009; Struelens *et al.,* 2009; Stefani *et al.,* 2012). The *spa* typing provides the reliable allocation of MRSA strains to the prevalent epidemic lineages (Strommenger *et al.,* 2008). Multilocus sequence typing is based on the sequence analysis of seven housekeeping genes [carbamate kinase (*arc*), shikimate dehydrogenase (*aro*E), glycerol kinase (*glp*F), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*) triosephosphate isomerase (*tpi*) and acetyl coenzyme A acetyltransferase (*yqi*L)] (Mehndiratta and Bhalla, 2012). The alleles at each of the seven loci define the allelic profile, which corresponds to the sequence types (Mehndiratta and Bhalla, 2012). The BURST (based upon repeated sequence types) algorithm is used to place strains of *S. aureus* that are similar at five or more of the seven housekeeping genes in the same clonal complex (CC) (Kennedy and DeLeo, 2009). The accessory gene regulator (*agr*) of *S. aureus* is referred to as the global regulator of staphylococcal virulence and controls a large set of genes, including extracellular virulence factors and surface proteins (Traber *et al.,* 2008). The polymorphism at the locus across two genes, *agr*C and *agr*D is used to classify the four *agr* groups in *S. aureus* (Nastaly *et al.,* 2010). Gomes *et al.* (2005) reported that isolates with different *agr* groups are most often recovered in distinct geographical locations. The different geographical distribution of distinct *agr* groups is evident of bacterial interference mediated by the *agr* operon (Nastaly *et al.,* 2010). Alternative typing assays, such as *spa*, *agr* and MLST typing can be accurately used to determine the genetic diversity of HA-MRSA and CA-MRSA isolates obtained from clinical specimens (Pantosti and Venditti, 2009).

Makgotlho *et al.* (2009) reported the clonal relatedness of HA-MRSA and CA-MRSA during 2006 to 2008 using conventional multiplex PCR (M-PCR) assays, a real-time M-PCR assay, *spa* typing and hypervariable region (HVR) typing. The *spa* types, *agr* groups, PFGE pulsotypes and MLST types were not reported for the HA-MRSA and CA-MRSA strains circulating in this clinical setting (Steve Biko Academic Hospital). It is, therefore, important to determine the genetic diversity of MRSA strains obtained from patients in this clinical setting in order to initiate strict surveillance and infection control policies that will help limit the spread of MRSA clones circulating in this hospital. This study is a follow-up of another departmental study where SCC*mec* and PFGE typing were used to determine the genetic relatedness of 194 clinical MRSA isolates obtained from Steve Biko Academic hospital (SBAH) in the Gauteng province from April 2010 to August 2011. The aim of this study was

to identify and determine the genetic diversity of HA-MRSA and CA-MRSA isolates that were circulating in the SBAH in the Gauteng province from April 2010 to August 2011 using *spa* typing, *agr* typing and MLST typing and to compare it to the gold standard method, PFGE.

The objectives of this study were:

- To culture and extract the DNA of MRSA isolates obtained from the NHLS diagnostic laboratory of the Department of Medical Microbiology, UP/NHLS
- To use *spa* typing to determine the genetic diversity of the MRSA isolates
- To use *agr* typing to determine the genetic diversity of the MRSA isolates
- To do MLST typing on a selected number of isolates, (selection based on the results by PFGE performed in another departmental study)
- Data analysis and comparisons and the construction of a dendrogramme

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

The most virulent *Staphylococcus* species is *Staphylococcus aureus* (*S. aureus*) (Boyle-Vavra and Daum, 2007). *Staphylococcus aureus* is the most isolated bacterial pathogen in hospitalised patients and the second most common bacterium isolated in the community (Boyle-Vavra and Daum, 2007). *Staphylococcus aureus* has various characteristics, such as its virulence factors and quorum sensing mechanisms, which enables it to cause a variety of infections in humans (Moellering, 2012). *Staphylococcus aureus* is associated with infections, such as septicaemia, wound sepsis, septic arthritis, post-surgical toxic shock syndrome, osteomyelitis and pneumonia with high rates of morbidity and mortality (Shittu and Lin, 2006).

In 1929, Fleming discovered penicillin, which was implemented for the use of *S. aureus* infections in the 1940s when commercial production of the antibiotic was possible (Barnes and Sampson, 2011). Penicillin resistant *S. aureus* isolates were observed in hospitals within two years after the introduction of penicillin (Deurenberg and Stobberingh, 2008). In 1959, the first semi-synthetic penicillin derivative namely methicillin was introduced for the treatment of penicillin resistant *S. aureus* infections (Johnson, 2011). However, by 1961 the first methicillin resistant *S. aureus* (MRSA) isolates were reported (Deurenberg and Stobberingh, 2008). Resistance to methicillin and other β-lactam antibiotics, such as penicillin and cephalosporins is caused by the acquisition of the *mec*A gene, which codes for a 78-kDa penicillin binding protein 2a (PBP2a or PBP2') (Argudin *et al.,* 2010). The *mec*A gene is located in a *mec* operon with its regulatory genes; *mec*I and *mec*RI (Berger-Bachi and Rohrer, 2002; Turlej *et al.,* 2011). The *mec* operon is located on a mobile genomic island called the staphylococcal cassette chromosome *mec* (SCC*mec*) (Deurenberg and Stobberingh, 2008). The specific origin of the SCC*mec* is unknown, but it is proposed that it was probably first acquired by horizontal gene transfer from coagulase-negative staphylococcal species such as *S. epidermidis, S. scruri* and *S. haemolyticus* (Hallin *et al.,* 2007; Turlej *et al.,* 2011).

Healthcare-associated MRSA (HA-MRSA) can be defined as MRSA infections obtained by inpatients within 48 h or more after hospitalisation, surgery, dialysis, long term care

indwelling devices or a history of previous MRSA infection (Labandeira-Rey *et al*., 2007). The Centers for Disease Control and Prevention (CDC) defined community-associated MRSA (CA-MRSA) as infections, which occur in otherwise healthy people without a recent history of hospitalisation or medical procedures and are associated with skin and soft tissue infections (Klevens *et al*., 2007). Risk factors for CA-MRSA include crowding, compromised skin, contaminated surfaces, shared items and poor hygiene (Kennedy and DeLeo, 2009). It is believed that direct contact with infected individuals is the major mode of transmission of HA-MRSA and CA-MRSA infections (Kennedy and DeLeo, 2009).

Phenotypic and genotypic data both play significant roles in the understanding of MRSA infections and the implementation of necessary infection control policies in hospitals (Shittu *et al.,* 2009a). Molecular techniques used in genotyping of MRSA strains include SCC*mec* typing, staphylococcal protein A typing (*spa*), accessory gene regulatory typing (*agr*), hyper-variable region typing (HVR), coagulase typing (*coa*), pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) (Strandén *et al.,* 2003). Pulsed-field gel electrophoresis is regarded as the gold standard for typing MRSA strains; this method is tedious, time-consuming, labour intensive and expensive to perform on a routine basis (Mediavilla *et al.,* 2012). However, other molecular techniques, such as *spa*, *agr* and MLST typing are able to overcome the limitations of PFGE with excellent reproducibility, quality-controlled data, a common nomenclature and utility in infection control (Stefani *et al.,* 2012). Molecular typing techniques are important tools to obtain epidemiological information regarding the most dominant MRSA clones circulating in a particular clinical setting (Stefani *et al.,* 2012).

2.2 History and background of *S. aureus*

Fossils provided evidence, which suggests that staphylococci have been in existence on earth for over a billion years (Moellering, 1995; Moellering, 2012). It was only identified as a bacterial pathogen in the 19th century (Moellering, 1995; Moellering, 2012). *Staphylococus aureus* was first described in 1881 as a cause of acute suppuration by Sir Alexandar Fleming (Oehler, 2013). In 1884, Rosenbach was able to describe the two colony types of *Staphylococcus* as *Staphylococcus albus* (white colonies), which was later renamed as *S. epidermidis* and *S. aureus* (yellow) (Oehler, 2013). In 1894, twenty-four years after the discovery of *S. aureus*, Dr Honore Van de Velde, discovered various enzymes and toxins that

contribute to the pathogenic ability of the bacteria (Lina *et al.,* 2005). During World War I, post-influenza staphylococcal pneumonia was reported among military personnel, which led to cyanosis and rapid progression to death (Chambers, 2001; Oehler, 2013). An 82% mortality rate was documented for patients treated for *S. aureus* septicaemia in the pre-antibiotic era (Diep *et al.,* 2006). The introduction of penicillin in the 1940s was revolutionary and mortality rate due to staphylococcal infections dropped tremendously (Oehler, 2013). However, strains of *S. aureus* resistant to penicillin were reported almost immediately and resistance to other antibiotics (β-lactam antibiotics) emerged within the next decade (Oehler, 2013).

2.3 Classification of *S. aureus*

The genus *Staphylococcus* belongs to the Gram-positive low G+C DNA content group (33% for *S. aureus*) of the bacterial phylum called the *Firmicutes* (Table 2.1) (Euzéby 2011). The genus *Staphylococcus* includes more than 60 species and subspecies that are ubiquitous in nature (Lamers *et al.,* 2012). The 16S rRNA gene is often used for the identification of staphylococcal species, though its utility is now limited due to the high level of similarities seen in various *Staphylococcus* species (Lamers *et al.,* 2012). Hence, more emphasis is placed on other genes, such as the *rpo*B (β-subunit of RNA polymerase), *dna*J (heat shock protein 40) and *tuf* (elongation factor Tu), which have been found to be important in the identification of staphylococcal species (Lamers *et al.,* 2012).

Table 2.1: Summary of the classification of *Staphylococcus aureus* (Euzéby, 2011)

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

The genus *Staphylococcus* is divided into two main groups, which are distinguished by their ability to coagulate plasma: these groups include coagulase negative staphylococci (CoNS) and coagulase positive staphylococci (Otto, 2010). The most important coagulase positive species is *S. aureus* (Otto, 2010). *Staphylococcus aureus* is a versatile and infectious

pathogen in humans (Lowy, 1998). *Staphylococcus aureus* bacteria are considered as opportunistic pathogens when access is gained into the host tissue (Lowy, 1998). The nose is the core ecological niche where *S. aureus* resides in human beings but the determinants of the carrier state is incompletely understood (Wertheim *et al.,* 2005). Coagulase negative staphylococci (CoNS) is becoming highly significant as nosocomial pathogen, especially in patients with indwelling foreign bodies, such as catheters, prosthetic heart valves and joint prostheses (Trülzsch *et al.,* 2007). Thirty-nine species of CoNS have been identified (Piette and Verschragreen, 2009). Sixteen of the CoNS species have been identified in specimens of human origin (Piette and Verschragreen, 2009). These 16 CoNS species are grouped into novobiocin-resistant species (*S. cohnii, S. saprophytcus, S. sciuri* and *S. xylosus*) and novobiocin-sensitive species (*S. auricularis, S. capitis, S. caprae, S. epidermidis, S. haemolyticus, S. hominis, S. lugdunensis, S. pasteuri, S. saccharolyticus, S. schleiferi, S. simulans* and *S. warneri*) (Piette and Verschragreen, 2009).

2.4 Characteristics and morphology of *S. aureus*

The staphylococcal cell wall is a unique, semi-rigid structure, which is made up of peptidoglycan, teichoic acids and surface proteins (Plata *et al.,* 2009). The staphylococcal cell wall is reported to consist of 50% peptidoglycan by weight (Lowy, 1998). The thick peptidoglycan structural unit in the cell wall is made up of two major components: (i) a disaccharide composed of N-acetylmuramic acid (NAM) and N-acetyl glucosamine (NAG), (ii) a pentapeptide covalently attached to NAM (Plata *et al.,* 2009; Atilano *et al.,* 2010). The teichoic acid (water soluble polymers that are covalently linked to peptidoglycan) protects the bacterium against environmental conditions, such as heat, antimicrobial fatty acids, cationic antibiotics and lytic enzymes (Bera *et al,* 2007). The members of the genus *Staphylococcus* are best described as facultative anaerobes, which generate energy through aerobic respiration and produces lactic acid by means of fermentation (Plata *et al.,* 2009). *Staphylococcus aureus* is differentiated from other staphylococcal species by its ability to produce coagulase, a surface enzyme, which interacts with prothrombin in the blood and coagulate plasma by converting fibrinogen into fibrin (Piette and Verschraegen, 2009; Plata *et al.,* 2009). The tube coagulase test is used to detect free coagulase, while the slide coagulase is used to detect the bound coagulase (Turnidge *et al.,* 2008). The slide coagulase test is rapid and easier to perform but some strains of *S. aureus* may yield a negative result (Turnidge *et al.,* 2008).

Hence, a negative slide coagulase test for a bacterium suspected to be *S. aureus* should be followed by a tube test (Turnidge *et al.,* 2008).

On solid agar *S. aureus* produces white pigmented to golden colonies (Plata *et al.,* 2009; Azeez-Akande 2010). The colonies produced by *S. aureus* are often 6 mm to 8 mm in diameter (Plata *et al.*, 2009). It has been reported that over 90% of *S. aureus* strains, isolated from infections in humans, are pigmented (Mishra *et al.,* 2011). Staphyloxanthin (STX) is an orange-red, membrane bound carotenoid, which plays a role in the ecological fitness of *S. aureus* (Mishra *et al.,* 2011). Carotenoids protect the bacterium against oxidants produced by the host immune system during infection and pathogenesis (Plata *et al.,* 2009; Mishra *et al.,* 2011). On blood agar plates, colonies of *S. aureus* are strongly beta-haemolytic due to its ability to lyse red blood cells (Ryan and Ray, 2004).

Staphylococcus aureus is non-motile, grows at a temperature range from 15^{o}C to 45^{o}C and is resistant to high concentrations of sodium chloride (up to 1.7 M) (Matouskova and Janout 2008; Jeyasekaran *et al.,* 2010; Nastaly *et al.,* 2010). The bacterial cells are coccal in shape, ranging from approximately 0.5 μm to 1.5 μm in diameter with some strains that are encapsulated (Green *et al.,* 2012). *Staphylococcus aureus* is able to resist 1% phenol for 15 minutes and withstand heat at 60^{o}C for 30 minutes (Bhanderi *et al.,* 2009).

2.4.1 Genomic characteristics of *S. aureus*

The staphylococcal genome is about 2.8 Mbp in size and its genome has about 2 700 protein coding sequences (CDSs) for regulatory and structural proteins (Plata *et al.,* 2009). The *S. aureus* genome consists of three components; the core genome, the core variable (CV) genes and the mobile genetic elements (MGEs) (McCarthy *et al.,* 2012). The core genome, which is well conserved in *S. aureus*, represents 75% of the genome and encodes the housekeeping genes (Plata *et al.,* 2009; McCarthy *et al.,* 2012). The CV genes represent 10% of the genome and code for proteins (surface proteins and secretory proteins) (McCarthy *et al.,* 2012). The MGEs, which make up 15% of the genome, are defined as fragments of DNA (plasmids, transposons and phages) which move between bacteria by horizontal genetic transfer mechanisms (McCarthy *et al.,* 2012). *Staphylococcus aureus* pathogenicity islands (SaPIs) are defined as large and distinct families of mobile phage-related pathogenicity islands that carry genetic elements, which contain groups of antimicrobial resistance genes as well as

chromosomal cassettes, plasmids, transposons and prophages (Baba *et al.,* 2008; Plata *et al.,* 2009).

To understand the mechanism of staphylococcal virulence and antibiotic resistance, whole genome sequencing of numerous *S. aureus* strains have been performed (Baba *et al.,* 2008). The first staphylococcal genomes to be sequenced were those of MRSA strains N315 (Japanese *S. aureus* strain) and Mu50 (MRSA strain with vancomycin intermediate resistance from a male Japanese baby), of which both were typed as the USA100 strain using PFGE typing (Baba *et al.,* 2008; DeLeo and Chambers, 2009). Kuroda *et al.* (2001) sequenced the whole genome of these two related strains (N3I5 and Mu50) using shotgun sequencing. The N315 and Mu50 strains have a low G+C content (33%) and comprise of five rDNA operons which makes it different from other *S. aureus* strains, which have rDNA operons (Kuroda *et al.,* 2001). The N315 and Mu50 genomes are made up of 2 595 and 2 695 open reading frames (ORF), respectively (Kuroda *et al.,* 2001; Baba *et al.,* 2008). These two strains have numerous remnant sequences of bacteriophages, pathogenicity islands, transposons and insertion sequence elements distributed over their genomes (Kuroda *et al.,* 2001). Each strain contained an additional distinct plasmid, which carries different antibiotic resistance genes (Steven, 2009).

The genomes of additional strains were sequenced in quick succession, since 2001 to 2009 with the publication of the whole genome sequences of: MW2, MRSA 252, MSSA 476, COL, USA300-or, FPR 3737, USA300-Hou-MR, NCTC 8325, ET3-1, H1, JH9 and the Newman strain (Steven, 2009). The *S. aureus* Newman strain was isolated in 1952 from a human infection and has been widely used in animal models of staphylococcal disease due to its various virulence phenotypes (Baba *et al.,* 2008). Thirty genes that are required for the pathogenesis was identified in the *S. aureus* Newman strain (Bae *et al.,* 2004). Well-characterised virulence genes and genes with unidentified function were reported to be involved in the pathogenesis of staphylococcal infection (Figure 2.1) (Bae *et al.,* 2004).

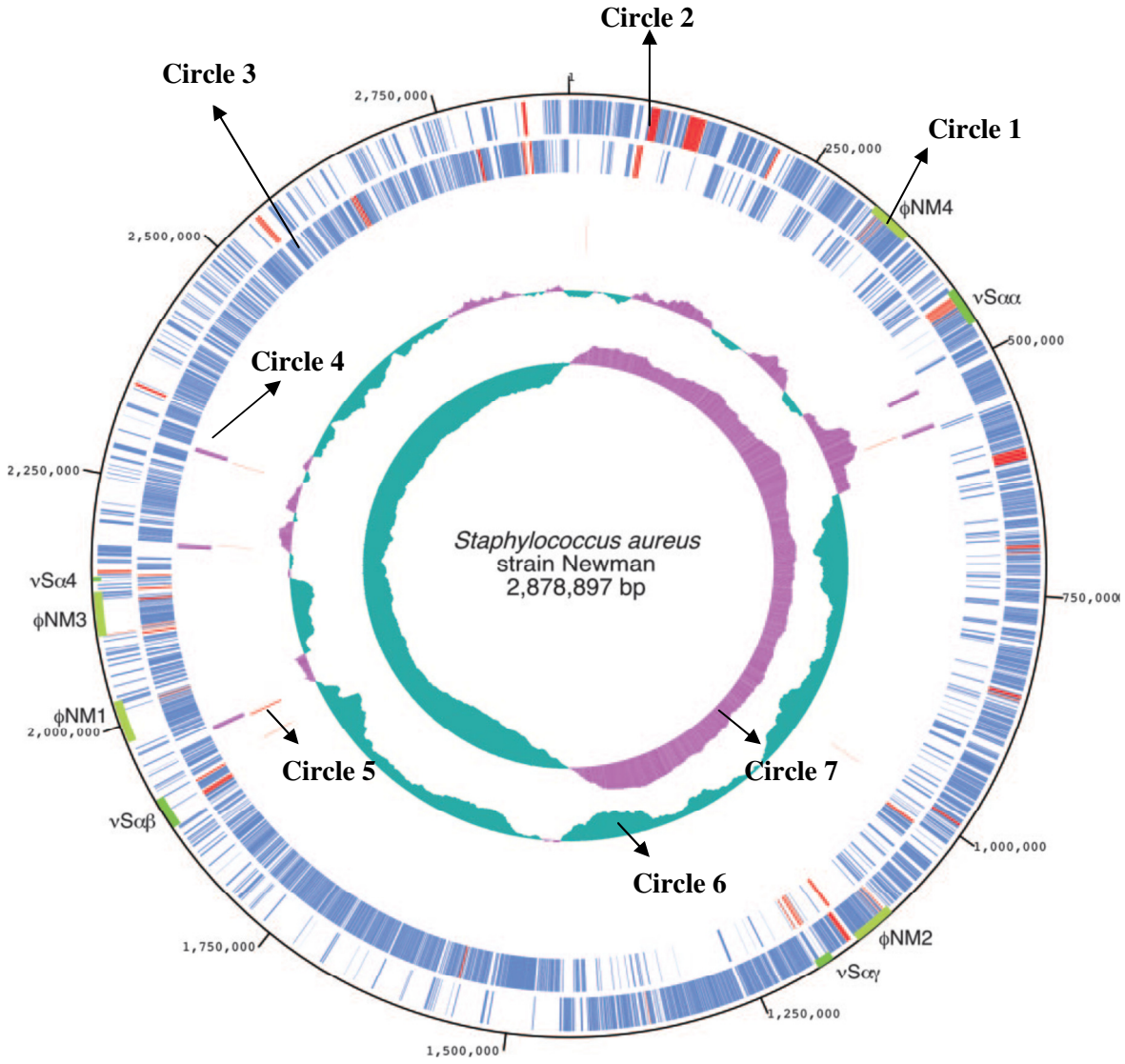

Figure 2.1: **Circular representation of the *S. aureus* Newman strain's chromosome. Green bars inside the first (outer) scale indicate the positions of genomic islands. The second circle shows open reading frames orientated in the forward direction; the third circle indicates those orientated in the reverse direction. The fourth and fifth circles show genes for rRNAs and transfer RNAs, respectively. The sixth circle represents G+C content values. Purple indicates domains with G+C contents higher than 50%. The seventh circle shows G+C skew, in which purple indicates a positive value (Baba *et al.*, 2008)**

The Newman genome was compared with the genomes of other *S. aureus* that have been sequenced (Baba *et al.,* 2008). The G + C content did not vary significantly (32.9%) but the size of the genomes differed by 5% when the JH9 strain with the largest genome was compared to the smallest RF122 (sizes ranged from 2.74 to 2.91 Mbp) (Baba *et al.,* 2008; Steven, 2009). Further research of the Newman strain's genome identified four prophages named ΦNM1 to ΦNM4 (Baba *et al.,* 2008). *Staphylococccus aureus* Newman variants, which lack any of the four prophages (ΦNM1 to ΦNM4), showed a reduction in its ability to cause an organ specific abscess after intravenous infection of mice (Baba *et al.,* 2008). This shows that these four prophages play a major role in the pathogenesis of staphylococcal infections (Baba *et al.,* 2008). Hence, unlike other staphylococcal strains, which carry some

of its virulence genes on the mobile pathogenicity island, virulence determinants in the Newman strain are more prominent in prophages (Baba *et al.,* 2002; Baba *et al.,* 2008). *Staphylococcus aureus* pathogenicity islands, *v*Saβ and *v*Saα, which have been found in all *S. aureus* sequenced genomes were also found in the Newman strain (Baba *et al.,* 2008). The *v*Saβ and *v*Saα harbours only the remnants of their integrase genes; it was thus assumed that *v*Saβ and *v*Saα are no longer mobile and must have played a role in the evolution of the pathogen (Baba *et al.,* 2008).

2.5 Epidemiology of MRSA infections

It has been noted that the frequency of MRSA infections continues to grow globally; the high incidence of infections due to *S. aureus* is attributed to the consequences of advances in patient care and the bacteria's ability to adapt to the changing environment (Boucher and Corey, 2008). Due to the changing epidemiology of infections with MRSA, accurate information is needed for the magnitude and scope of MRSA infections in the general population (Klevens *et al.*, 2007). Methicillin resistant *Staphylococcus aureus* infections were initially detected in hospitals and have emerged in the community and livestock, which means MRSA, cannot be exclusively considered as a healthcare-associated problem (Stefani *et al.,* 2012). There are about 170 000 estimated MRSA infections in the European healthcare system annually, with more than 5 000 fatalities and an estimate, which indicates more than one million inpatient days with high additional cost (Köck *et al.,* 2011).

Moet *et al.* (2007) gave a report of the data collected from three continents during a seven year period by the SENTRY Antimicrobial Surveillance programme, which monitored the aetiology of skin and soft tissue infections (SSTIs). The incidence of methicillin resistance in *S. aureus* was 35.9% in North America, 29.4% in Latin America and 22.8% in Europe between 1988 to 2004 (Moet *et al.,* 2007; Bassetti *et al.,* 2009). Methicillin resistant *S. aureus* infection rates varied in European countries, ranging from 0.8% to 50% from 1988 to 2004 (Moet *et al.,* 2007; Bassetti *et al.,* 2009). In 2007, the European Antimicrobial Resistance Surveillance System (EARSS) reported that MRSA accounted for 25% to 50% of invasive infections in Bulgaria, Croatia, France, Greece, Turkey, Hungary, Israel, Italy, Portugal, Spain, Turkey and the United Kingdom (EARSS *et al.,* 2007; Bassetti *et al.,* 2009). Figure 2.2 shows the worldwide prevalence of HA-MRSA.

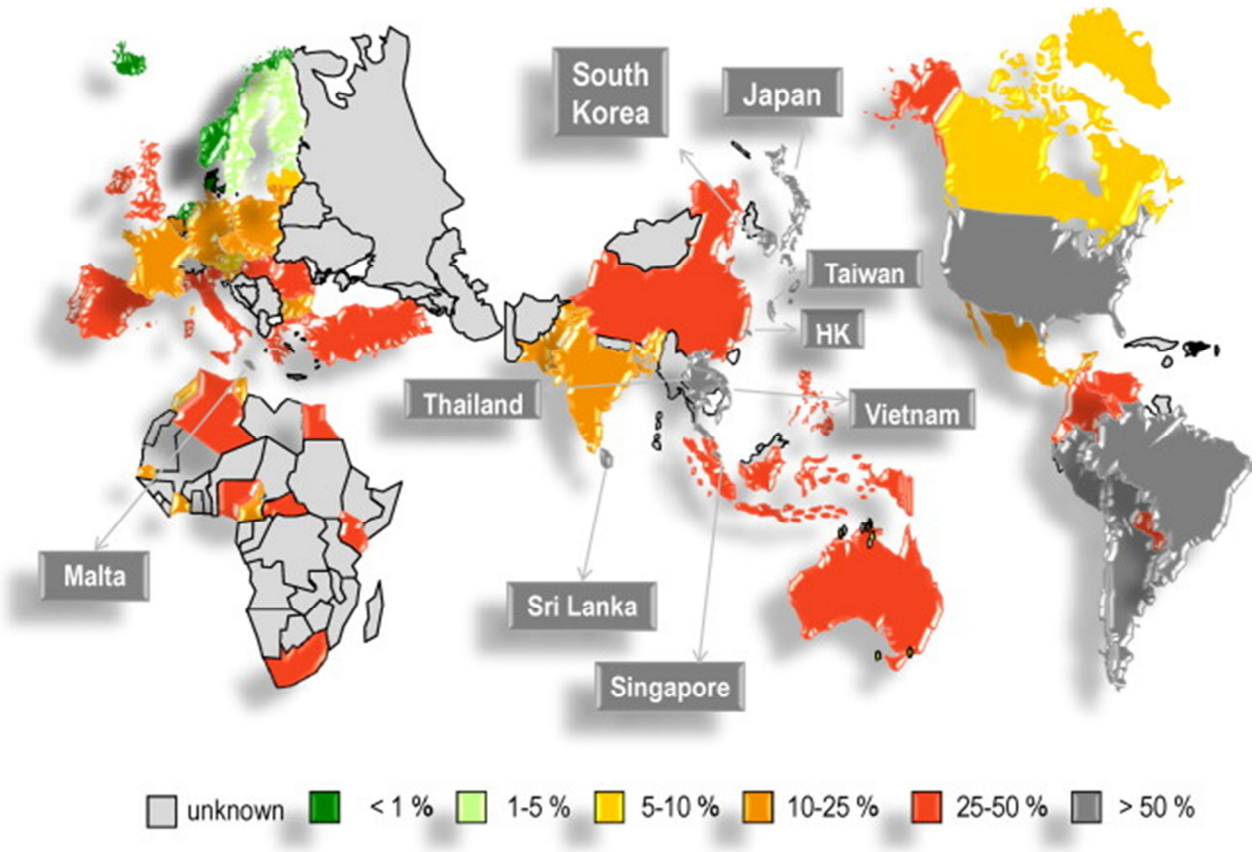

Figure 2.2: Worldwide prevalence of healthcare-associated methicillin resistant *S. aureus* infections (Stefani *et al.*, 2012)

Kesah *et al.* (2003) reported the prevalence of MRSA from eight African countries between 1996 and 1997. The prevalence of MRSA was comparatively high in Cameroon, Kenya and Nigeria ranging from 21% to 30% and below 10% in Algeria and Tunisia (Kesah *et al.,* 2003). According to Ramdani-Bouguessa *et al.* (2001) the prevalence rate of MRSA in Algeria increased to 14% in 2001. Ojulong *et al.* (2009) reported the prevalence of MRSA to be 31.5% in *S. aureus* isolated from surgical site infections in Mulago hospital, Kampala and Uganda. Different studies have reported prevalence rates of MRSA in South Africa (Shittu and Lin, 2006; Shittu *et al.,* 2009b; Moodley *et al.,* 2010; Jansen van Rensburg *et al.,* 2011). Methicillin resistant *S. aureus* was first reported in one of the earliest studies carried out in South Africa in a hospital in Durban in 1978 (Shittu and Lin, 2006). A study by Shittu and Lin (2006) in the KwaZulu-Natal province of South Africa reported 26.9% of 227 (61/227) *S. aureus* isolates obtained to be MRSA. The prevalence rates in Johannesburg and Cape Town were reported to be 33% and 43%, respectively (Shittu and Lin, 2006). A prospective prevalence survey was conducted by Heysell and colleagues in 2008 in the adult tuberculosis (TB) wards of the Church Of Scotland Hospital, a provincial government district hospital in

Tugela Ferry, KwaZulu-Natal, South Africa (Heysell *et al.,* 2011). The study was carried out to determine the prevalence of MRSA colonisation in patients admitted with TB in the endemic human immunodeficiency virus (HIV) area and to describe the dynamics of transmission and the possible patterns of resistance among the MRSA isolates (Heysell *et al.,* 2011). Interestingly, 90% of all isolates of *S. aureus* from the nares of the HIV-infected patients co-infected with TB were MRSA (Heysell *et al.,* 2011). Nosocomial transmission was suggested by similarities of antibiotic resistance, previous history of hospitalisation and a possible further acquisition during hospitalisation (Heysell *et al.,* 2011).

2.5.1 Healthcare-associated MRSA and Community-associated MRSA

Methicillin resistant *S. aureus* was considered as a healthcare-associated pathogen, until the first case of CA-MRSA infection was reported in 1982 in drug-addicted patients who had not been hospitalised earlier (Nastaly *et al*, 2010). In 1993, a high incidence of CA-MRSA was reported from the remote Kimberley region of Western Australia, this prompted a review of surveillance data from 1983 to 1992, which indicated a major increase in all the regions of the state (Udo *et al.,* 1993; Riley and Rouse, 2005; Nastaly *et al.,* 2010). In 1999, the CDC reported the death of four children due to CA-MRSA infection (Nastaly *et al.,* 2010). The rapid spread of CA-MRSA has been reported in almost all geographical regions of the world since 1982 (Bassetti *et al.,* 2009). The spread of CA-MRSA is more prominent in the USA and this has been attributed to the USA300 strain (Otto, 2012). The success and spread of CA-MRSA, in particular the USA300 strain, is believed to be due to its higher virulence and transmissible features, compared to the traditional HA-MRSA (Otto, 2012; Udo, 2013). The gene spermidine acetyltransferase (*spe*G*)* is found in the arginine catabolic mobile element (ACME) of the USA300 strain (Joshi *et al.,* 2011; Otto, 2012; Hellmark *et al.,* 2013). The *spe*G gene is responsible for the transfer of resistance to spermidine (aliphatic cation that are synthesised by all living organisms) and other polyamine molecules produced by living cells, except for *S. aureus* (Otto, 2012). Polyamine hypersensitivity differentiates *S. aureus* from other bacteria and is exhibited by all tested strains excluding those belonging to the group of USA300 strains (Joshi *et al.,* 2011). It has been suggested that the *spe*G may be responsible for the high colonisation capacity of the USA300 strain (Otto, 2012). Table 2.2 shows the clinical and genetic differences between HA-MRSA and CA-MRSA.

Table 2.2: Clinical and genetic differences between HA-MRSA and CA-MRSA strains

Characteristics	HA-MRSA	CA-MRSA
Clinical manifestation	Pneumonia, urinary tract, blood stream, surgical site (Chavez and Decker, 2008; Robinson *et al.,* 2008)	Skin and soft tissue infections "spider bite", necrotising pneumonia, sepsis (Chavez and Decker, 2008; Robinson *et al.,* 2008)
Risk groups	Elderly people, healthcare workers, preterm neonate, long-term hospitalised patients (Chavez and Decker, 2008; Robinson *et al.,* 2008)	Young drug users, prisoners, athletes, soldiers, men who have sex with men, selected ethnic populations (Chavez and Decker, 2008; Robinson *et al.,* 2008)
Transmission	Person-to-person spread: healthcare staff (nurses, doctors, surgeons) Environment-to-person spread (hospital equipment) (Chavez and Decker, 2008; Robinson *et al.,* 2008)	Person-to-person spread: shared facilities (towels, pools) Environment-to-person spread (shared sports equipment) (Chavez and Decker 2008; Robinson *et al.,* 2008)
Resistance to antimicrobial agent	Resistant to multiple antibiotics (Frey *et al.,* 2003)	Susceptible to multiple antimicrobial agents, β-lactam resistant (Frey *et al.,* 2003; Hauang *et al.,* 2006)
SCC*mec* types	I, II, III and VI (Valsesia *et al.,* 2010).	IV, V, VII, VII, VIII, IX, X and XI (Kennedy and DeLeo, 2009; Valsesia *et al.,* 2010)
PVL production	Produced by both HA-MRSA and CA-MRSA (David and Daum, 2010)	Produced by both HA-MRSA and CA-MRS (David and Daum, 2010)
Main strain type	USA100 and USA200 (Bassetti *et al.,* 2009)	USA300 and USA400 (Bassetti *et al.,* 2009)

Two main hypotheses suggested that the high virulence of CA-MRSA strains are the increase in the expression of the core genome-encoded virulence genes, such as cytolysins, α-toxin, other virulent determinants and most likely the presence of the Panton-Valentine leukocidin (PVL) (Vandenesch *et al.,* 2003; Liu, 2009; Otto 2012). However, the role of the PVL gene remains controversial due to its presence in HA-MRSA and MSSA (Salaam-Dreyer, 2010).

Panton-Valentine leukocidin was discovered in 1894 by Van de Velde and was linked to SSTIs by Panton and colleagues in 1932 (David and Daum, 2010). At the onset of the CA-MRSA outbreak epidemics, PVL was found in all CA-MRSA strains, while it was mostly absent in HA-MRSA strains (Vandenesch *et al.,* 2003; Otto 2010). Schlievert *et al.* (2010) reported that CA-MRSA (USA300 and USA400) and community-associated methicillin sensitive *S. aureus* (CA-MSSA) often produce high inflammatory cytolysins, such as α-toxin, γ-toxin, δ-toxin and the PVL toxin. New findings by researchers pose major doubt regarding the role of PVL as a virulence factor in CA-MRSA disease (Otto, 2010; Otto, 2012). New strains of CA-MRSA have been discovered, which lacks the PVL gene and still show significant virulence characteristics (Otto, 2012). Isogenic PVL gene deletion mutants also

did not confirm the impact of PVL on CA-MRSA virulence in various animal models (Otto, 2012). However, Löffler *et al.* (2010) reported that the expression of PVL by staphylococcal strains confers strong cytotoxicity against neutrophils isolated from human cells, but not in Java monkey or simian cells.

2.5.2 Important global MRSA clones

Various clones have been discovered, since the detection of the first MRSA isolates in 1959 (DeLeo and Chambers, 2009; Otto, 2010). The human-associated *S. aureus* strains can be grouped into ten main clonal complex lineages and various minor lineages (Lindsay *et al.,* 2012; McCarthy and Lindsay, 2012). However, the MRSA strains detected worldwide belong to only five major clonal complexes (CC5, CC8, CC22, CC30 and CC45) (Figure 2.3) (Diep *et al.,* 2006; Otto, 2012).

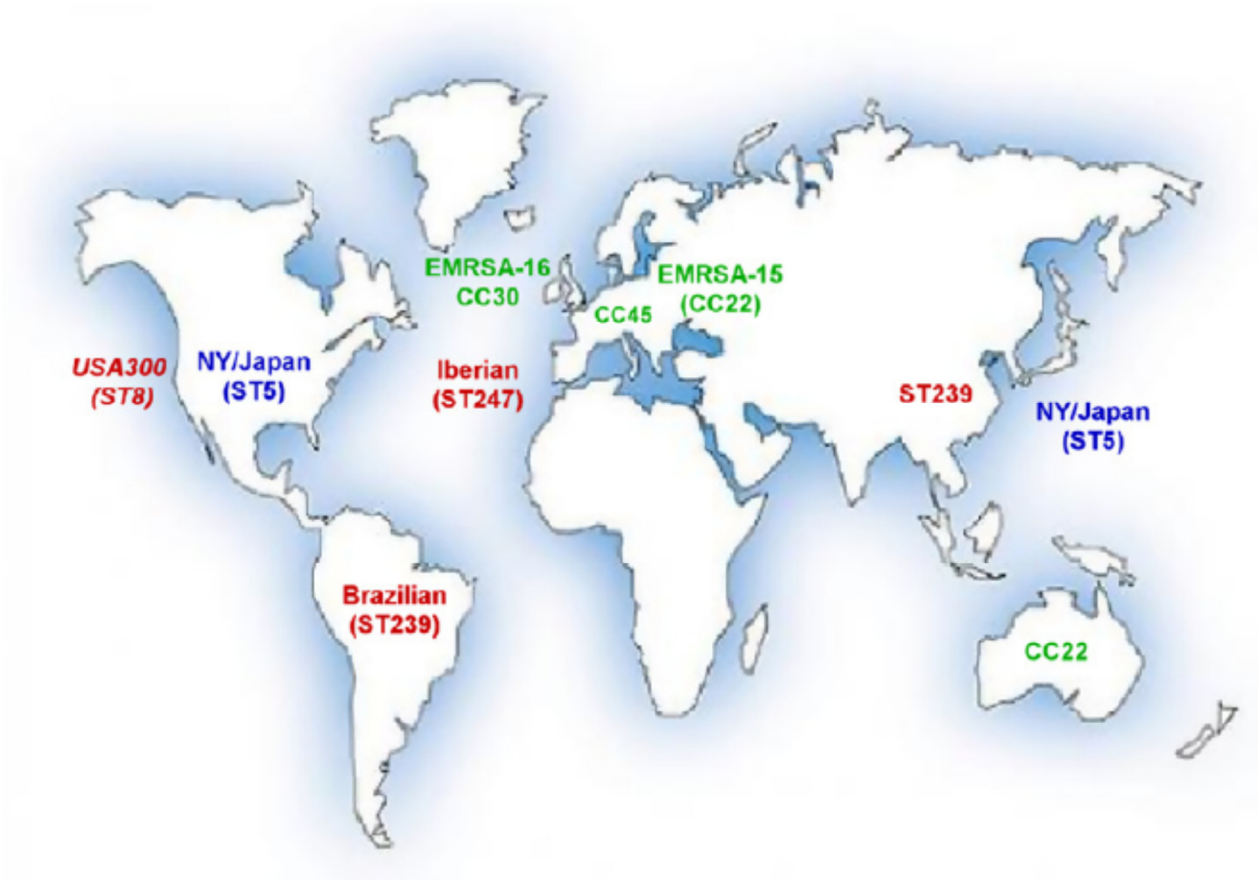

Figure 2.3: **The important global MRSA clones which is currently predominant in large geographical locations around the world. Most clones belong to clonal complexes 5 (shown in blue) or 8 (shown in red). Other predominant clones belong to CCs 22, 30 and 45 (Otto, 2012)**

Well-known MRSA clones include (i) the archaic clone *spa* types t008, t009, t194-SCC*mec*I-[Sequence type 250 (ST250)] [clonal complex 8 (CC8)] discovered in the UK in the 1960s; (ii) the Iberian clone *spa* types t008, t051, t052, t054, t200-SCC*mec*IA-ST247(CC8) that was reported from Spain in 1989; (iii) the Brazilian clone *spa* types t030, t037, t234, t387, t388-SCC*mec*IIIA-ST239(CC8) reported in Brazil in 1992; (iv) the epidemic methicillin resistant *Staphylococcus aureu*s EMRSA-16 clone *spa* types t018, t253, t418, t419-SCC*mec*II-ST36(CC30) that was reported in the UK in 1993 and (v) the New York/Japan clone *spa* types t001, t002, t003, t010, t045, t053, t062, t105-SCC*mec*II-ST5(CC5) that was reported in the US in 1998 (Yamamoto *et al*., 2010; Campanile *et al.,* 2010). Other important clones are the Berlin, Hungarian, Paediatric and the Russian clones, all of which lacking the PVL gene (Yamamoto *et al.,* 2010). The Iberian, Hungarian, EMRSA-15, Brazilian, NewYork/Japan and paediatric clones are described as the pandemic clones due to their dissemination internationally (Aires de Sousa *et al.,* 2005; Yamamoto *et al.,* 2010).

Shittu *et al.* (2011) reported four clones of MRSA in the South-West of Nigeria (*spa* type t451-SCC*mec*V-ST8; *spa* type t008-SCC*mec*IV-ST94; *spa* type t002-SCC*mec*V-ST5 and *spa* type t064-SCC*mec*V-ST8). Moodley *et al.* (2010) identified five major clones in South Africa [(PFGE type A-*spa* type t045-SCC*mec*I-ST5(CC5), PFGE type D-*spa* type t037-SCC*mec*III-ST239(CC8), PFGE type F-*spa* type t064-SCC*mec*IV-ST612(CC8), PFGE type K-*spa* type t012-SCC*mec*II-ST612(CC8) and PFGE type T-*spa* type t012-SCC*mec*II-ST36(CC30)]. The five major clones identified by Moodley and colleagues (2010) were widespread in South Africa. However, type PFGE-D-*spa* type t037-SCC*mec*III-ST239 (CC8) was not detected in the Eastern and Western Cape provinces (Moodley *et al.,* 2010). During March 2001 to August 2003, Shittu and colleagues (2009b) performed a study on 61 MRSA isolates obtained from 13 healthcare institutions in the KwaZulu-Natal (KZN) province, South Africa. Three major PFGE pulsotypes were reported [PFGE type A-*spa* type t064-SCC*mec*IV-ST1173, PFGE type F-*spa* type t037- SCC*mec*III-ST239 and PFGE type G-*spa* type t045-SCC*mec*III-ST5] (Shittu *et al.,* 2009b). The pandemic clone t037-ST239-SCC*mec*III, which was found in Brazilian hospitals, was also identified by Shittu *et al.* (2009b) and Moodley and colleagues (2010) in South Africa. Makgotlho *et al.* (2009) reported a prevalence of 67% (65/97) of SCC*mec*II, 14.4% (14/97) of SCC*mec* type III and 4% (4/97) of SCC*mec*IVd in MRSA isolates obtained from the Steve Biko Academic Hospital in Pretoria, South Africa. The *spa* types, sequence types (ST) and PFGE types were not provided by Makgotlho *et al.* (2009). Hence there is a paucity of information regarding the

spa types, ST/CC and the pulsotypes of MRSA circulating in the Pretoria region of South Africa.

2.6 Pathogenesis and virulence of MRSA strains

Staphylococcus aureus is associated with virulence factors that allow the bacterium to survive severe conditions in the human host (Liu, 2009). Infections of *S. aureus* are established by various surface proteins called the microbial surface components recognising adhesive matrix molecules (MSCRAMMs), which mediates adherence to the host's tissue (Gordon and Lowy, 2008). Colonisation by MRSA is enhanced by biofilm formation, surface adhesins and antiphagocytic microcapsules (Skrupky *et al.,* 2009). Once an infection is established, *S. aureus* produces a variety of virulence factors that mediate disease (Skrupky *et al.,* 2009). Figure 2.4 shows some of the virulence factors produced by *S. aureus* strains.

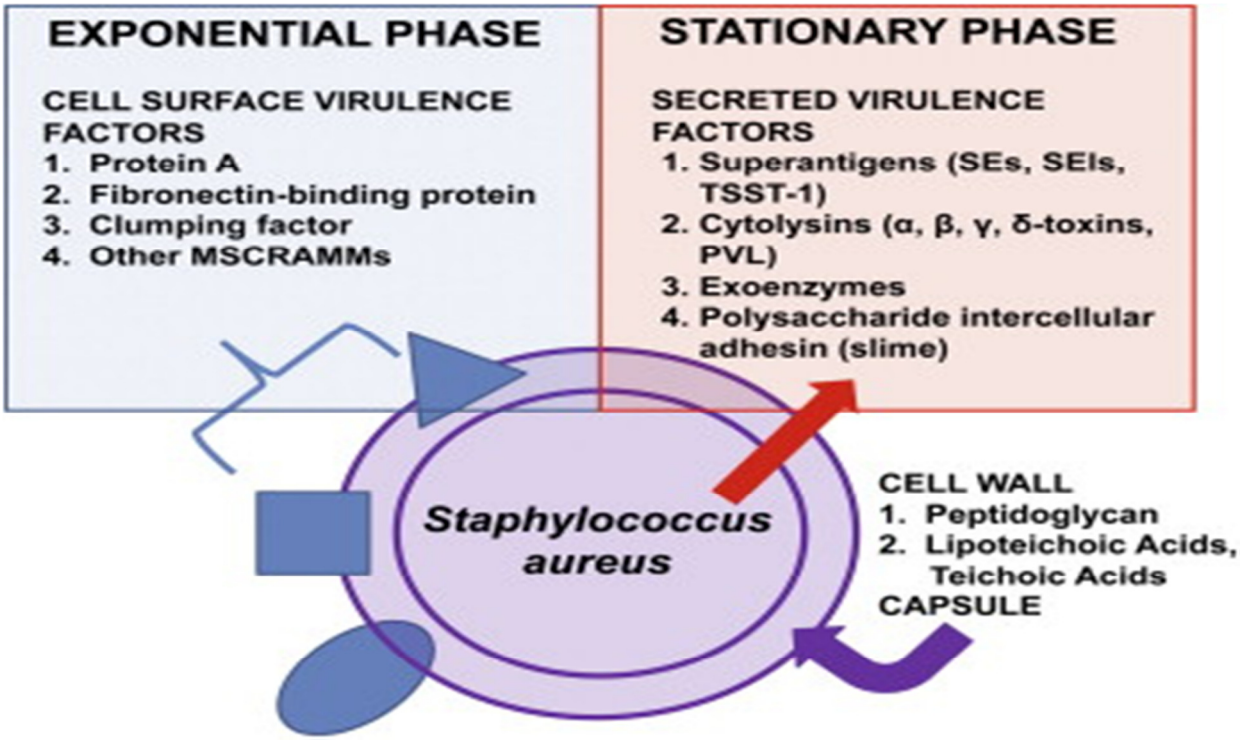

Figure 2.4: Virulence factors produced by *S. aureus* strains. The bacteria produce cell surface virulence factors (microbial surface components recognising the adhesive matrix molecules, MSCRAMMs) during the exponential phase, while producing exoproteins and exopolysaccharides during the post exponential/stationary phase (Schlievert *et al.,* 2010)

The newly discovered SasX protein, which is encoded on a mobile genetic element and occurs in ST239 MRSA strains are the most common source of MRSA infection in Asia (Otto, 2012). The SasX protein has been shown to play a major role in the formation of biofilms,

nasal colonisation, immune evasion and virulence in animal models (Otto, 2012). The SasX protein has been described as an important factor responsible for the pathogenic success and spread of the ST239 strains (Otto, 2012). Table 2.3 shows selected *Staphylococcus aureus* virulence factors and associated clinical syndromes.

Table 2.3: Selected *Staphylococcus aureus* virulence factors: arginine catabolic mobile elements (ACME), community-associated methicillin resistant *S. aureus* (CA-MRSA), chemotaxis inhibitory protein of staphylococci (CHIPS) and extracellular adherence protein (Eap) (Gordon and Lowy, 2008)

Type of virulence factors	Selected factors	Genes	Associated clinical syndromes
Involved in attachment	MSCRAMMS (e.g., clumping factors, fibronectin-binding proteins, collagen and bone sialoprotein-binding protein)	*clf*A, *clf*B, *fnb*A, *fnb, can, sdr, bbp*	Endocarditic, osteomyelitis, septic arthritis, prosthetic-device and catheter infections
Involved in persistence	Biofilm accumulation (polysaccharide intracellular adhesion), small-colony variants and intracellular persistence	*ica* locus, *hem*B mutation	Relapsing infections and cystic fibrosis
Involved in evasion/destruction of host defences	Leukocidins (eg, PVL and γ-toxin) capsular polysaccharide, Protein A, CHIPS, Eap and phenol-soluble modulins	*ℓuk*S-PV, *ℓuk*F-PV, *hlg, cap* 5 and 8 gene clusters, *spa, chp, eap, psm-α* gene cluster	Invasive skin infections and necrotising pneumonia (CA-MRSA strains that cause these often harbour the PVL toxin gene) and abscesses (associated with capsular polysaccharide)
Involved in tissue invasion / penetration	Proteases, lipases, nucleases, hyaluronate lyase, phospholipase C and metalloproteases (elastase)	*v*8, *hys*A, *hla, plc, sep*A	Tissue destruction and metastatic infections
Involved in toxin-mediated disease and sepsis	Enterotoxins, toxic shock syndrome toxin, exfoliative toxins A and B, α-toxin, peptidoglycan and lipoteichoic acid	*sea-q* (no *sef), tst*H, *eta, etb, hla*	Food poisoning, toxic shock syndrome, scalded skin syndrome, bullous impetigo and sepsis syndrome
With poorly defined role in virulence	Coagulase, ACME and bacteriocin	*Arc* cluster, *opp-3* cluster, *bsa*	

Expression of virulence factors in *S. aureus* is controlled by a complex staphylococcal regulatory network, namely the accessory gene regulator (*agr*) system (Gould *et al.,* 2012). These genes are known to vary between strains (Gould *et al.,* 2012). The up- and down-regulation of secreted factors occur when the *agr* gene is active (Skrupky *et al.,* 2009). It has been suggested that the arginine catabolic mobile element (ACME) may play a role in the pathogenesis of *S. aureus* by conferring an enhanced ability of CA-MRSA strains to colonise the skin of healthy people (David and Daum, 2010). This enables CA-MRSA strains to

spread more easily in the community (David and Daum, 2010). Though, no scientific data exist to support this hypothesis (David and Daum, 2010).

The Brazilian epidemic clonal complex (BECC A_1) strain was compared to MSSA and sporadic MRSA clones in an *in vitro* experiment (Gordon and Lowy, 2008). The BECC A_1 strains formed biofilms and shared an increasing ability to adhere to polystyrene, epithelial bronchial cells and were able to invade cells (Amaral *et al.,* 2005).

2.7 Antimicrobial resistance of MRSA

A major public health concern, particularly in tertiary hospitals and other healthcare settings is the rise of antibiotic resistant bacteria (Mulvey and Simor, 2009). Ribosomal RNA or genes in bacteria, encodes proteins that makes it possible for bacteria to become resistant against the actions of antibiotics (Mulvey and Simor, 2009). This resistance can either be acquired or intrinsic (Mulvey and Simor, 2009). The evolution of resistance in *S. aureus* emerged in the 1940s after the introduction of penicillin (Stefani and Goglio, 2010). Penicillin resistant clone type 80/81, which is now known as the ST30-CA-MRSA-IV Southwest clone type, emerged in the 1950s with the production of PVL and caused serious infections in hospitals and communities (Stefani and Goglio, 2010). Methicillin resistant *S. aureus* is synonymous with multi-drug resistant *S. aureus* because many MRSA strains have developed resistance to a wide range of antibiotics (Foster, 2004, Shittu *et al.,* 2009b). Hence, the most important antibiotic resistance mechanism acquired by *S. aureus* strains is methicillin resistance, which is now associated with multi-drug resistance (Stefani and Goglio, 2010). Methicillin resistant *Staphylococcus aureus* strains are seen all around the world due to clonal spread (Stefani and Goglio, 2010; Liu *et al.,* 2012).

2.7.1 Penicillin resistance of MRSA

Penicillin was the first antibiotic used for the treatment of *S. aureus* infections (Barber and Rowzwadowska-Dowzenko, 1948; Otto 2012). In 1944, Kirby demonstrated that penicillin was inactivated by penicillin resistant *S. aureus* strains (Lowy, 2003; Gaze *et al.,* 2008). It is estimated that about 90% to 95% of clinical *S. aureus* strains are resistant to penicillin worldwide (Sakoulas and Moellering, 2008). Resistance to penicillin is due to the production of penicillinase, a β-lactamase, which was discovered before the clinical use of penicillin

(Otto, 2012). This enzyme is often produced by staphylococci when the bacterial cells are exposed to a β-lactam antibiotic, an agent which includes penicillin and its derivatives (Kernodle, 2000). Staphylococcal resistance to penicillin is mediated by the *bla*Z gene which encodes the extracellular enzyme β-lactamase (Lowy, 2003). The enzyme β-lactamase, hydrolyses the beta-lactam ring of penicillin, rendering the antibiotic inactive (Lowy, 2003; Malachowa and DeLeo, 2010). The *bla*Z gene, is a transposable gene located on a plasmid (*pBw*15) and transposon *Tn*4002 (Gillespie *et al.,* 1988; Makgotlho *et al.,* 2009). The *bla*Z gene is controlled by two adjacent regulatory genes *bla*R1 and *bla*I (Lowy 2003; Malachowa and DeLeo, 2010). It has been documented that 96% of all *S. aureus* strains carry the β-lactamase plasmid (McMurray *et al.,* 1990).

2.7.2 Methicillin resistance of MRSA

Methicillin is a penicillin derivative that was developed in 1959 by Beecham laboratories for the treatment of infections caused by penicillin resistant *S. aureus* (Kennedy and DeLeo, 2009). Methicillin resistant *Staphylococcus aureus* occurs when methicillin sensitive *S. aureus* (MSSA) strains exogenously acquire the SCC*mec*, which is transmitted among staphylococcal species as a mobile element (Hanssen and Ericson Solid, 2006; Tsubakishita *et al.,* 2010). Staphylococcal cassette chromosome *mec* in *S. aureus* strains usually integrates at a unique sequence site of the chromosome called the bacterial chromosomal attachment site (*attBscc*) (Hiramatsu *et al.,* 2001; Ito *et al.,* 2001; Turlej *et al.,* 2011). Resistance to β-lactams seen in MRSA strains, results from the expression of the PBP2a protein (penicillin binding protein) encoded in the *mec*A gene, which resides on the staphylococcal cassette chromosome *mec* (SCC*mec*) (Zapun *et al.,* 2007). The PBP2a is a high molecular weight class B transpeptidase which has a low affinity for all β-lactam agents and catalyses a transpeptidation reaction, which is an important step in the cross-linkage of peptidoglycan chains (Lowy 2003; Chen *et al.,* 2009; Marais *et al.,* 2009). The transpeptidation reaction involves the formation of acyl enzyme intermediate which is comparable to that seen in other serine transferases such as trysin (Lupoli *et al.,* 2011).

Eleven SCC*mec* types have been recognised, with all sharing similarities in their backbone structure (Turlej *et al.,* 2011). The backbone structure of the different SCC*mec* types are characterised by the presence of the *mec* gene complex (Turlej *et al.,* 2011). The *mec* gene complex encodes the *mec*A, the *ccr* gene complex encodes site-specific recombinase(s) used

for the movement of the element and the three regions, which borders the *ccr* and *mec* gene complex known as the joining region (J) (Hiramatsu *et al.,* 2001; Chongtrakool *et al.,* 2006; Tsubakishita *et al.,* 2010; Turlej *et al.,* 2011). The *mec* complex is divided into four classes A, B, C and D by the absence or presence of insertion sequences (ISs) in the *mec*R1 gene, though all *mec* gene complexes possess the IS*431*R found downstream of the *mec*A gene (Figure 2.5) (Tsubakishita *et al.,* 2010).

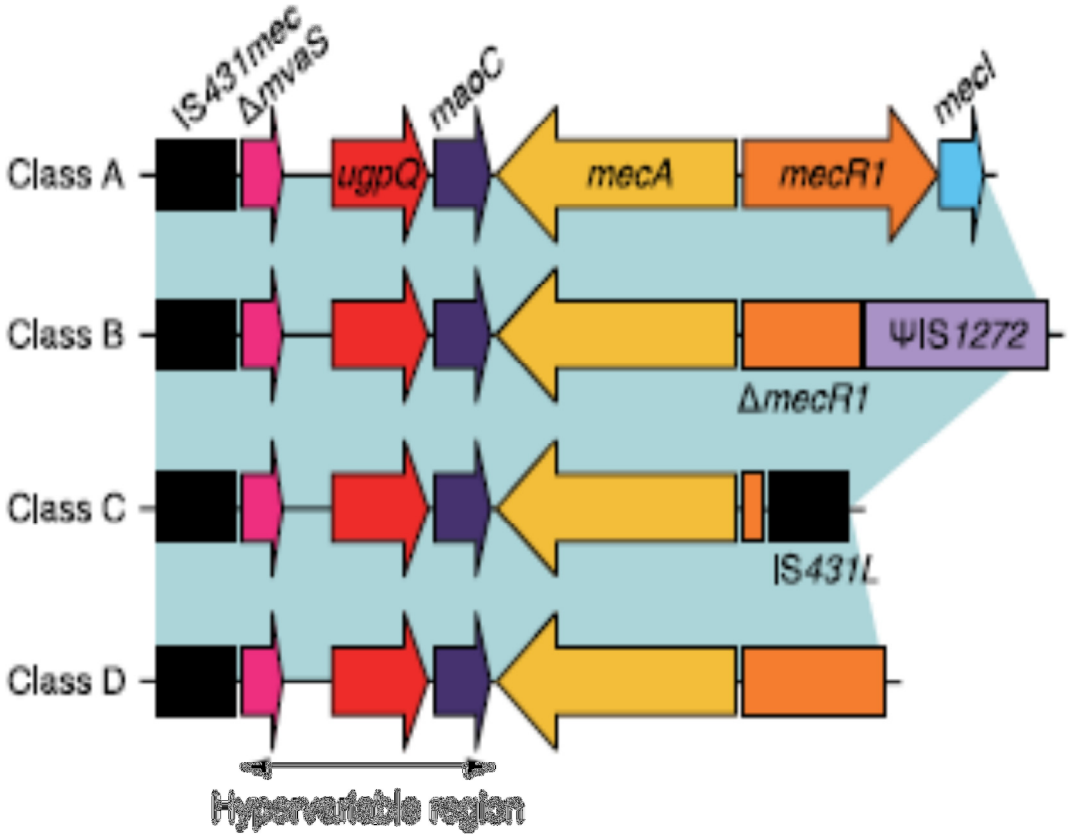

Figure 2.5: **Basic structure of the representative *mec* gene complexes. The class A *mec* gene complex is composed of intact *mec*RI and *mec*I genes, encoding the signal transducer and repressor of the *mec*A gene, respectively. The class B and C *mec* gene complexes have ΨIS*1272* and IS*431*L integrated in *mec*RI respectively, which resulted in the partial deletion of the *mec*RI and complete deletion of the *mec*RI genes. Class D has no IS element, but parts of the *mec*RI gene and complete *mec*I genes are deleted. All *mec* gene complexes contain IS*431mec* (also called IS*431*R) (Tsubakishita *et al.,* 2010)**

The class A *mec* complex is referred to as a prototype structure with the gene order IS*431mec*-*mec*A-*mec*RI-*mec*I, while classes B, C and D are derived from the class A *mec* gene complex (Katayama *et al.,* 2000; Tsubakishita *et al.,* 2010). Regulatory elements *mec*I and *mec*RI control the transcription of the *mec*A gene (Tsubakishita *et al.,* 2010). The *mec*RI, a membrane-bound signal transduction protein, senses the presence of β-lactams and activates

its cytoplasmic domain (Zapun *et al.,* 2007). The cleavage of the *mec*I repressor occurs when the intracellular domain of *mec*RI is activated (Zapun *et al.,* 2007). A new *mec*A homologue designated as *mec*C/*m*ecA$_{LGA251}$ was described in *S. aureus* (Medhus *et al.,* 2013). The occurrence of the *mec*C gene has been confirmed in different countries in Northern Europe (UK, Denmark, Ireland, Germany, France, Sweden and Norway) (Medhus *et al.,* 2013).

There are two *ccr* gene complexes, one which carries two adjacent *ccr* genes, *ccr*A and *ccr*B and the other carries the *ccr*C gene (Turlej *et al.,* 2011). The *ccr*C gene is carried by only the SCC*mec* type III, V and VII (Kennedy and DeLeo, 2009). The largest SCC*mec* element is the SCC*mec* type III, which is composed of two cassettes, the SCC*mercury* (*ccr*C) and SCC*mec* type III (*ccr*AB) (Zhang *et al.,* 2012). The variations in the J regions (J1, J2 and J3) are used for subtyping SCC*mec* type IV (Turlej *et al.,* 2011; Medina *et al.,* 2012). The J1 region is located between the chromosomal left junction and the *ccr* complex, while the J2 is the region from the *ccr* genes to the *mec* complex (Medina *et al.,* 2012). The J3 is the region between the *mec* complex to the left *orf*X of the SCC*mec* (Deurenberg *et al.*, 2009; Medina *et al.,* 2012).

The SCC*mec* element can be typed and subtyped into eleven different types and eleven (SCC*mec* type IV) subtypes, which differ in size, such as SCC*mec* type I (34.3 kb), type II (53.0 kb), type III (66.9 kb), type IV ranging from 20.9 to 24.3 kb, type V(28. 0 kb), type VI (24.0 kb), VII (41.3 kb), type VIII (32 kb), type IX (43.7 kb), X (50.8 kb) and XI (30.0 kb) (Deurenberg and Stobberingh, 2008; Vanderhaeghen *et al.,* 2012). The SCC*mec* element is characterised as follows: type I (class B *mec* gene complex and *ccr*AB type 1), type II (class A *mec* gene complex and *ccr*AB type 2), type III (class A *mec* gene complex and *ccr*AB type 3), type IV (class B *mec* gene complex and *ccr* AB type 2), type V (class C2 *mec* gene complex and *ccr*C), type VI (class B *mec* gene complex and *ccr* AB type 4), type VII (class C *mec* gene complex and *ccr*C), type VIII (class A and *ccr*AB type 4), type IX (class C *mec* gene complex and *ccr*A1B1), type X (class C *mec* gene complex and *ccr*A1B6) and type XI (class E *mec* gene complex and *ccr*A1B3) (Hanssen and Ericson Sollid 2006; Turlej *et al.,* 2011; Laurent *et al.,* 2012). Figure 2.6 shows the classification scheme for SCC*mec* scheme for SCC*mec* types by *mec* complex type and *ccr* complex.

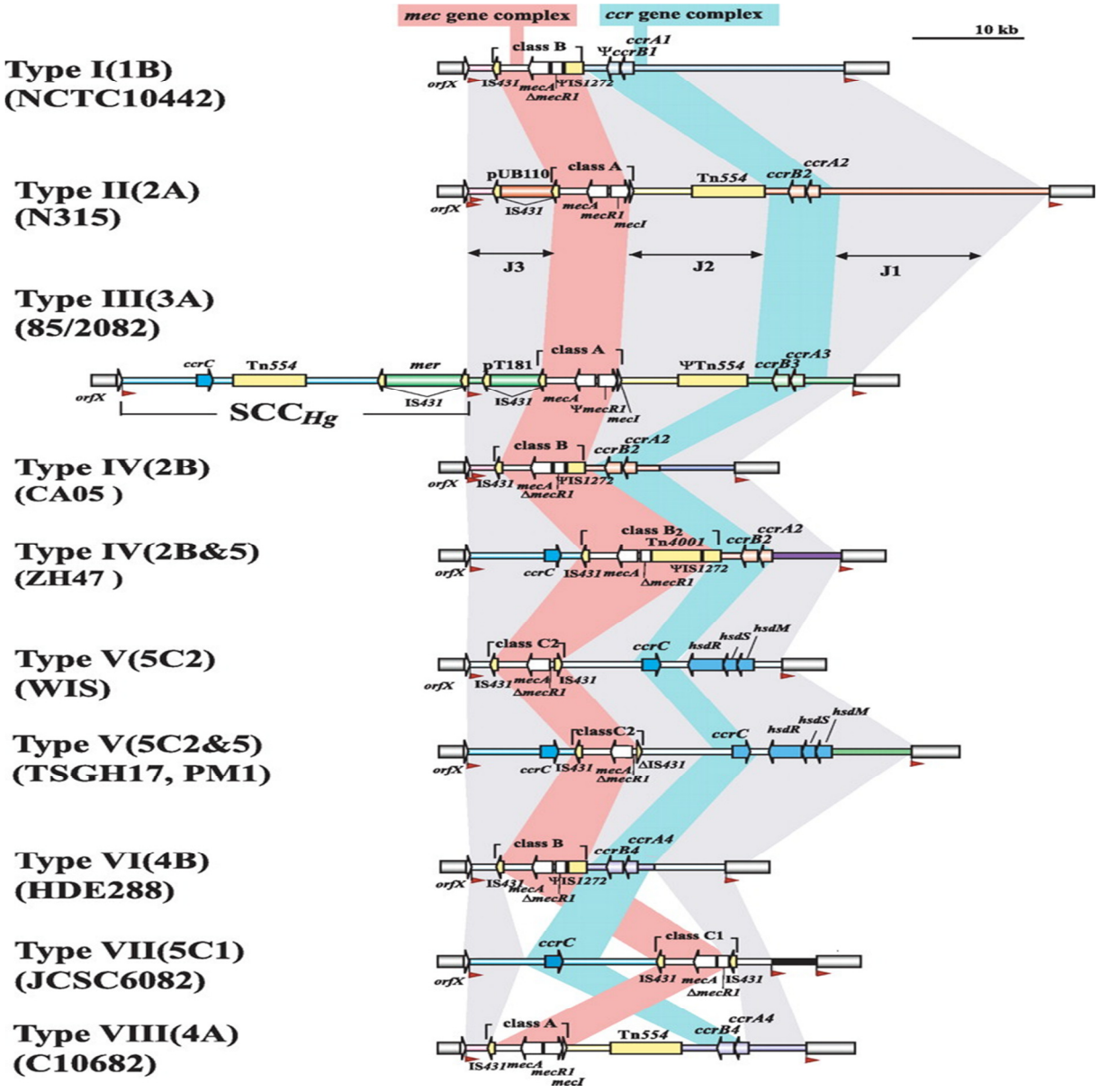

Figure 2.6 **Classification scheme for SCC*mec* types by *mec* complex and *ccr* complex type of MRSA (David and Daum, 2010)**

The SCC*mec* types I, IV, V, VI and VII are resistant to β-lactam antibiotics (penicillins, cephalosporins, monobactams and carbapenems), while SCC*mec* type II, III and VIII are resistant to multiple classes of antibiotics (tetracycline, lincosamide, tobramycin and streptogramin (Deurenberg and Stobberingh, 2008; Sangappa and Thiagarajan, 2012). The SCC*mec* type IV (CA-MRSA) has been strongly linked with strains of MRSA causing infections in patients who have no HA-MRSA risk factors (David and Daum, 2010). The SCC*mec* type IV is regarded as the smallest and most mobile of the SCC*mec* types (Millheiriço *et al.,* 2007b). Due to the enhanced mobility of the SCC*mec* type IV eleven subtypes (IVa to IVk) which differ mainly in the J1 region have been described (Millheiriço *et*

al., 2007b; Turlej *et al.,* 2011). The SCC*mec* type IV is associated with CA-MRSA, it is also found in some nosocomial MRSA clones, such as the EMRSA-15 which is endemic in the UK (Millheiriço *et al.,* 2007b).

2.7.3 Vancomycin resistance of MRSA

Vancomycin is a glycopeptide, which was introduced in 1958 for the treatment of Gram-positive bacterial infections (Sakoulas, 2006; Tiwari, 2009). However, the use of vancomycin was minimised because of concerns about toxicity to certain organs, such as the kidney and liver (Sakoulas, 2006; Tiwari, 2009). The use of vancomycin as an antimicrobial has increased tremendously in the past 20 years, due to the rise in the prevalence of MRSA and CoNS (Tiwari, 2009). Reduced susceptibility seen in glycopeptides due to the selective pressure of the antibiotics from years of use has resulted in two different phenotypes: vancomycin-intermediate resistant *Staphylococcus aureus* strains (VISA) and heteroresistant vancomycin-intermediate *Staphylococcus aureus* (hVISA) (Stefani and Goglio, 2010). The *S. aureus* hVISA isolates are more often found than the VISA isolates (Nannini *et al.,* 2010). The hVISA strains are reported to have a small subpopulation of one bacterium per 10^5 to 10^6 colony units, which is able to grow at a vancomycin concentration of ≥4 μg/ml (Nannini *et al.,* 2010). The first glycopeptide intermediate *S. aureus* (GISA/VISA) was isolated in a paediatric patient in Japan in 1996 (Sakoulas and Moellering, 2008). Though the incidence of VISA or vancomycin-resistant *Staphylococcus aureus* (VRSA) remains low, there is an increase in reports of hVISA from different parts of the world (Song *et al.,* 2004). Shittu and Lin (2006) described the antimicrobial susceptibility patterns of 227 clinical isolates of *S. aureus* in the Kwazulu-Natal province of South Africa. All *Staphylococcus aureus* isolates were susceptible to vancomycin and 26.9% of isolates studied were confirmed as MRSA (Shittu and Lin, 2006). Glycopeptides act by inhibiting cell wall synthesis of *S. aureus* by binding with high affinity to the D-Ala-D-Ala C-terminus of late peptidoglycan precursors and using these precursors in transglycosylases, D, D-carboxypeptidases and transpeptidases (Figure 2.7) (Sakoulas and Moellering, 2008).

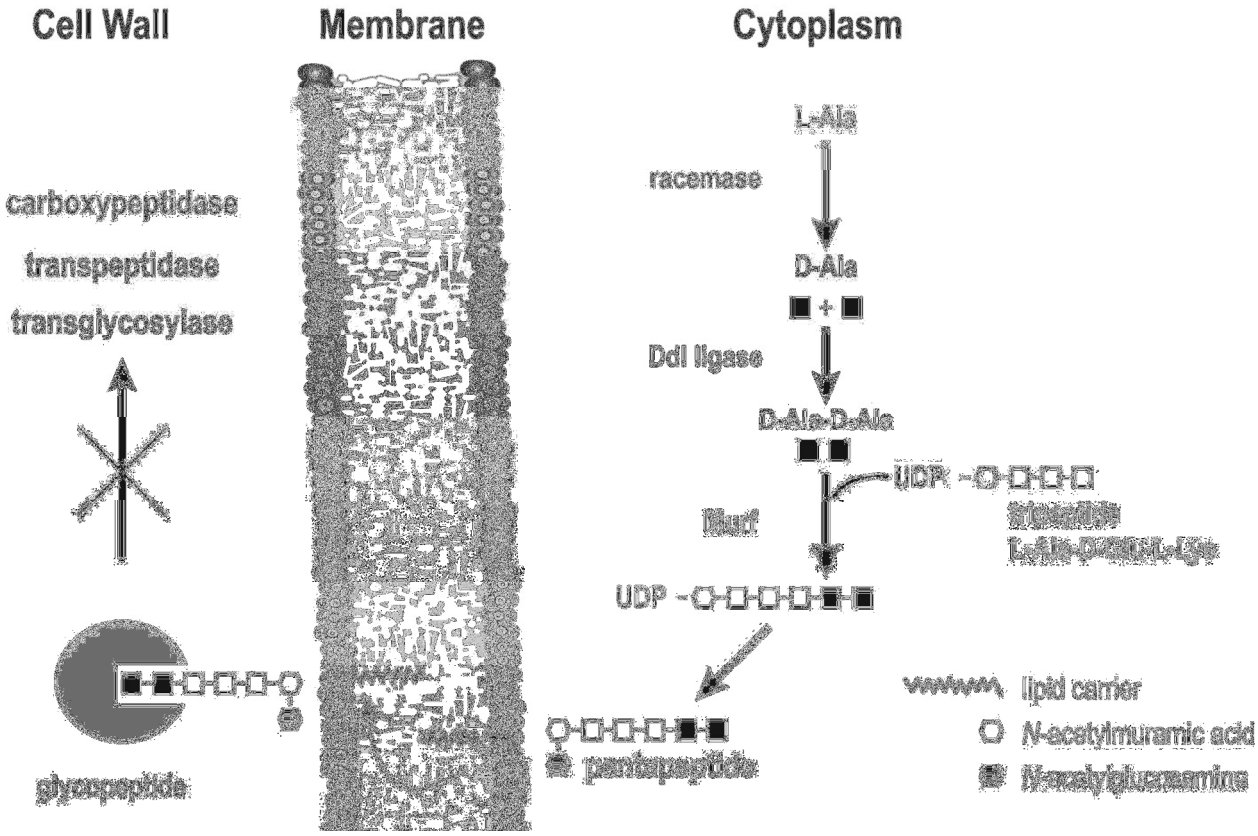

Figure 2.7: **Peptidoglycan biosynthesis and mechanism of action of vancomycin. Binding of vancomycin to the C-terminal D-Ala-D-Ala of the late peptidoglycan precursors inhibits transglycosylases, transpeptidases and D,D-carboxypeptidases (Sakoulas and Moellering, 2008)**

Researchers predicted that the acquisition of the *van*A resistant gene by *S. aureus* from enterococci will lead to the emergence of highly VRSA (Tenover, 2006). It has been hypothesised that strains of *S. aureus* that express *agr* dysfunction may acquire an intrinsic survival advantage (Tsuji *et al.,* 2011). These strains often demonstrate vancomycin tolerance and a tendency to develop heterogeneous resistance under vancomycin selective pressure (Tsuji *et al.,* 2011). The prevalence of *agr* dysfunction among CA-MRSA is reported to be relatively low (3.5% to 9%) compared to HA-MRSA, which displays a high prevalence of *agr* dysfunction (Appelbaum 2007; Tsuji *et al.,* 2011). The established breakpoints given by Clinical and laboratory Standard Institute (2013) for vancomycin are: resistant (VRSA) ≥ 32 μg/ml, intermediate (VISA) 8 to 16 μg/ml and susceptible ≤ 4 μg/ml.

2.8 Diseases caused by MRSA

Methicillin resistant *Staphylococcus aureus* has undergone rapid changes in the past four decades, to become the main cause of healthcare and community-associated infections

worldwide (Boucher *et al.,* 2010). It has been estimated that MRSA accounts for >50% of all *S. aureus* infections in hospitals worldwide (Boucher *et al.,* 2010). Research showed that 80% of infections with CA-MRSA manifest with pyogenic skin infections and SSTI (Yamamoto *et al.,* 2010). Secondary infections seen with CA-MRSA include: endocarditis, brain abscesses, pelvic abscesses, osteomyelitis and arthritis (Yamamoto *et al.,* 2010).

Staphylococcus aureus is the most frequently isolated pathogen from all forms of blood stream infections (Shorr *et al.,* 2006; Del Rio *et al.,* 2009). A report of a positive blood culture for *S. aureus* should initiate an empiric MRSA antibiotic treatment regimen and follow-up blood cultures 48 h to 72 h later (Cosgrove and Fowler, 2008; Boucher *et al.,* 2010). Bacteraemia caused by MRSA poses a major clinical challenge, as infections due to MRSA are usually associated with a worse patient outcome compared to infections caused by MSSA (Rasmussen *et al.,* 2011).

Staphylococcus aureus is the principal cause of endocarditis that occurs in structurally normal heart valves (Lesse and Mylotte, 2006; Naber, 2009). An intravascular device (ID) is the most common source of nosocomial *S. aureus* infective endocarditis (Fowler, 1999; Naber, 2009). Patients suffering from *S. aureus* infective endocarditis (IE) are usually clinically debilitated and develop multiple organ failure, severe sepsis and major neurological complications (Cabell *et al.,* 2002; Nadji *et al.,* 2005; Del Rio *et al.*, 2009).

Other infections caused by *S. aureus* include toxic shock syndrome (TSS), staphylococcal food poisoning (SFP) and staphylococcal scalded skin syndrome (SSSS). Toxic shock syndrome is a multisystemic disease, which was first reported in 1978 (Ortega *et al.,* 2010). It is a disease that is characterised by the rapid onset of high fever, erythematous rash, mucosal hyperaemia, hypotension, desquamation and multi-organ involvement (Ortega *et al.,* 2010). Toxic shock syndrome became prominent in the early 1980s in association with the use of tampons, in previously healthy women (Lappin and Ferguson, 2009; Silversides *et al.*, 2010). Non-menstrual TSS seen in men, children and women has been associated with other staphylococcal enterotoxins following the growth of *S. aureus* in other body sites and the blood stream (Larkin *et al.,* 2009). *Staphylococcus aureus* produces various exotoxins that play a major role in the pathogenesis of TSS (Silversides *et al*, 2010). These exotoxins are the staphylococcal enterotoxins (SEs), toxic shock syndrome toxin-1 (TSST-I) and the staphylococcal enterotoxin-like toxins (SEIs) (Silversides *et al.*, 2010). These toxins are all

virulence factors acting as superantigens (Silversides *et al.,* 2010). Staphylococcal food poisoning (SFP) is defined as the intoxication, which occurs from the consumption of food containing large amounts of one or more enterotoxins (Dinges *et al.,* 2000; Le Loir *et al.,* 2003; Argudin *et al.,* 2010). Symptoms associated with SFP are nausea, vomiting, abdominal cramps and diarrhoea, usually after a short incubation period (Le Loir *et al.,* 2003; Ortega *et al.,* 2010).

Staphylococcal scalded skin syndrome (SSSS) is described as a life-threatening, blistering skin disease, which is caused by certain strains of *S. aureus* that produce an exfoliative toxin (ET) (Ruocco *et al.,* 2011). Staphylococcal scalded skin syndrome is also known as Ritters von Ritterschein in infants less than one month old or Ritter's disease (Bukowski *et al.,* 2011). It is reported that three serotypes of ETs exist; the ETA, ETB and ETD serotypes (Nishifuji *et al.,* 2008; Yamamoto *et al,* 2010). The ETA serotype is more prevalent in Africa, Europe and the USA and is expressed by over 80% of the toxin producing strains (Bukowski *et al.,* 2011). The ETA and ETB serotype cause SSSS in infants and bullous impetigo in children (Yamamoto *et al.*, 2010).

2.9 Treatment and prevention of MRSA infections

The world continues to look for urgent control measures of the pandemic H1N1 influenza virus, while the impact of pandemic MRSA infections continues to go virtually unnoticed (Jarvis, 2009). Infections caused by MRSA are associated with morbidity and mortality, linked with high personal and public cost (Beibei *et al.,* 2010).

Vancomycin (administered intravenously) remains a standard treatment regimen for patients with serious HA-MRSA and CA-MRSA infections, such as endocarditis, septic shock and osteomyelitis (Beibei *et al.,* 2010). However, the emergence of *S. aureus* isolates, which are resistant to vancomycin and the adverse effects noticed with the use of vancomycin have limited its use as a choice of treatment (Stryjewski and Corey, 2009; Beibei *et al.,* 2010). Linezolid (the first available oxazolidinone) is active against a broad range of antibiotic susceptible and resistant Gram-positive bacteria that include MRSA (Beibei *et al,* 2010). Linezolid is used for the treatment of HA-MRSA and CA-MRSA infections as well as complicated skin and soft tissue infections and MRSA ventilator-associated pneumonia (Stryjewski and Corey, 2009). Commonly recommended inexpensive oral agents for the

treatment of CA-MRSA infections include: clindamycin, long-acting doxycycline, minocycline, co-trimoxazole and fusidic acid (DeLeo *et al.,* 2010).

Different countries apply different measures to control MRSA healthcare facility endemicity (Jarvis, 2009). In Denmark, Western-Australia and Holland HA-MRSA infections are kept at a low level by the successful application of active detection and isolation (ADI) measures (Jarvis, 2009). High-risk patients are screened for MRSA, barrier precautions are applied to those who are MRSA positive and healthcare worker hand hygiene is enforced (Muto *et al.,* 2003; Jarvis, 2009).

Human MRSA infections are classified into three groups based on their presumed sources; HA-MRSA, CA-MRSA and HA-MRSA with community inception (Price *et al.,* 2012). However, a recent classification has been added, which identifies human MRSA cases associated with exposure to livestock (LA-MRSA). Livestock-associated MRSA represents a unique clone characterised as ST398 by MLST typing (Golding *et al.,* 2010). The rapid spread of MRSA infection in the hospital, community and livestock shows that MRSA transmission occurs in everyday life, leisure activities, cross-border commuting, livestock transport and exposure to contaminated food products (Köck *et al.,* 2010). Hence, the right precautions, especially hand hygiene along with isolation, proper education and patient decolonisation in selected circumstances are major components of MRSA prevention and control (Humphreys *et al.,* 2009). Investigations are being carried out to develop vaccines, such as the capsular polysaccharide-protein conjugate vaccine and antibodies to the ligand-binding domains of various MSCRAMMS (Daum and Spellberg, 2012).

2.10 Diagnostic identification of MRSA from clinical specimens

Rapid and reliable methods for the identification of MRSA are important in order to make the right choice for therapy and to prevent the undue use of antibiotics (Kaya *et al.,* 2009). Identification of MRSA is based on phenotypic and genotypic methods (Taiwo, 2009). Phenotypic methods used for the identification of MRSA include culture (β-haemolytic white to yellowish colonies on blood agar), Gram-staining (provides a rough classification of the bacteria detected), the DNase test, the catalase test (differentiates between staphylococci and streptococci) and the coagulase test (differentiates *S. aureus* from other staphylococcal

species) (Weile and Knabbe, 2009; Kateete *et al.,* 2010). *Staphylococcus aureus* colonies are further subjected to various antimicrobial susceptibility testing methods, such as the disc diffusion, automated systems [Microscan (Dade Behring), Vitek2 (bioMérieux, Marcy I'Etoile, France) and BD Phoenix (Becton Dickinson)] and the latex agglutination kits, which are available commercially (Brown *et al.,* 2005; Taiwo, 2009). However, these phenotypic methods are time consuming and require two to three days for MRSA strains to be considered as positive (Kramer *et al.,* 2010).

Several molecular based techniques make use of the stable genotypic characteristics, which are not affected by *in vitro* testing conditions (Weile and Knabbe, 2009). These molecular methods are able to identify *S. aureus* strains carrying the *mec*A gene and by using typing assays the possible relationships between the strains can be determined (Kim, 2009).

2.10.1 Phenotypic methods for the characterisation of MRSA isolates

Detection of MRSA with culture media, such as blood agar has gradually been replaced with selective chromogenic agars, such as ChromID (bioMérieux, Marcy I'Etoile, France), MRSA select (Bio-Rad Laboratories, Belgium), CHROMagar MRSA (CHROMagar Microbiology, France; BD Diagnostics, Belgium) and MRSA Ident agar (Heipha GmbH, Eppelheim, Germany) (Malhotra-Kumar *et al.,* 2010). Some of these chromogenic agars are able to produce results within 24 h eliminating the need for further sub-culturing and biochemical tests (Malhotra-Kumar *et al.,* 2010).

Antibiotic susceptibility testing (AST) methods include the disc diffusion, E-test, broth microdilution and the agar screening method (Brown *et al.,* 2005; Taiwo, 2009; Karami *et al.,* 2011). The disc diffusion method (Kirby-Bauer) has proven to be a reliable method used for the detection of methicillin resistance in most routine laboratories (Taiwo, 2009). Karami *et al.* (2011) reported that the oxacillin disk diffusion test has limited accuracy and precision for the detection of MRSA. This is because some strains of *S. aureus* are referred to as borderline oxacillin resistant *S. aureus* (BORSA), which means that these bacteria are hyperproducers of β-lactamases (Mathew *et al.,* 2010). The BORSA strains usually appear as oxacillin resistant but do not possess the genetic mechanism for such resistance (Mathew *et al.,* 2010). The CLSI in 2006 recommended the use of cefoxitin testing as a marker for the detection of methicillin resistance rather than the commonly used oxacillin, since cefoxitin is a better

inducer of the *mec*A gene (Karami *et al.,* 2011). The heterogeneous nature of methicillin resistance in *S. aureus* restricts the precision of the phenotypic antimicrobial susceptibility testing methods used for the detection of MRSA (Kaya *et al.,* 2009). Another phenotypic method used for the detection of MRSA is the latex agglutination assay ((Malhotra-Kumar *et al.,* 2010). The latex agglutination assay is a rapid test, which is approved by the Food and Drug Administration (FDA) for the detection of *S. aureus* and MRSA isolates (Malhotra-Kumar *et al.,* 2010). Different studies have shown that the latex agglutination test has a 100% sensitivity and specificity (Cuevas *et al.,* 2003; Lee *et al.,* 2004). Though Brown *et al.* (2005) reported that any test, which involves the clumping factor might give false positive results.

The most commonly used automated systems include the BD Phoenix (Becton, Dickinson Germany), Vitek2 (bioMérieux Mary I'Etoile, France), Autobac (Pfizer, New York) and MicroScan system (Siemens, Sacramento, California) (Taiwo, 2009). Manufacturers of automated systems have adapted their instruments to maximise the detection of *mec*A resistance (Junkins *et al.,* 2009). Junkins and colleagues (2009) reported an improved accuracy of the Vitek2 (bioMérieux Mary I'Etoile, France) and Phoenix systems with the addition of cefoxitin, which was placed in the test panels to detect the *mec*A gene. Iraz *et al.* (2012) reported that automated systems for detection of MRSA were more accurate than other known phenotypic methods.

2.10.2 Molecular identification and characterisation assays for MRSA isolates

Molecular identification of infectious diseases is the fastest growing field of interest in most clinical diagnostic laboratories (Weile and Knabbe, 2009). There are various molecular methods available, each with unique advantages and disadvantages (Hall *et al.,* 2012). Molecular techniques, such as conventional and real-time polymerase chain reaction (PCR) assays (Conceição *et al.,* 2010) make use of the stable genotypic characteristics of bacteria (Weile and Knabbe, 2009). Genes, which may code for toxins help in defining the pathogenic characteristics of a bacterium (Weile and Knabbe, 2009). The genomes of bacteria provide useful information on conserved regions, which allows identification of bacterial species by using genotyping assays (Weile and Knabbe, 2009). The most important genotyping methods used for MRSA are SCC*mec* typing, staphylococcal protein A typing (*spa* typing), pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) (Conceição *et al.,* 2010). It has been suggested that harmonisation of the genotyping methods by using

standardised protocols is needed to establish surveillance networks and facilitate global MRSA control (Mehndiratta and Bhalla, 2012).

2.10.2.1 Conventional PCR assays for the detection of MRSA isolates

The use of conventional PCR assays in most clinical diagnostic laboratories is gradually replacing phenotypic methods, which are time consuming and labour intensive (Al-Talib *et al.,* 2009). Polymerase chain reaction assays have a shorter turn-around time compared to the phenotypic methods (Van Hal *et al.,* 2007). Pillai *et al.* (2012) reported that the frequently used phenotypic tests are not totally reliable for the detection of methicillin resistance in *S. aureus* due to the low level of sensitivity and specificity of these tests (Pillai *et al.,* 2012). Pillai *et al.* (2012) recommended the use of conventional PCR assays for the detection of the *mec*A gene due to rapidity, sensitivity and accuracy (Pillai *et al.,* 2012). Various PCR assays have been developed, which helps to identify the *Staphylococcus* genus, species, virulence factors and antibiotic resistance genes (Al-Talib *et al.,* 2009). Al-Talib *et al.* (2009) developed a pentaplex PCR assay, which was found to be rapid and gave results within 4 h, which is crucial for the detection of *Staphylococcus* species (Al-Talib *et al.,* 2009).

2.10.2.2 Real-time PCR assays for the detection of MRSA isolates

Although conventional PCR assays are more rapid than the phenotypic methods, it is still considered complex, since these assays require preparation and amplicon characterisation using gel electrophoresis (Suhaili *et al.,* 2009). The development of real-time PCR assays with the use of fluorescent-labelled probes with amplification techniques has made molecular assays in the clinical microbiology laboratory easier (Weile and Knabbe, 2009). Real-time PCR assays make use of fluorescent-labelled probes, such as Scorpion probes, TaqMan hydrolysis probes, fluorescence resonance energy transfer (FRET) and minor groove binding probes (Weile and Knabbe, 2009). The use of closed-tube systems in real-time PCR assays has limited the risk of contamination in clinical diagnostics (Weile and Knabbe, 2009). Real-time PCR assays are more rapid than conventional PCR assays; however, these assays are more expensive, cannot monitor amplicon size and have limited multiplexing capabilities because the number of channels varies between different real-time PCR platforms (Bankowski and Anderson, 2004; Conterno *et al.,* 2007).

2.10.2.3 Staphylococcal cassette chromosome *mec* typing of MRSA isolates

Staphylococcal cassette chromosome *mec* typing is used to differentiate *S. aureus* clones determined by MLST or PFGE typing (DeLeo and Chambers, 2009). The organisation of the *mec*A and different cassette chromosome recombinase (*ccr*) genes helps to dictate the specific SCC*mec* types (Kennedy and DeLeo, 2009). Variations seen in the J regions (three non-essential components of SCC*mec* element) within the same *mec-ccr* complex are used to define the SCC*mec* subtypes (Coombs *et al.,* 2010). A conventional or multiplex PCR (M-PCR) assay is used to determine the structural types of the SCC*mec* (Taiwo, 2009). The genetic diversity observed in MRSA and SCC*mec* subtypes provide evidence that supports the idea that the acquisition of the SCC*mec* by *S. aureus* occurred several times by horizontal gene transfer (Kennedy and DeLeo, 2009).

Various M-PCR assays have been developed over several years by different researchers (Ito *et al.,* 2001, Olivera and De Lencastre, 2002; Zhang *et al.,* 2005; Milheiriço *et al.,* 2007a; Milheiriço *et al.,* 2007b; Boye *et al.,* 2007; Kondo *et al.,* 2007; McClure *et al.,* 2010; Zhang *et al.,* 2012). Each SCC*mec* typing method targets different structures in the SCC*mec*, which makes it difficult for the various assays to agree with one another (Kim, 2009). Chongtrakool *et al.* (2006) suggested that the classification of the SCC*mec* element should be based on the *ccr* complex types. These authors also suggested that the classes of the *mec* gene and subtypes should be classified based on differences found within the J region (Chongtrakool *et al.,* 2006).

2.10.2.4 Staphylococcal protein A typing of MRSA isolates

Protein A was historically known as Jensen's antigen A and is one of the MSCRAMM surface proteins covalently anchored to the cell of *S. aureus* (Dossett *et al.,* 1969). The *spa* locus is composed of 2 150 bp with a molecular weight of 42 000 kilo Dalton (42 kDa) and carries the IgG fibronectin (Fc)-binding region and X-region (Majeed *et al,* 2012). The Fc-binding region and the X-region found in the *spa* gene, code for protein A in *S. aureus* and contain different functional regions, which are highly polymorphic (Schmitz *et al.,* 1998). The Fc-binding region of the *spa* gene is made up of two to five repeat sequences (each 160 bp in size) and the X region is made up of 15 repeat sequences (each 24 bp in size) (Schmitz *et al.,* 1998). Frenay *et al.* (1994) developed the *spa* typing technique, which is based on the

variation of the polymorphic X region of *S. aureus* (Deurenberg and Stobberingh, 2008). The polymorphic X region is located upstream of the region, which codes the cell wall attachment sequence (Ruppitsch *et al.,* 2006). Staphylococcal protein A typing has shown to have a discriminative power between that of PFGE and MLST (Deurenberg and Stobberingh, 2008).

A public *spa* type database and the Ridom *spa* server with differential assignment are often used for naming the *spa* types (Yamamoto *et al.,* 2010). The Ridom StaphType (Ridom GmbH, Würzburg, Germany) software allows data management and retrieval. The software is used for the assignment of new *spa* types with an automatic quality control of DNA sequence chromatogrammes (Harmsen *et al.,* 2003). Currently, the *spa* server database consists of 5 501 *spa*-types, consisting of 311 *spa*-repeats from a total number of 92 900 isolates typed in 66 countries (Deurenberg *et al.,* 2009; Salaam-Dreyer, 2010). The *spa* typing technique has many practical advantages, which includes high throughput, less labour intensive than PFGE, reproducibility between laboratories and full portability of data (Struelens *et al.,* 2009). Staphylococcal protein A typing is the main typing method used to study the molecular evolution and outbreaks of MRSA (Deurenberg and Stobberingh, 2008; Struelens *et al.,* 2009). Limitations associated with *spa* typing are the loss of discriminatory power, which were observed because of the related *spa* repeat patterns within different clonal lineages (Deurenberg *et al.,* 2009). This makes the use of additional typing techniques such as PFGE necessary (Kim, 2009).

2.10.2.5 Accessory gene regulator typing of MRSA isolates

The accessory gene regulator (*agr*) is the most comprehensively characterised two-component signal transducer in *S. aureus* (Gordon *et al.,* 2013). The *agr* locus is found on the *S. aureus* genome and is thought to be part of the core genome (Thoendel, 2012). Wesson *et al.* (1998) reported that *agr*-dependent factors mediate the induction of apoptosis by *S. aureus.* The locus of the *agr* system contains transcription elements and two promoters (P2 and P3) (Novick, 2003). The P2 transcript is coded by four genes known as *agr*ABCD, which encode a two-component system and its auto-inducing peptide (AIP) (Novick, 2003; Nastaly *et al.,* 2010). The effector molecule of the *agr* system is a 514-nt transcript derived from the P3 promoter called RNA III, which also carries the *hld* cistron that codes for delta-haemolysin (Plata *et al.,* 2009). The products of the four genes establish a quorum-sensing mechanism, which activates the response regulators P2 (RNA III) and P3 (RNA III) (Novick *et al.,* 2003;

Nastaly *et al.*, 2010). The polymorphisms observed within the *agr*BCDA operons in *S. aureus* define four *agr* groups (*agr*-I, *agr*-II, *agr*-III and *agr*-IV). It is documented that 71% of all MRSA strains belong to *agr* group I, which makes it the most prevalent *agr* group worldwide (Nastaly *et al.,* 2010). The *agr* group II is usually isolated in patients with endocarditis, while TSST-1 producing MRSA strains belong to *agr* group III (Nastaly *et al.,* 2010). The exfoliatin producing strains, which cause SSSS, belong to *agr* group IV (Nastaly *et al.,* 2010).

2.10.2.6 Pulsed-field gel electrophoresis typing of MRSA isolates

The pulsed-field gel electrophoresis typing technique is regarded as the gold standard for the typing of MRSA strains (Conceição *et al.,* 2010). The PFGE typing technique was developed in 1983 by Schwartz and Cantor (Trindade *et al.,* 2003). The PFGE typing technique is a variation of conventional agarose gel electrophoresis in which an alternating electrical current is passed across the gel periodically (Mehndiratta and Bhalla, 2012). An international MRSA database was established by the CDC based upon PFGE profiles of genomic DNA digested with the endonuclease restriction enzyme (RE) *Sma*I (Kennedy and DeLeo, 2009). The band patterns obtained can be analysed with the GelCompar*II* software using the Dice coefficient and unweighted pair group method with arithmetic mean (UPGMA) to construct a dendrogramme (Cernnone *et al.,* 2012). Clusters are often defined using 80% similarity as the cut-off value (DeLeo and Chambers, 2009). The unique banding pattern obtained for each *S. aureus* strain provides segregation into the specific USA or PFGE types (Kennedy and DeLeo *et al.,* 2009). The PFGE technique is accurate and has a high discriminatory power (Conceição *et al.,* 2010). The disadvantage of the PFGE typing technique is that it is time consuming, labour intensive and difficulty exist in comparing the results obtained in different laboratories due to variations in PFGE protocols (Deurenberg and Stobberingh, 2008; Conceição *et al.,* 2010; Taiwo, 2009).

2.10.2.7 Multilocus sequence typing of MRSA isolates

Multilocus sequence typing (MLST) was first proposed in 1998 as a genotyping method, which enables the characterisation of bacterial isolates in a homogeneous, reproducible and convenient manner (Bravo and Procop, 2009). Multilocus sequence typing is a highly

discriminatory typing method, which has proven to be the best method to study the molecular evolution of *S. aureus* (Deurenberg and Stobberingh, 2008). Multilocus sequence typing compares the nucleotide sequences of seven housekeeping genes present in all isolates of a particular known species (Singh *et al.,* 2006). The internal fragments (500 bp) of seven housekeeping genes of *S. aureus* [carbamate kinase (*arc*), shikimate dehydrogenase (*aro*E), glycerol kinase (*glp*F), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*) triosephosphate isomerase (*tpi*) and acetyl coenzyme A acetyltransferase (*yqi*L)] are sequenced (Deurenberg and Stobberingh, 2008). The DNA sequences are compared to those already published on the MLST website (http://www.mlst.net) (Kim, 2009). The combination of alleles identified is unequivocally associated to a number that corresponds to the sequence type (Pantosti and Venditti, 2009). An MLST allelic profile of 3-3-1-12-4-4-16 is referred to as ST247 and an allelic profile of 2-2-2-2-3-3-2 is defined as ST36 (Deurenberg and Stobberingh, 2008; Kim, 2009). The algorithm based upon repeated sequence types (BURST) is used to determine the clonal complexes (CCs) (Deurenberg and Stobberingh, 2008). Isolates found in one CC have at least five of the seven housekeeping genes identical (Kim, 2009). A putative ancestor of a CC is defined as the sequence type with the largest number of single locus variants (Deurenberg and Stobberingh, 2008).

Data from recent studies have suggested that recombination might occur more repeatedly within CCs than between CCs (Basic-Hammer *et al,* 2010). To test this hypothesis and to understand how genetic diversity is produced in *S. aureus* Basic-Hammer *et al.* (2010) analysed 182 isolates of *S. aureus* with MLST and five core adhesion genes (*clf*A, *clf*B, *fnb*A, *map* and *sdr*C). The study by Basic-Hammer *et al.* (2010) did not confirm the suggestion that recombination occurs more frequently within CCs than between CCs (Basic-Hammer *et al.,* 2010). However, the study highlighted the significance of recombination on the evolution of highly clonal *S. aureus* strains (Basic-Hammer *et al.,* 2010). The study suggested that when recombination is pooled with demographic mechanisms and selection, it may favour the quick formation of new CCs (Basic-Hammer *et al.,* 2010). A disadvantage of MLST is that it is mostly suitable for research purposes since it is too expensive to be used as part of routine diagnostic analysis or for resource poor settings (Al Nakib *et al.,* 2011).

2.11 Summary

The bacteria belonging to the *Staphylococcus* genus are ubiquitous in nature and are colonisers of epithelial cells (Stefani *et al.,* 2012). *Staphylococcus aureus* is the most virulent species of the staphylococcal species due to the vast level of pathogenicity and virulence of this species in humans and animals (Otsuka *et al.,* 2007). It is reported that 20% to 30% of the world's population is constantly colonised with *S. aureus* in the anterior nares with 60% to 100% of individuals being transiently colonised at some point in their lives (Lamers, 2012). Resistance to β-lactam antibiotics and other antibiotics make the prevention and treatment of *S. aureus* difficult (Llarrull *et al.,* 2009). Methicillin resistant *Staphylococcus aureus* is defined as a multi-drug resistant bacterium circulating in clinical settings (Khanna *et al.,* 2008). Previously, MRSA was established as a healthcare pathogen, but the emergence of the bacterium in the community has further heightened public health concerns (Liu, 2009).

The epidemiology of infectious diseases, such as MRSA infections depends on various typing methods used as tools for the characterisation and differentiation of isolates based on their phenotypic or genotypic characteristics (Faria *et al.,* 2008). Conventional methods used for screening MRSA strains are based on the use of culture with or without prior broth enrichment (Stürenburg, 2009). Susceptibility testing is usually performed with the disc diffusion method or with an automated system (Stürenburg, 2009). Although phenotypic methods, such as culture, catalase, coagulase and DNase tests are still regarded as standard methods in most clinical diagnostic laboratories, molecular based methods are highly sensitive and provide rapid results (Kennedy and DeLeo, 2009; Marlowe and Bankowski, 2011).

Different molecular typing techniques are applied for the typing of MRSA strains (Goering *et al.,* 2008). These molecular typing techniques include: SCC*mec*, *spa, agr*, PFGE and MLST typing (Faria *et al.,* 2008). The PFGE typing technique is regarded as an excellent technique for typing MRSA strains (Faria *et al.,* 2008). Compared to other typing techniques the PFGE has a good discriminatory power and interlaboratory exchange of data is possible when standardised protocols are used (Rasschaert *et al.,* 2009). The disadvantage of PFGE typing, especially in outbreaks, is that this technique is labour intensive and technically demanding (Narukawa *et al.,* 2009; Hallin *et al.,* 2012). Other rapid methods, such as the *spa, agr* and MLST typing techniques have gained popularity, since these methods are able to overcome

the disadvantages of the PFGE typing technique (Wolters *et al.,* 2011). These typing techniques offer good interlaboratory reproducibility and easy data handling (Wolters *et al.,* 2011). The *spa* typing technique has been shown to be an important method for outbreak investigation and studies for global population structure of MRSA strains (Wolters *et al.,* 2011). Multilocus sequence typing has resulted in various searchable databases of most clinically important species, such as *S. aureus* (Harris *et al.,* 2010). Multilocus sequence typing of MRSA isolates is useful for long term studies due to the low mutation rate of the seven housekeeping genes (Vindel *et al.,* 2009). Protein expression during the transition in *S. aureus* from the exponential to stationary phase is controlled by global regulators and the accessory gene regulator (*agr*) (Loughman *et al.,* 2009).

Limited data is available regarding the clones of MRSA circulating in the Pretoria region of South Africa. Hence, the aim of this study was to determine the genetic diversity of 194 MRSA isolates obtained from the Steve Biko Academic Hospital (an academic hospital situated in the Gauteng province of South Africa) from April 2010 to August 2011 using three genotyping techniques (*spa, agr* and MLST). The information provided by this study will help in making recommendations regarding surveillance and monitoring.

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CHAPTER 3

Genetic diversity of methicillin resistant *Staphylococcus aureus* strains in the Pretoria region in South Africa

The editorial style of the Journal of Clinical Microbiology and Infection was followed in this chapter

3.1 ABSTRACT

Methicillin resistant *Staphylococcus aureus* (MRSA) causes severe infections in humans. This study aimed to identify and characterise 194 clinical MRSA isolates obtained from a tertiary academic hospital in the Pretoria region collected during April 2010 to August 2011. One of the isolates was contaminated and excluded from the study. The 193 isolates were typed using three PCR-based genotyping techniques (*spa, agr* and MLST typing). The strains were separated into 12 distinct clusters (A to L) with subclusters by *spa* typing. Three of the four *agr* groups were identified in this study: 84.5% (163/193) as *agr* group I, 7.3% (14/193) as *agr* III, 4.7% (9/193) as *agr* II, 3.6% (7/193) as *agr* I and III. No *agr* group IV was detected. Representative isolates were selected for *spa* sequencing and MLST typing. The *spa* types and sequence types identified in this study are in agreement with previously characterised genotypes in South Africa and worldwide. The sequence types (ST) obtained corresponded to the frequently described pandemic clonal complexes, which have been reported worldwide *spa* type t012-ST36/CC30, *spa* type t037-ST239/CC8, *spa* type t891-ST22/CC22 and *spa* type t1257-ST612/CC8. The detection of an epidemic clone (EMRSA-15) t891-ST22/CC22 harbouring the PVL gene as well as a pandemic clone t037-ST239/CC8 in this clinical setting is alarming. The results from this study emphasise the need for strict infection control policies to prevent possible outbreaks of these epidemic and pandemic clones that are already circulating in this clinical setting.

Keywords: ***mec*A, MRSA, *spa, agr,* MLST**

3.2 INTRODUCTION

Staphylococcus aureus (*S. aureus*) is one of the most important and virulent bacterial pathogens, which is responsible for infections seen in healthcare and community settings (Kim, 2009). The bacterium causes diseases ranging from mild infections of the skin and soft tissue to life threatening sepsis (Havaei *et al.*, 2010). The emergence of *S. aureus* strains, which are resistant to methicillin [methicillin resistant *Staphylococcus aureus* (MRSA)] and other antimicrobial agents, such as rifampicin and chloramphenicol are of major concern (Dulon *et al.*, 2011). Infections caused by MRSA were previously associated with healthcare settings (healthcare-associated MRSA) but the emergence of community-associated MRSA (CA-MRSA) worsened the challenges associated with MRSA (Nastaly *et al.,* 2010; Moussa *et al*, 2012). Community-associated MRSA strains are associated with severe infections that can result in necrotising fasciitis or even death in otherwise healthy people outside of the healthcare setting (Wang *et al.,* 2007). Community-associated MRSA differs from the HA-MRSA in various ways: (i) the lack of traditional risk factors associated with MRSA among patients, (ii) a susceptibility pattern with resistance to few antimicrobial agents and (iii) the inclusion of specific virulence factors (Weber, 2005; Lo and Wang, 2011).

Community-associated MRSA has been reported to be a major cause of skin and soft tissue infections (SSTI), endocarditis and necrotising pneumonia (Otto, 2012). Panton-Valentine leukocidin (PVL) is a toxin, which is lethal to leukocytes and is often reported in strains of CA-MRSA (Boakes *et al.,* 2011; Aschbacher *et al.*, 2012). Genome sequencing of two CA-MRSA strains (USA300 and USA400), showed that 20% of the unique genomic contents of CA-MRSA strains, resulted from horizontal acquisition of different mobile genetic elements, such as prophages and pathogenicity islands (Diep and Otto, 2008). These mobile genetic elements are not present in conventional HA-MRSA strains such as COL, N315 and MRSA252 (Diep and Otto, 2008). However, Li *et al.* (2009) reported that for the clonal complex 8 (CC8) lineage (predominantly the USA300), the differential expression of core genome-encoded virulence factors rather than mobile genetic elements (MGEs) have a profound effect on the evolution of virulence. This was based on observations that: (i) the high virulence capacity of USA300 was established in its progenitor strain USA500 before the possession of other virulent determinants on MGEs, (ii) virulence factor expression were comparable among clones within the specific CC8 lineages and (iii) differential distribution of

virulence genes among CC8 strains tested did not explain the observed differences in virulence by gene presence (Li *et al.,* 2009).

It has been noted that CA-MRSA strains has successfully integrated into the healthcare environment, which makes it highly essential to distinguish between the origin of different strains (HA-MRSA and CA-MRSA) (Skrupky *et al.,* 2009). Different types of staphylococcal cassette chromosome *mec* elements (SCC*mec*) have been identified in *S. aureus* strains (Basset *et al.,* 2013). It was reported that methicillin susceptible *S. aureus* (MSSA) strains evolved to become MRSA by the acquisition of the SCC*mec*, which carries the *mec*A gene (Chongtrakool *et al.*, 2006). Staphylococcal cassette chromosome *mec* element eleven (SCC*mec* XI), which carries a new *mec*C element was recently discovered in humans and animals from several European countries (Basset *et al.,* 2013). The *mec*C has a 70% sequence homology with *mec*A genes and has raised concerns about the detection of MRSA carrying this element (Basset *et al.,* 2013). Eleven SCC*mec* types and four subtypes of SCC*mec* type IV has been identified, which differs in the structure and size of the SCC*mec* chromosome (Turlej *et al.,* 2011; Basset *et al.,* 2013). Healthcare-associated MRSA strains harbour primarily SCC*mec* types I, II, III and VI (Al-Rawahi *et al.,* 2007). Healthcare-associated MRSA strains are multi-drug resistant (Otter and French, 2008). Community-associated MRSA carries the SCC*mec* types IV, V, VII, VIII, IX, X and XI and are resistant to only β-lactam antibiotics and sensitive to non-β-lactam antibiotics (Turlej *et al.,* 2011).

The precise and rapid determination of methicillin resistance is of key importance in the diagnosis of infections caused by *S. aureus* (Anand *et al.,* 2009). Phenotypic techniques used for the detection of MRSA are time consuming and the likelihood of misidentifying MRSA based on phenotypic testing has been reported in the past (Bhutia *et al.,* 2012). Molecular-based methods are used to improve the limitations of phenotypic methods by providing rapid identification and characterisation of these pathogens (Stefani *et al.,* 2012). Furthermore, different genotyping techniques are available to discriminate between clinically relevant *S. aureus* isolates, although it does not always form part of the routine diagnostic analysis (Trindade *et al,* 2003). The criteria for the evaluation of these genotyping techniques are divided into: (i) the performance criteria (typeability, reproducibility, discriminatory power and agreement between typing techniques) and (ii) convenience criteria (versatility, rapidity, ease of execution and interpretation) (Trindade *et al.,* 2003).

Genotyping methods used for typing *S. aureus* strains are: pulsed-field gel electrophoresis (PFGE), SCC*mec* typing, staphylococcal protein A typing (*spa* typing) and multilocus sequence typing (MLST) (Deurenberg and Stobberingh, 2008; Skrupky *et al.,* 2009). Pulsed-field gel electrophoresis is regarded as the gold standard method for typing *S. aureus* strains and particularly for isolates, which are MRSA (Tenover and Goering, 2009). The PFGE typing assay is based on a variation of standard gel electrophoresis using an alternating voltage gradient, which ensures a better resolution of large DNA molecules (Kim, 2009). The PFGE typing assay has a good discriminatory power and reproducibility at the inter-laboratory level when standardised protocols are used (Rasschaert *et al.,* 2009). This technique (PFGE) has been used to identify and document major clones of MRSA (Brazilian, Hungarian, Iberian, New York/Japan and paediatric) worldwide (Faria *et al.,* 2008). It has; however, been reported that PFGE is tedious, labour intensive and time consuming (Mehndiratta and Bhalla, 2012). Genotyping methods, such as *spa, agr* and MLST are more discriminatory methods for detecting evolutionary changes and transmission events between strains of *S. aureus* (Mehndiratta and Bhalla, 2012).

Different studies have documented MRSA strains in South Africa (Shittu *et al.,* 2009; Moodley *et al.,* 2010; Jansen van Rensburg *et al.,* 2011). However, information regarding the genetic diversity of MRSA in the Pretoria region using *spa*, *agr* and MLST typing is limited. This study is a follow-up of another study where PFGE, the gold standard and SCC*mec* typing has been used to determine the genetic relatedness of 194 clinical MRSA strain. The aim of this study was to identify and determine the genetic diversity of healthcare-associated MRSA (HA-MRSA) and community-associated MRSA (CA-MRSA) isolates that are circulating in a tertiary academic hospital using *spa*, *agr* and MLST typing. The results obtained were compared to that of PFGE to possibly find (i) a more rapid and easier genotyping method and (ii) to determine clones of MRSA circulating in this clinical setting in order to make recommendations regarding surveillance and monitoring.

3.3 MATERIALS AND METHODS

3.3.1 Study setting, collection and confirmation of MRSA isolates

This was a semi-quantitative study conducted at the Research Division of the Department of Medical Microbiology University of Pretoria (UP) / National Health Laboratory Service

(NHLS). One hundred and ninety four MRSA isolates collected from April 2010 to August 2011 which have been analysed by the Diagnostic Laboratory, Department of Medical Microbiology UP/NHLS were included in this study. *Staphylococcus aureus* isolates were received as MRSA positive after the routine diagnostic analysis was performed. The isolates were confirmed as MRSA using the DNase test, Kirby-Bauer disc diffusion method and Vitek2 system (bioMérieux, Mary l'Etoile, France). The plates were incubated at 37^{o}C for 18 h to 24 h. A Gram-stain was performed to confirm that the isolates were not contaminated. The isolates were stored in 50% glycerol (Merck, Darmstadt, Germany) at -70^{o}C (New Brunswick Scientific, England) for future use (Appendix C).

The PFGE and SCC*mec* genotyping of these MRSA isolates was part of another MSc study conducted in the department (S189/2010). The same 194 isolate were used for this study. This study was approved by the Student Ethics Committee of the University of Pretoria (Protocol number S175/2011).

3.3.2 Total DNA extraction of the MRSA isolates

Bacterial DNA extraction of the 194 MRSA isolates was performed using the ZR Fungal/Bacterial DNA MiniPrep (Zymo Research, Thermo Scientific, USA), according to the manufacturer's instructions, which was optimised with the addition of β-mercaptoethanol to the Fungal/Bacterial DNA binding buffer to a final concentration of 0.5% (v/v) (Appendix B).

3.3.3 Multiplex-PCR assay for the detection of the 16S rRNA, *mec*A and PVL genes of the MRSA isolates

A multiplex PCR assay was performed on the clinical isolates based on the method described by McClure *et al.* (2006). Three primer pairs specific for the 16S rRNA, *mec*A and *luk*-PV genes were used (Table 3.1). A CA-MRSA strain (ATCC CA05) served as a positive control. The primer pair concentrations of 0.2 µM, 0.24 µM and 0.2 µM from McClure *et al.* (2006) was modified to 2 µM for each primer. The PCR assay included 5 µℓ of the DNA template in a 50 µℓ final volume. The M-PCR assay reaction compositions consisted of 25 µℓ of 2X QIAGEN M-PCR master mix (Qiagen, Germany), 5 µℓ of 10X primer mix and 15 µℓ of RNase-free water. The PCR programme for the G-storm thermocycler (Vacutec, UK) included an initial activation step at 95°C for 15 min, followed by 35 cycles of denaturation at

94ºC for 45 s, annealing at 57ºC for 90 s, extension at 72ºC for 90 s and a final extension step at 72ºC for 10 min. Analysis of the PCR products is shown in Section 3.3.7.

3.3.4 PCR assay for the *spa* typing of the MRSA isolates

The PCR assay included three primers (SPA1, SPA2 and SPA3), that selectively amplified the repetitive sequence region (Fc binding region and the X region) of the *S. aureus* specific protein A gene. The primers used for the *spa* typing was obtained from a previously published study (Schmitz *et al.,* 1998). The primer concentrations were modified for the PCR assay (TaKaRa Ex*Taq* PCR kit, Takara Biotechnology, Japan). The primer pair concentration was adjusted to 0.32 µM for each primer. The primer pair concentration and primer sequences are shown in Table 3.2.

The PCR reaction set-up included 5 µℓ of the DNA template in a final volume of 25 µℓ. The reaction composition consisted of 0.125 µℓ (TaKaRa *Taq* DNA polymerase, Takara Biotechnology, Japan), 2 µℓ of dNTPs (2.5 mM each), 2.5 µℓ of 10X PCR buffer (10 mM Tris-HCℓ, pH 8.3, 500 mM KCℓ and 15 mM $MgC\ell_2$), 0.32 µM of each primer (one forward primer SPA1 and two reverse primers SPA2 and SPA3) and 15 µℓ of RNase-free water. The PCR amplification was performed using the G-storm thermocycler (Vacutec, UK) according to the method by Schmitz *et al.* (1998), with the following modifications: an initial activation step at 94^{o}C for 15 min followed by 35 cycles of denaturation at 94^{o}C for 30 s, annealing at 52^{o}C for 40 s and extension at 72^{o}C for 50 s followed by a final extension step at 72^{0}C for 5 min. The analysis of the amplified PCR products is discussed in section 3.3.7.

Ten representative isolates were selected for *spa* sequencing based on the results from PFGE. The isolates were: 2, 71, 100, 125, 131, 133, 134, 143, 165 and 183. The X region of the *spa* gene was amplified with primers from a previously published study (Larsen *et al.,* 2008). The primer concentrations and primer sequences are shown in Table 3.3. The PCR assay included 3 µℓ of prepared DNA template in a 25 µℓ final reaction mixture. The reaction composition consisted of 12.5 µℓ of the 2X QIAGEN M-PCR master mix (Qiagen, Germany), 2 µM of each primer and 7 µℓ of RNase-free water (Qiagen, Germany). The PCR cycling programme for the G-storm thermocycler (Vacutec, UK) included an initial activation step of 95^{o}C for 15 min followed by 32 cycles of denaturation of 94^{o}C for 30 s, annealing at 56^{o}C for 30 s and extension at 72^{o}C for 1 min followed by a final extension at 72^{o}C for 7 min. The analysis of

the amplified PCR products is discussed in section 3.3.7. The amplicons after gel electrophoresis were sent to Inqaba biotec for sequencing.

3.3.5 Duplex PCR assay for the *agr* typing of the MRSA isolates

The PCR assay for *agr* typing consisted of a forward primer (used for all reactions) and four reverse primers (*agr* group I, II, III and IV) as described by Shopsin *et al.* (2003). To differentiate between the similar sized products of the reverse primers of *agr* group I (440 bp) plus III (406 bp) and *agr* group II (572 bp) plus *agr* group IV (588 bp) duplex PCR assays were performed for each isolate. The duplex PCR assays were performed using (i) the forward primer and two reverse primers for *agr* group I and *agr* group II and (ii) the forward primer and two reverse primers for *agr* group III and group IV.

The primer concentrations and nucleotide sequences are shown in Table 3.4. Each duplex PCR assay included 5 µℓ of prepared DNA template in a final reaction of 25 µℓ mixture. The reaction composition consisted of 12.5 µℓ Maxima® Hot Start Green PCR Master Mix (2X), supplied in 2X hot start PCR buffer, Mg^{2+} and dNTPs (Fermentas, Thermo Scientific, USA). The PCR amplification was performed using the G-storm thermocycler (Vacutec, UK) with modification to the cycling profile of Shopsin and colleagues (2003), with an initial activation step at 95°C for 4 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min and a final extension step at 72°C for 10 min. Analysis of the amplified PCR products is as described in section 3.3.7.

3.3.6 Multilocus sequence typing analysis of selected MRSA isolates

The fragments of seven housekeeping genes [carbamate kinase (*arc*), shikimate dehydrogenase (*aro*E), glycerol kinase (*glp*F), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*) triosephosphate isomerase (*tpi*) and acetyl coenzyme A acetyltransferase (*yqi*L)] were amplified using the primers described by Enright *et al.* (2000). The primer concentrations and nucleotide sequences are shown in Table 3.5. The PCR assay included 3 µℓ of prepared DNA template in a final volume of 25 µℓ. The reaction composition consisted of 12.5 µℓ of the 2X QIAGEN M-PCR master mix (Qiagen, Germany), 2 µM of each primer (forward and reverse) and 7 µℓ of RNase-free water (Qiagen, Germany). The PCR amplification was performed using the G-storm thermocycler (Vacutec, UK), with

modifications to the cycling profile by Enright *et al* (2000), with an initial activation step of 95°C for 15 min followed by 32 cycles of denaturation of 94°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 1 min and a final extension step at 72°C for 7 min. Analysis of the amplified PCR product was as described in section 3.3.6. The amplicons after gel electrophoresis were cleaned using the Zymoclean gel DNA recovery kit (Fermentas, Thermo Scientific, USA) (Appendix B) and sent to Inqaba biotec for sequencing.

3.3.7 Analysis of the PCR products

Amplified products were visualised for specific fragment size bands under UV illumination (TFM-26 Ultra Transilluminator, UVP, Upland, California) after electrophoretic separation at 100 V for 1 h on a 3% (m/v) SeaKem® LE agarose gel (Lonza, Rockland, USA) for *spa* typing in 1X TBE buffer [45 mM Tris-borate (pH 8.0) (Sigma Chemical co, USA), 0.5 M EDTA (Promega, Madison, USA)] and a 100 bp molecular weight marker (Fermentas, Thermo Scientific, USA) was included as a reference in the first and last lane. A 1% (m/v) SeaKem® LE agarose gel (Lonza, Rockland, USA) was used for multiplex PCR assay for 16S rRNA, *mec*A, PVL, *agr* and MLST analysis and a 50 bp molecular weight marker (Fermentas, Thermo Scientific, USA) was included as reference in the first and last lane. The agarose gel used contained 5 µℓ of a 20 µg/ml of ethidium bromide stock solution (Promega, Madison, USA) (Appendix B). Images were captured using a digital gel documentation system (DigiDoc-It Imaging System, UVP, Upland, Sweden).

3.3.8 Analysis of data

The sample size of 194 randomly collected MRSA isolates was analysed using percentages. The clonal diversity of the MRSA strains were grouped into clusters from the dendrogramme and expressed as percentages.

The resulting *spa* patterns obtained after gel electrophoresis were analysed with the GelCompar*II* software (Applied Maths, Belgium) programme using the Dice Coefficient and the unweighted pair group method with arithmetic mean (UPGMA) to construct a dendrogramme which showed the genetic diversity of MRSA strains analysed. A 60% similarity value was used as a cut-off value for cluster definition to group isolates into clusters

and a 80% similarity was used as a cut-off value for cluster definition to group isolates into subclusters.

The *spa* types were determined using the Ridom StaphType software (Ridom GmbH, Würzburg, Germany). After providing the input sequence (ABI format), Ridom StaphType software (Ridom GmbH, Würzburg, Germany) attaches to each called base a quality value, which corresponds to a sequence error probability (Harmsen *et al.,* 2003). The software constructs a consensus sequence, detects the *spa* repeats and assigns a *spa* type (Harmsen *et al.,* 2003)

The amplicons for the MLST typing were sequenced in both directions (forward and reverse). The sequences obtained for each of the housekeeping genes were analysed using the CLC main workbench version 6.0 (CLC, Denmark). The sequences were uploaded on the MLST database (http://saureus.mlst.net/sql/multiplelocus) where allelic profiles and sequence types were assigned.

3.4 RESULTS

3.4.1 Demographics

The MRSA isolates were obtained from female and male patients (aged: 1 day to 78 y with an average of 39 y) in a range of wards, which included: surgery, paediatric, cardiothoracic, outpatients, intensive care unit, anti-retroviral clinic, oncology, urology, gynaecology, nephrology and internal medicine. The MRSA isolates were recovered from blood cultures (39%, *n*=75/194), pus swabs (23%, *n*=44/194), central venous pressure tips (13%, *n*=25/194), tissue (8%, *n*=16/194), endotracheal aspirate (Luki) (6%, *n*=12/194), sputum (4%, *n*=7/194), unknown (3%, *n*=6/194), urine (2%, *n*=4/194), briviac tip (1%, *n*=2/194), bone fragment (0.5%, *n*=1/194), catheter tip (0.5%, *n*=1/194) and cerebrospinal fluid (0.5%, *n*=1/194). The MRSA isolates were subcultured on blood agar plates (Oxoid, England) to obtain single colonies for Gram-staining.

3.4.2 Prevalence of the 16S rRNA, *mec*A and PVL genes

All 194 MRSA isolates were 100% (194/194) positive for the 16S rRNA (756 bp) and *mec*A (310 bp) genes. Only one of the isolates (isolate 71) was positive for the PVL (433 bp) gene (Figure 3.3).

3.4.3 Characterisation of MRSA isolates using *spa* typing

Out of the 193 isolates, six isolates were untypeable with *spa* typing. All of these isolates were excluded from this study and the remaining 187 MRSA isolates revealed twelve distinct clusters designated A to L and four outliers (Figure 3.1). The majority of the isolates belonged to cluster A [23.5% (44/187)] and B [22.9% (43/187)], followed by E [13.4% (25/187)], D [10.2% (19/187)], G [8.6% (16/187)], K [4.3% (8/187]), C [3.7% (7/187)], J [3.2% (6/187)], L [2.7% (5/187)], H [2.1% (4/187)], F [1.6% (3/187)] and I [1.6% (3/187)], while the four outliers made up for 2.1% (4/187). Cluster A had five subclusters (A1 to A5), cluster B had four subclusters (B1 to B4), cluster C and D had two subclusters each (C1 and C2; D1 and D2, respectively), cluster E had four subclusters (E1 to E4) and cluster G and K had two subclusters (G1 and G2; K1 and K2, respectively).

Four *spa* types were identified from the *spa* sequencing. Three isolates (2, 100 and 143) belonged to *spa* type t1257 with the *spa* repeats-pattern: 11-19-34-05-17-34-24-34-22-25. Isolates 125, 131 and 133 belonged to *spa* type t037 with *spa* repeats-pattern: 15-12-16-02-25-17-24. Isolate 71 was identified as, *spa* type t891 with *spa* repeats-pattern: 26-23-13-23-31-05-17-25-17-25-28 while isolate 183 was identified as, *spa* type t012 with *spa* repeats-pattern: 15-12-16-02-16-02-25-17-24-24. Isolates 134 and 165 remained untypeable (Table 3.6).

3.4.4 Characterisation of MRSA isolates using *agr* typing

One contaminated MRSA isolate was excluded from the *agr* typing. A total of 193 MRSA isolates were analysed using the *agr* assay. The *agr* typing assay identified the majority of isolates as follow: 84.5% (163/193) as *agr* group I, 7.3% (14/193) as *agr* group III, 4.7% (9/193) as *agr* group II and 3.6% (7/193) as *agr* group I and III (Figures 3.2, 3.3 and 3.4). No

agr group IV isolates were detected. Table 3.7 shows the distribution of the *agr* groups among the *spa* clusters identified from the dendrogramme.

3.4.5 Characterisation of MRSA isolates using multilocus sequence typing

Ten representative isolates were selected for MLST typing, three of the isolates namely numbers 125, 134 and 165 were untypeable by *spa* typing (Table 3.). Isolate 71 belonged to ST22, isolate 100 to ST612, isolates 131 and 133 belonged to ST239, isolate 143 to ST612 and isolate 183 to ST36. Sequence type 22 with allelic profile 7-6-1-5-8-8-6 belonged to clonal complex 22 (CC22), ST36 with allelic profile 2-2-2-2-3-3-2 belonged to CC30, ST239 with allelic profile 2-3-1-1-4-4-3 and ST612 with allelic profile 3-3-1-1-4-88-83 both belonged to CC8. The *tpi* gene for isolate 125 had a new allele number (240) (2-3-1-1-4-240-3). The allele number for the *yqi*L gene for isolate 2 was not found on the MLST website (3-3-1-1-4-88-?). The allelic numbers for *arc*C, *aroE* and *pta* gene for isolates 134 and 165 was not found on the MLST website.

3.4.6 Antimicrobial susceptibility for the ten representative isolates for *spa* sequencing and MLST analysis using the Vitek2 automated system (bioMérieux, Mary l'Etoile, France)

The Vitek2 automated system (bioMérieux, Mary l'Etoile, France), showed that two of the isolates (2 and 71) were resistant to glycopeptides (teicoplanin and vancomycin). The interpretation of the antibiotic susceptibility profiles was performed according to the clinical laboratory standards institute (CLSI) guidelines of 2013. However, the E-test micro-dilution showed that isolate (2 and 71) were sensitive to teicoplanin (0.75 μg/ml) and vancomycin (1.0 μg/ml).

In this study the two isolates (131 and 133), which belonged to ST239, showed resistance to the following antibiotics: ciprofloxacin (≥ 8 μg/ml), clindamycin (≤ 0.25 μg/ml), erythromycin (≥ 8 μg/ml), gentamicin (≥ 16 μg/ml), oxacillin (≥ 4 μg/ml), tetracycline (≥ 16 μg/ml) and trimethoprim-sulfamethoxazole (≥ 320 μg/ml). However, both isolates (131 and 133) were sensitive to teicoplanin (≤ 0.5 μg/ml) and vancomycin (2 μg/ml; ≤ 0.5 μg/ml).

The two isolates (100 and 143) identified as ST612 in this study showed resistance to the following antibiotics (ciprofloxacin (≥ 8 μg/ml), clindamycin (≤ 0.25 μg/ml), erythromycin (≥ 8 μg/ml), gentamicin (≥ 16 μg/ml), tertracycline (≥ 16 μg/ml), trimethoprim-sulfamethoxazole (≥ 320 μg/ml), oxacillin (≥ 4 μg/ml) and rifampicin (≥ 32 μg/ml). The isolates (100 and 143) were sensitive to teicoplanin (≤ 0.5 μg/ml) and vancomycin (1 μg/ml; ≤ 0.25 μg/ml). Isolate (71) identified as ST22, was resistant to the following antibiotics (clindamycin (≥ 8 μg/ml), erythromycin (≥ 8 μg/ml), fusidic acid (≥ 32 μg/ml), gentamicin (≥ 16 μg/ml), teicoplanin (≥ 32 μg/ml), tertracycline (≥ 16 μg/ml), trimethoprim-sulfamethoxazole (≥ 320 μg/ml), oxacillin (≥ 8 μg/ml), rifampicin (≥ 32 μg/ml) and vancomycin (≥ 32). Isolate 183 identified as (ST36/CC30) was resistant to (ciprofloxacin (≥ 8 μg/ml), clindamycin (≥ 8 μg/ml), erythromycin (≥ 8 μg/ml), moxifloxacin (≥ 8 μg/ml) and oxacillin (≥ 4 μg/ml). This isolate was sensitive to teicoplanin and vancomycin.

Isolate 125 was resistant to (ciprofloxacin (≥ 8 μg/ml), clindamycin (≤ 0.25 μg/ml), erythromycin (≥ 8 μg/ml), moxifloxacin (≥ 8 μg/ml), oxacillin (≥ 4 μg/ml), rifampicin (≥ 16 μg/ml), tetracycline (≥ 16 μg/ml), tigecycline (0.5 μg/ml) and trimethoprim-sulfamethoxazole (≥ 320 μg/ml). This isolate (125) showed an intermediate resistance to fusidic acid (4). Isolate (125) was sensitive to teicoplanin (2) and vancomycin (2). Isolate 2 was resistant to (clindamycin (≥ 8 μg/ml), erythromycin (≥ 8 μg/ml), fusidic acid (≥ 32 μg/ml), gentamicin (≥ 16 μg/ml), rifampicin (≥ 32 μg/ml), oxacillin (≥ 4 μg/ml), tetracycline (8 μg/ml), teicoplanin (≥ 32 μg/ml) and vancomycin (≥ 32 μg/ml).

The two isolates (134 and 165) were resistant to (clindamycin (≤ 0.25 μg/ml), erythromycin (≥ 8 μg/ml), gentamicin (≥ 16 μg/ml), rifampicin (≥ 32 μg/ml), oxacillin (≥ 4 μg/ml), tetracycline (≥ 16 μg/ml; 2 μg/ml), trimethoprim-sulfamethoxazole (80 μg/ml; 160 μg/ml). Isolate 165 showed an intermediate resistance to fusidic acid (4 μg/ml) and ciprofloxacin (2 μg/ml). The two isolates [134 (2 μg/ml) and 165 (8 μg/ml)] were sensitive to teicoplanin and vancomycin.

3.5 DISCUSSION

In the current study the molecular typing methods *spa, agr* and MLST were used for the characterisation of MRSA isolates. Ten representative isolates were chosen for the *spa* sequencing and MLST analysis based on the specific PFGE pulsotypes obtained for the same

clinical isolates (Maphanga, 2013). Two isolates (125 and 170) were untypeable by PFGE. Maphanga, (2013) identified eleven pulsotypes A to K from the PFGE, with pulsotype A being the major pulsotype [(57% (110/191)] having subtypes A1 to A6. The ten representative isolates were chosen from PFGE pulsotype A (131 and 133), subtypes of pulsotype A [A3 (isolate 71) and A6 (isolate 100)], pulsotype B (isolate 143), subtype of pulsotype B (B2 isolate 134), pulsotype E (isolate 2), pulsotype I (isolate 165) and pulsotype J (isolate 183). Isolate 125 was chosen as the tenth representative isolate.

The *spa* typing revealed twelve distinct clusters (A to L) with subclusters from clusters A (A1 to A5), B (B1 to B4), C (C1 and C2), D (D1 and D2), E (E1 to E4), G (G1 and G2) and K (K1 and K2). This study indicated six *spa* non-typeable MRSA isolates. These six non-typeable isolates may have been attributed to *S. aureus* strains that do not produce the surface protein A, lacking the *spa* gene. This is in agreement with different studies that have reported strains of *S. aureus* without the *spa* gene (Guzman *et al.,* 1992; Larsen *et al.,* 2008; Makgotlho *et al.,* 2009). Guzman *et al.* (1992) reported 5% (10/196) of *S. aureus* strains that lacked the protein A gene. However, it was documented that two of the isolates were misidentified as *S. hyicus* and *S. intermedius* (Guzman *et al.,* 1992). Larsen *et al.* (2008) reported 1.2% (9/759) of *S. aureus* isolates that lacked the protein A gene after identification by coagulase test and PCR detection for the *nuc* and *fem*A gene. A study by Makgotlho *et al.* (2009), reported 5.2% (5/97) non-typeable MRSA isolates.

Although the *spa* typing depicted a higher discriminatory power compared to PFGE in this study, six isolates (Isolates 52, 125, 134, 138, 139 and 165) were untypeable using *spa* typing, while only two isolates (Isolates 125 and 170) were untypeable with PFGE typing. Similarities were noted in the PFGE and *spa* typing methods with some isolates sharing the same PFGE pulsotypes and *spa* cluster. According to the *spa* typing results 61.3% of the isolates which belonged to cluster A were identified as PFGE pulsotype A, 27.2% with *spa* cluster A belonged to subtypes of PFGE pulsotype A, 57.1% with *spa* cluster J belonged to PFGE pulsotype J. Three of the six untypeable isolates (138, 139 and 165) belonged to PFGE pulsotype I. Faria *et al.* (2008) reported that the *spa* and PFGE typing provided an excellent result for strains of MRSA. Faria *et al.* (2008) concluded that *spa* and PFGE typing provide the basic discriminatory power needed for local and long-term epidemiological surveillance. Different studies have however, reported the *spa* typing method to be rapid, less labour intensive and easier to interpret than the PFGE (Palavecino 2007; Vaino *et al,* 2011). The *spa*

typing can be used to investigate outbreaks and the molecular evolution of MRSA strains (Vaino *et al.,* 2011).

Staphylococcal protein A sequencing was performed to compare the *spa* types with the profiles of international clones. The *spa* types (t012, t037, t891 and t1257) identified in this study have been reported worldwide including South Africa (Table 3.5) (Jansen van Rensburg *et al.,* 2011; Moodley *et al.,* 2010; Salam-Dreyer, 2010). The *spa* types t012 and t037 was reported by Jansen van Rensburg *et al.* (2011) in Cape Town from six different hospitals. Moodley *et al.* (2010) reported *spa* types t012, t037 and t891 from isolates collected between August 2005 to November 2006 at 15 state and eight private diagnostic microbiology laboratories in the nine provinces of South Africa. The four *spa* types identified in this study (t012, t037, t891 and t1257) were also identified at Tygerberg hospital, Western Cape Province in South Africa by Salam-Dreyer (2010). Staphylococcal protein A type t891 had the longest *spa* repeats-pattern (26-23-13-23-31-05-17-25-17-25-28), while *spa* type t037 had the shortest *spa* repeats-pattern (15-12-16-02-25-17-24). Isolates with the same sequence type (ST), belonged to the same *spa* types. Staphylococcal protein A type t012 (15-12-16-02-16-02-25-17-24-24) belonged to ST36 in this study, which is in agreement with the study conducted by Moodley *et al.* (2010). Three isolates (125, 131 and 133) identified as ST239 belonged to *spa* type t037 (15-12-16-02-25-17-24). The *spa* type t037 is most often associated with the pandemic Brazilian/Hungarian clone ST239 (Monecke *et al.,* 2011). This is in agreement with previous studies conducted by Shittu *et al.* (2009) and Moodley *et al.* (2010) that reported ST239 with *spa* type t037 in South Africa. Three isolates (2, 100 and 143) identified as ST612 belonged to *spa* type t1257. The *spa* type t1257 was one of the major *spa* types detected by Salam-Dreyer (2010). Isolate 125, which was untypeable by the *spa* typing PCR assay and PFGE was identified as *spa* type t1257 with *spa* sequencing, while isolates 134 and 165 remained untypeable. The only PVL positive isolate in this study ST22 belonged to *spa* type t891 (26-23-13-23-31-05-17-25-17-25-28). Moodley *et al.* (2010) detected only one PVL positive MRSA, which also belonged to the same *spa* type t891, ST22 (CC22).

The *agr* group IV, which is common among exfoliatin producing MRSA (causes staphylococcal scalded skin syndrome) strains was not detected in this study (Nastaly *et al.,* 2010). The recovery of three of the four *agr* groups in this study is in agreement with the reported data from Shopsin *et al.* (2003). A rapid PCR-based method was used to determine

the *agr* groups in *S. aureus* isolates obtained from a healthy population of children and their guardians in New York City, New York (Shopsin *et al.,* 2003). Some studies have detected all four *agr* groups, which might be due to ecological and geographical distribution (Sakoulas *et al.,* 2002; Nastaly *et al.*, 2010). Accessory gene regulator group I (84.5%) had the highest prevalence in this study. Most of the published studies have shown that 71% of all MRSA strains belong to *agr* group I, which makes it the most prevalent genotype (Sakoulas *et al.,* 2002; Nastaly *et al.*, 2010). Accessory gene regulator II, which is common in patients with endocarditis, is associated with reduced susceptibility to vancomycin and had a prevalence of 4.7% in this study. Accessory gene regulator I and III have been described as molecular markers of community-associated MRSA (Skrupky *et al.,* 2009; Nastaly *et al*, 2010). This study showed a high prevalence of *agr* group I (84.5%), followed by *agr* group III (7.3%) indicating that most of the isolates were CA-MRSA, which is not in agreement with the SCC*mec* typing on the same clinical isolates (Maphanga, 2013). The results from SCC*mec* typing assays showed that 75% (145/194) belonged to HA-MRSA, while 25% (49/194) belonged to CA-MRSA (Appendix C) (Maphanga, 2013). These conflicting results emphasize the unspecific nature of the *agr* typing method to differentiate between HA-MRSA and CA-MRSA. This is supported by the findings by Wright *et al.* (2005) and Diep *et al.* (2006) that reported that there was no obvious pattern to the distribution of *agr* allotypes among MRSA lineages. A study by Holmes and colleagues (2005) reported that the majority of the United Kingdom epidemic healthcare-associated MRSA strains belonged to *agr* group I. Van Leeuwen *et al.* (2000) reported that the pathogenic ability of *S. aureus* is dependent on the production of exoproteins which is regulated by the *agr* operon. In most toxin mediated diseases, the *agr* alleles and toxin genes occurred with their parent strains and horizontal transfer played a marginal role (Jarraud *et al.,* 2002).

Strains of MRSA are often resistant to multiple classes of antimicrobial agents including aminoglycosides, macrolides-lincosamides-streptogramins (MLS) and tetracyclines (Denis *et al.,* 2006). The use of glycopeptides as an antimicrobial agent has increased in the past 20 years, due to the rise in MRSA and coagulase negative staphylococci (CoNS) (Tiwari, 2009). Glycopeptides are used to treat Gram-positive bacterial infections that are resistant to other known antimicrobial agents such as the β-lactams (Khane *et al.,* 2005). However, the need for effective new agent for the treatment of MRSA infections is now apparent due to emergence of MRSA strains with reduced susceptibility to these glycopeptides (Denis *et al.,* 2006). In this study the antimicrobial susceptibility testing including the minimum inhibitory

concentration (MICs) obtained using the Vitek2 automated system (bioMérieux, Mary l'Etoile, France) showed that two isolates (2 and 7) were resistant to glycopeptides (teicoplanin and vancomycin). However, the E-test micro-dilution showed that isolate (2 and 71) were sensitive to teicoplanin (0.75 µg/ml) and vancomycin (1.0 µg/ml). The discrepant results obtained emphasize the importance of the accurate performance of the antibiotic resistance protocol for the Vitek2 automated system (bioMérieux, Mary l'Etoile, France) to prevent false positive results

The dominant pulsotype from the PFGE typing technique [57% (110/191)] corresponded to the pulsotype A, which was identified as *spa* type t037-ST239 in this study. Isolates 131 and 133 identified as ST239/CC8 (2-3-1-1-4-4-3) belonged to PFGE A-SCC*mec*(II+SCC*mercury*)-*spa* type t037/ClusterB-*agr*III and PFGE A-SCC*mec*IVa-*spa* type t037/ClusterB-*agr*I respectively. The ST239 with an allelic profile of 2-3-1-1-4-4-3 is described as the oldest pandemic MRSA clone, which is resistant to multiple antibiotics and accounts for 90% of all HA-MRSA in China, Thailand, Turkey, Mainland Asia and South America (D'Souza *et al.,* 2010; Harris *et al.,* 2010).

The pandemic clone t037-ST239-MRSA-III (pulsotype A) has previously been reported in Cape Town, South Africa (Jansen van Rensburg *et al.,* 2011) and by Moodley *et al.* (2010). Pulsotype PFGE D-SCC*mec* III-*spa*CC12-ST239 was reported as one of the major clones circulating in the Pretoria region of South Africa (Moodley *et al.,* 2010). This pandemic clone ST239-MRSA-III, also referred to as the Brazilian/Hungarian clone, was the second major clone reported from healthcare facilities in the KwaZulu-Natal province, South Africa, between 2001 and 2003 (Shittu *et al.,* 2009). Sequence type 239 is a single locus variant of USA300, which is thought to have evolved from ST8 (D'Souza *et al.,* 2010). The TW20 clone, a variant of ST239 MRSA, was isolated as the cause of an outbreak in an intensive care unit in London (Harris *et al.,* 2010). According to Cirlan *et al.,* 2005, ST 239 is able to withstand background mutations without changing its ecological success. The nature of these changes does not seem to affect its potential to spread and it might even be considered that the mutation rate of ST239 may be part of its success in coping with different nosocomial ecosystems (Cirlan *et al.,* 2005).

Isolates 100 and 143 identified as ST612/CC8 (3-3-1-1-4-88-83) belonged to PFGE A6-SCC*mec*IVd-*spa* t1257/ClusterA-*agr*I and PFGE B-SCC*mec*IVa-*spa* type t1257/ClusterA –

*agr*III, respectively. Clone ST612-MRSA-IV has been described as an infrequent clone isolated only in South Africa and Australia (Moodley *et al.,* 2010; Jansen van Rensburg *et al.,* 2011). Based on the genetic variation shown by PFGE and the number of *spa* types identified for ST612-MRSA-IV Jansen van Rensburg *et al.* (2011) suggested that: (i) it was an old clone, which has accumulated genetic variation over time and (ii) has undergone clonal expansion.

Isolate 71, which was the only PVL positive isolate from the 193 isolates analysed was identified as ST22/CC22 (7-6-1-5-8-8-6) and belonged to PFGE A3-SCC*mec*V-*spa* type t891/ClusterD-*agr*I. The low prevalence of the PVL gene [0.5% (1/194)] detected in this study is in agreement with a previous national study conducted by Moodley *et al.* (2010) during August 2005 to November 2006 in South Africa. The PVL positive MRSA detected by Moodley *et al.* (2010) was *spa* type t891/ST22/CC22 carrying the SCC*mec* type IV. Salam-Dreyer (2010), reported PVL positive MSSA with *spa* type t891 in South Africa. The SCC*mec* types IV and V are often found in strains of CA-MRSA (Deurenberg and Stobberingh, 2008). Nadiq *et al.* (2010) reported the increase in SCC*mec* type IV and V and a decrease in SCC*mec* type III MRSA strains between 2006 to 2009 in Mumbai, India. Sequence type ST22/CC22 was initially isolated in the southeast of England in 1991 and referred to as the epidemic MRSA-15 (EMRSA-15) strain (Holden *et al.,* 2013). The spread of the strain was rapid in the UK and accounted for 60% of HA-MRSA bacteraemia in England in 2000 (Holden *et al.,* 2013). Large nosocomial outbreaks of PVL positive ST22/CC22 have been reported in other parts of the world such as Germany, Ireland, India and Hong Kong (Monecke *et al.,* 2011). Sequence type 22-MRSA-IV is also known as the UK-EMRSA-15, Irish AR06, Barnim Epidemic strain or Spanish type E13, which has spread worldwide (Monecke *et al.,* 2011). Sporadic cases of PVL negative ST22/CC22 have been reported in Saxony and South Africa (Moodley *et al.,* 2010; Monecke *et al.,* 2011). The presence of EMRSA-15 in epidemiologically unrelated settings suggests a polyphyletic origin, given that PVL-positive CC22-methicillin sensitive *S. aureus* (MSSA) are widespread (Monecke *et al.,* 2011). Studies have shown that ST22-MRSA has frequently demonstrated its ability to succeed and replace other established MRSA clones (Hsu *et al.,* 2005; Amorim *et al.,* 2007; Witte *et al.,* 2008).

Infections caused by MRSA continue to be a problem, with the dissemination of HA-MRSA in the community and the increase in reports of CA-MRSA causing healthcare-associated

infections (Marchese *et al.,* 2009). In addition ST22/CC22 have been reported in healthcare and community settings as well as in animals (horses, cats and dogs) (Monecke *et al.,* 2011). The PVL positive ST22 identified in this study belonged to *agr* group I, which is in agreement with studies that has reported *agr* I positive ST22 (Tristan *et al.,* 2007; Nadiq *et al.,* 2010). The possession of the PVL gene by ST22/CC22 (EMRSA-15) enhances the virulence of this clone that is already highly transmissible and poses an important health concern worldwide (Boakes *et al.,* 2010).

Isolate 183 identified as ST36/CC30 (2-2-2-2-3-3-2) belonged to PFGE J-SCC*mec*II-*spa* type t012/Cluster I-*agr*I and III. The ST36 is also referred to as EMRSA-16 (USA200) and has been reported in Australia, Austria, Belgium and Germany (Deurenberg and Stobberingh, 2008). Moodley *et al.* (2010) and Jansen van Rensburg *et al.* (2011) reported ST36/CC30 in Cape Town and the Pretoria region in South Africa. Sequence type 36 is one of the most predominant nosocomial clones circulating in the UK (Deurenberg and Stobberingh, 2008). In the 1990s, CC30 was mostly isolated in the UK and Ireland (Monecke *et al.,* 2011). Although, it has been reported that CC30 has become increasingly rare worldwide, it is still being reported in Malta and South Africa (Monecke *et al.,* 2011).

The *tpi* gene for isolate 125 had a unique allele number (240) (2-3-1-1-4-240-3). Hence, the ST was not found on the MLST database (http://saureus.mlst.net/sql/multiplelocus.asp), though the allelic profile of the other six housekeeping genes (*arc*, *aro*E*, glp*F, *gmk, pta* and *yqi*L) (2-3-1-1-4-?-3) for this isolate was similar to that of ST239 (2-3-1-1-4-4-3). Further molecular investigation showed that isolate 125 was a mix culture consisting of *S. aureus* and *S. haemolyticus.* The allelic number for the *yqi*L gene for isolate 2 was not found on the MLST database (http://saureus.mlst.net/sql/multiplelocus.asp), (3-3-1-1-4-88-?). However, the six allelic numbers of the *arc*, *aro*E*, glp*F, *gmk, pta* and *tpi* genes of isolate 2 were similar to that of ST612, which is 3-3-1-1-4-88-83. The *spa* type of isolate 2, which was t1257, corresponded to those of isolates which belonged to ST612 in this study. The allele numbers for the *arc*, *aro*E and *pta* genes for isolates 134 and 165 were not found on the MLST database. These allele numbers could be new ones of sequence types yet to be described.

3.6 CONCLUSION

This study showed that the combination of different genotyping assays, such as *spa, agr* and MLST typing can accurately be used to determine the genetic diversity of MRSA strains. In comparison to PFGE, these PCR-based typing assays (*spa, agr* and MLST) were rapid and less labour intensive. The *spa* typing method in this study showed a high discriminatory power compared to the PFGE typing method used in the previous study on the same clinical isolates. The high prevalence of *agr* I, which is a molecular marker for community-associated MRSA, could suggest the possibility of healthcare-associated MRSA with community onset. Though, it has been reported that there is no obvious pattern associated with the distribution of *agr* allotypes among MRSA lineages (Wright *et al.,* 2005; Diep *et al.,* 2006). The sequence types and *spa* types identified in this study by MLST and *spa* typing are clones of MRSA that have been previously reported in South Africa and worldwide. Three of the sequence types ST22/CC22, ST36/CC30, ST239/CC8 identified in this study are among the predominant HA-MRSA MLST clonal complexes reported worldwide (Monecke *et al,* 2011). The prevalence of these HA-MRSA clones suggests that some MRSA strains have unique abilities to survive in hospital environments and once established in healthcare settings are difficult to eradicate. The fourth sequence type (ST612/CC8) identified in this study has been described as an infrequent clone isolated in South Africa and Australia. The detection of a pandemic clone ST239/CC8 and an epidemic clone ST22/CC22 harbouring the PVL gene is alarming. Hence, it is important to implement strict infection control measures and conduct surveillance to prevent the outbreak of these clones in this clinical setting.

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Table 3.1: Oligonucleotide sequences of the primers used in the M-PCR assay for the detection of the 16S rRNA, *mec*A and PVL genes of the MRSA isolates (McClure *et al*., 2006)

Primer	Oligonucleotide Sequence (5'- 3')	Target gene	Amplicon Size (bp)	Conc (µM)
Staph 756F Staph 756R	-AACTCTGTTATTAGGGAAGAACA- -CCACCTTCCTCCGGTTTGTCACC-	16S rRNA	756	0.2
MecA1-F MecA2-R	-GTAGAAATGACTGAACGTCCGATAA- -CCAATTCCACATTGTTTCGGTCTAA-	*mec*A	310	0.2
Luk-PV-1F Luk-PV-2R	-ATCATTAGGTAAAATGTCTGGACATGATCCA- - GCATCAAGTGTATTGGATAGCAAAAGC-	*lukS/*F-PV	433	0.2

Table 3.2: Oligonucleotide sequences of primers used for the *spa* typing of MRSA isolates (Schmitz *et al.,* 1998)

Primer	Oligonucleotide sequence 5'- 3'	Concentration	Position
SPA 1	-GATTTTAGTATTGCAATACATAATTCG-	0.4 µM	114-140
SPA 2	-CCACCAAATACAGTTGTACCG-	0.4 µM	1702-1682
SPA 3	-CTTTGGATGAAGCCGTTGCGTTG-	0.4 µM	1088-1066

Table 3.3: Oligonucleotide sequences of primers used for the *spa* sequencing of MRSA isolates (Larsen *et al.,* 2008)

Primer	Oligonucleotide sequence 5'- 3'	Concentration
1095F	-AGACGATCCTTCGGTGAGC-	0.25 µM
1517R	-GCTTTTGCAATGTCATTTACTG-	0.25 µM

Table 3.4: Oligonucleotide sequences of primers used for the *agr* typing of MRSA isolates (Shopsin *et al.,* 2003)

Primer	Oligonucleotide sequence 5'-3'	Target gene	Amplicon size (bp)	Conc (µM)
Pan-*agr*B (F)	-ATGCACATGGTGCACATGC-	*agr*B	-	0.2
agr I (R)	-GTCACAAGTACTATAAGCTGCGAT-	*agr*D	440	0.2
agr II (R)	-GTATTACTAATTGAAAAGTGCCATAGC-	*agr*C	572	0.2
agr III (R)	-CTGTTGAAAAAGTCAACTAAAAGCTC-	*agr*D	406	0.2
agr IV (R)	-CGATAATGCCGTAATACCCG-	*agr*C	588	0.2

Table 3.5: Oligonucleotide sequences of primers used for the MLST typing of selected MRSA isolates (Enright *et al.,* 2000)

Primer	Oligonucleotide Sequence 5'-3'	Target gene	Amplicon Size (bp)	Conc (µM)
arcC-fd *arcC*-Rv	-TTGATTCACCAGCGCGTATTGTC- -AGGTATCTGCTTCAATCAGCG-	Carbamate kinase (*arc*C)	500	0.5
aroE-fd *aroE*-Rv	-ATCGGAAATCCTATTTCACATTC- -GGTGTTGTATTAATAACGATATC-	Shikimate dehydrogenase (*aro*E)	500	0.5
glpF-fd *glpF*-Rv	-TGGTAAAATCGCATGTCCAATTC- -CTAGGAACTGCAATCTTAATCC-	Glycerol kinase (*glp*F)	500	0.5
gmk-fd *gmk*-Rv	-ATCGTTTTATCGGGACCATC- -TCATTAACTACAACGTAATCGTA-	Guanylate kinase (*gmk*)	500	0.5
pta-fd *pta*-Rv	-GTTAAAATCGTATTACCTGAAGG- -GACCCTTTTGTTGAAAAGCTTAA-	Phosphate acetyltransferase (*pta*)	500	0.5
tpi-fd *tpi*-Rv	-TCGTTCATTCTGAACGTCGTGAA- -TTTGCACCTTCTAACAATTGTAC-	Triosephosphate isomerase (*tpi*)	500	0.5
yqiL-fd *yqiL*-Rv	-CAGCATACAGGACACCTATTGGC- -CGTTGAGGAATCGATACTGGAAC-	Acetyl coenzyme A acetyltransferase (*yqi*L)	500	0.5

Table 3.6: Ridom sequence *spa* types, sequence types and geographical distribution of *spa* types identified in this study (http://spaserver.ridom.de)

Spa types	Geographical Distribution	Ridom sequence of *spa* types	Sequence type
t012	Australia, Belgium, Canada, Cyprus, Denmark, Finland, France, Germany, Iceland, Italy, Jordan, Latvia, Lebanon, Netherlands, New York, Norway, Poland, SA, Spain, Sweden, Switzerland, UK and USA	15-12-16-02-16-02-25-17-24-24	ST36
t037	Australia, Belgium, Bulgara, Canada, China, Croatia, Denmark, France, Germany, Iceland, Italy, Jordan, Latvia, Lebanon, Malaysia, Netherlands, New York, Norway, Poland, SA, Spain, Sweden, Switzerland, Taiwan and UK	15-12-16-02-25-17-24	ST239
t891	Denmark, Finland, Germany, Norway, SA, Sweden and Switzerland	26-23-13-23-31-05-17-25-17-25-28	ST22
t1257	Denmark, Germany, Norway and SA	11-19-34-05-17-34-24-34-22-25	ST612

Table 3.7: Distribution of the *agr* groups among the *spa* clusters identified from the dendrogramme

***agr* groups**	***spa* CLUSTERS**												**OUTLIERS FROM *spa* DENDOGRAMME**	**UNTYPEABLE BY *spa* TYPING**
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**		
agr I 84.4% (163/193)	24.0% (39/163)	22.1% (36/163)	3.1% (5/163)	10.4% (17/163)	13.4% (22/163)	1.8% (3/163)	9.8% (16/163)	4/163, (2.4%)	-	-	4.9% (8/163)	2.5% (4/163)	2.5% (4/163)	3.1% (5/163)
agr II 4.7% (9/193)	11.1% (1/9)	44.4% (4/9)	-	-	11.1% (1/9)	-	-	-	-	-	11.1% (1/9)	11.1% (1/9)	-	11.1% (1/9)
agr III 7.3% (14/193)	21.0% (3/14)	29.0% (4/14)	7.0% (1/14)	-	-	-	-	-	7.0% (1/14)	29.0% (4/14)	7.0% (1/14)	-	-	-
agr I and III 3.6% (7/193)	14.3% (1/7)	-	-	14.3% (1/7)	28.5% (2/7)	-	-	-	28.5% (2/7)	14.3% (1/7)	-	-	-	-
agr IV	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 3.8: Vitek2 automated system (bioMérieux, Mary l'Etoile, France) antimicrobial susceptibility testing (AST) for the ten representative isolates for *spa* sequencing and MLST typing

	ISOLATE NUMBER	VITEK2 AUTOMATED SYSTEM (bioMérieux, Mary l'Etoile, France) RESULT	RESISTANT (R) ANTIBIOTICS (μg/ml)	SUSCEPTIBLE (S) ANTIBIOTICS (μg/ml)	INTERMIDIATE (I) ANTIBIOTICS (μg/ml)
1	2	MRSA	Clindamycin (≥ 8), Erythromycin (≥ 8), Fusidic Acid (≥ 32), Oxacillin (≥ 4), Rifampicin (≥ 32), Teicoplanin (≥ 32), Tetracycline (8) and Vancomycin (≥ 32)	Ciprofloxacin (≤ 0.5), Gentamicin (≤ 0.5), Moxifloxacin (≤ 0.25), Tigecycline (0.5) and Trimethoprim-Sulfamethoxazole (≤ 10)	
2	71	MRSA	Clindamycin (≥ 8), Erythromycin (≥ 8), Fusidic Acid (≥ 32), Gentamicin (≥ 16), Rifampicin (≥ 32), Teicoplanin (≥ 32), Tetracycline (≥ 16), Trimethoprim-Sulfamethoxazole (≥ 320), Oxacillin (≥ 4) and Vancomycin (≥ 32)	Ciprofloxacin (≤ 0.5), Moxifloxacin (≤ 0.25) and Tigecycline (0.5)	
3	100	MRSA	Ciprofloxacin (≥ 8), Clindamycin (≤ 0.25), Erythromycin (≥ 8), Gentamicin (≥ 16), Tetracycline (≥ 16), Trimethoprim-Sulfamethoxazole (≥ 320), Oxacillin (≥ 4) and Rifampicin (≥ 32)	Fusidic Acid (≤ 0.5), Linezolid (1), Moxifloxacin (1), Mupirocin (≤ 2), Teicoplanin (≤ 0.5), Tigecycline (≤ 0.12) and Vancomycin (1)	
4	125	MRSA	Ciprofloxacin (≥ 8), Clindamycin (≤ 0.25), Erythromycin (≥ 8), Gentamicin (≥ 16), Moxifloxacin (≥ 8), Tetracycline (≥ 16), Trimethoprim-Sulfamethoxazole (≥ 320), Oxacillin (≥ 4) and Rifampicin (≥ 32),	Linezolid (2), Mupirocin (≤ 2), Teicoplanin (2), Tigecycline (0.5) and vancomycin (2.0)	Fusidic Acid (4)
5	131	MRSA	Ciprofloxacin (≥ 8), Clindamycin (≤ 0.25), Erythromycin (≥ 8), Gentamicin (≥ 16), Moxifloxacin (2), Oxacillin (≥ 4), Tetracycline (≥ 16) and Trimethoprim-Sulfamethoxazole (≥ 320)	Fusidic Acid (≤ 0.25), Linezolid (2), Rifampicin (≤ 0.5), Teicoplanin (≤ 0.5), Tigecycline (0.25) and Vancomycin (2)	

Table 3.8: Vitek2 automated system (bioMérieux, Mary l'Etoile, France) antimicrobial susceptibility testing (AST) for the ten representative isolates for *spa* sequencing and MLST typing (continued)

	ISOLATE NUMBER	VITEK2 AUTOMATED SYSTEM (bioMérieux, Mary l'Etoile, France) RESULT	RESISTANT (R) ANTIBIOTICS (μg/ml)	SUSCEPTIBLE (S) ANTIBIOTICS (μg/ml)	INTERMIDIATE (I) ANTIBIOTICS (μg/ml)
6	133	MRSA	Ciprofloxacin (≥ 8), Clindamycin (≤ 0.25), Erythromycin (≥ 8), Gentamicin (≥ 16), Oxacillin (≥ 4) , Tetracycline (≥ 16) and Trimethoprim-Sulfamethoxazole (≥ 320)	Fusidic Acid (≤ 0.5), Linezolid (2) , Moxifloxacin (1), Rifampicin (≤ 0.5), Teicoplanin (≤ 0.5) , Tigecycline (0.25) and Vancomycin (≤ 0.5)	
7	134	MRSA	Clindamycin (≤ 0.25), Erythromycin (≥ 8) , Gentamicin (≥ 16) , Oxacillin (≥ 4), Tetracycline (≥ 4) and Trimethoprim-Sulfamethoxazole (80)	Ciprofloxacin (≤ 0.5), Fusidic Acid (≤ 0.5), Linezolid (1), Moxifloxacin (≤ 0.25), Mupirocin (≤2), Teicoplanin (2), Tigecycline (0.25) and Vancomycin (2)	
8	143	MRSA	Ciprofloxacin (≥ 8), Clindamycin (≤ 0.25), Erythromycin (≥ 8), Gentamicin (≥ 16), Oxacillin(≥ 4) , Rifampicin (≥ 32), Tetracycline (≥ 16) and Trimethoprim-Sulfamethoxazole (≥ 320)	Fusidic Acid (≤ 0.5), Linezolid (2), Moxifloxacin (≤ 2) , Mupirocin (≤ 2) , Teicoplanin (≤ 0.5),, Tigecycline (≤ 0.12) and Vancomycin (≤ 0.5)	
9	165	MRSA	Clindamycin (≤ 0.25), Erythromycin (≥ 8), Gentamicin (≥ 16), Oxacillin (≥ 4) , Rifampicin (≥ 32),Tetracycline (2) and Trimethoprim-Sulfamethoxazole (160)	Linezolid (1), Moxifloxacin (0.5),Teicoplanin (8), Tigecycline (≤ 0.12) and Vancomycin (2)	Ciprofloxacin (2) and Fusidic Acid (4)
10	183	MRSA	Ciprofloxacin (≥ 8), Clindamycin (≥ 8), Erythromycin (≥ 8) , Moxifloxacin (≥ 8) and Oxacillin (≥ 8)	Fusidic Acid (≤ 0.25) , Gentamicin (≤ 0.5), Linezolid (≥ 8), Tetracycline (≤ 1), Teicoplanin (2), Tigecycline (≤ 0.5), Rifampicin (≤ 0.5) and Vancomycin (≤ 0.0.5)	

Table 3.9: Results of the ten representative isolates for MLST typing showing the presence of the PVL gene, PFGE pulsotypes, *spa* cluster and subcluster, *spa* types, *agr* groups, sequence types (ST), allelic profiles, clonal complexes (CCs) and the distribution of the sequence types identified worldwide

ISOLATE NUMBER	PVL	*spa* CLUSTER	*spa* SUBCLUSTER	*spa* sequencing types	*agr* GROUP	PFGE	MLST typing			WORLDWIDE DISTRIBUTION
							ST	Allelic Profile	CC	
2	Neg	A	A1	NA	I	E	NA	3-3-1-1-4-88-?		NA
71	Pos	D	D1	891	I	A3	22	7-6-1-5-8-8-6	22	Europe, Indonesia, SA and Canada
100	Neg	A	A5	1257	I	A6	612	3-3-1-1-4-88-83	8	SA and Austraila
125	Neg	NT	NT	037	I	NT	NT	2-3-1-1-4-240-3	NT	NA
131	Neg	B	B4	037	III	A	239	2-3-1-1-4-4-3	8	Asia, Austraila, SA, South America and Europe
133	Neg	B	B1	037	I	A	239	2-3-1-1-4-4-3	8	Asia, Austraila, SA, South America and Europe
134	Neg	NT	NT	NA	I	B2	NT	?-?-1-1-?-4-83-	NT	NA
143	Neg	A	A1	1257	III	B	612	3-3-1-1-4-88-83	8	SA and Austraila
165	Neg	NT	NT	NA	I	I	NT	?-3-1-1-?-4-3	NT	NA
183	Neg	I	I	012	I, III	J	36	2-2-2-2-3-3-2	30	USA, UK, Austraila, Canada and SA

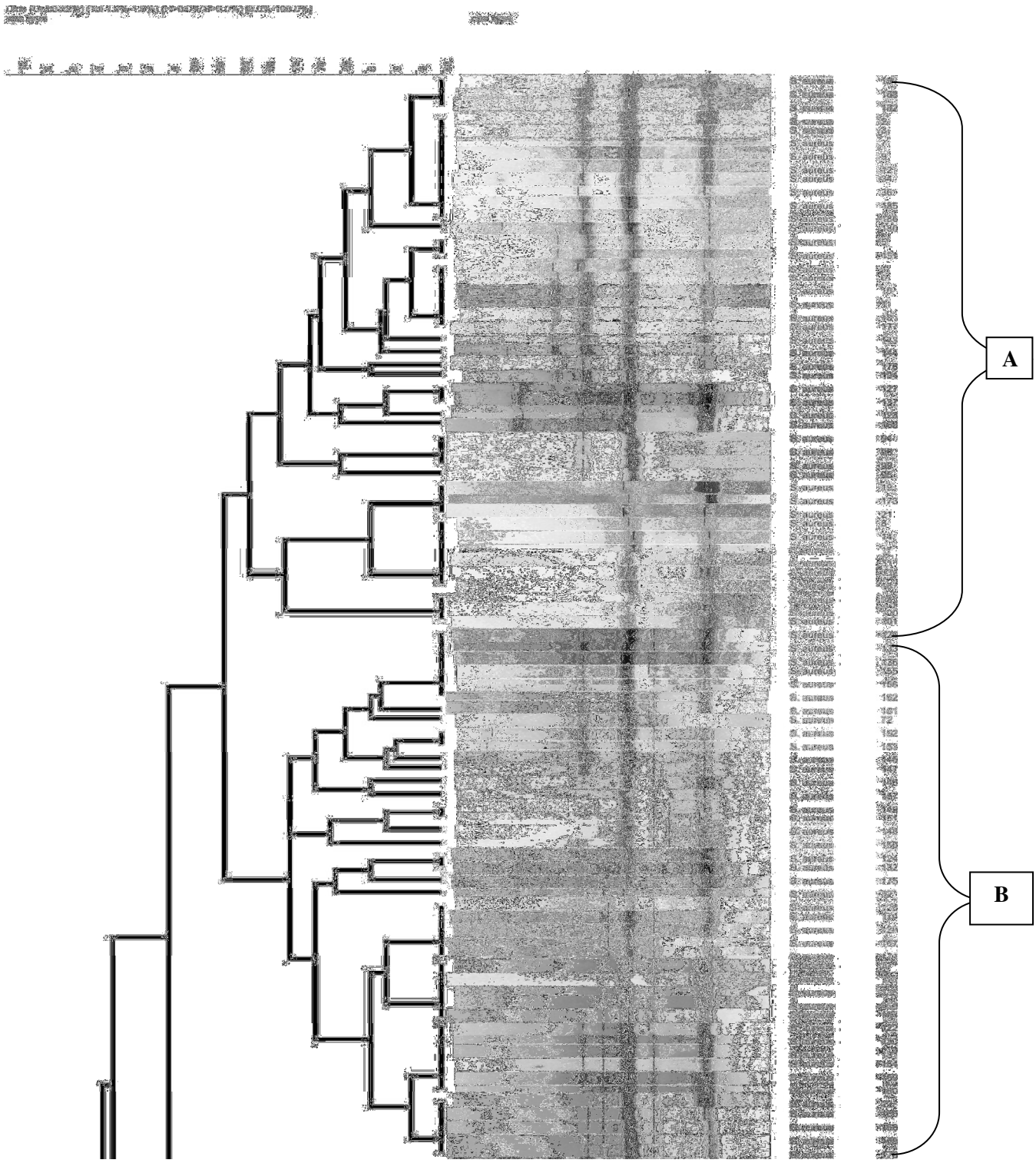

Figure 3.1: **Dendogramme obtained for *spa* typing, depicting clonal relatedness of the 187 typeable (Clusters A to L) MRSA clinical isolates obtained from the Steve Biko Academic Hospital, Gauteng, South Africa**

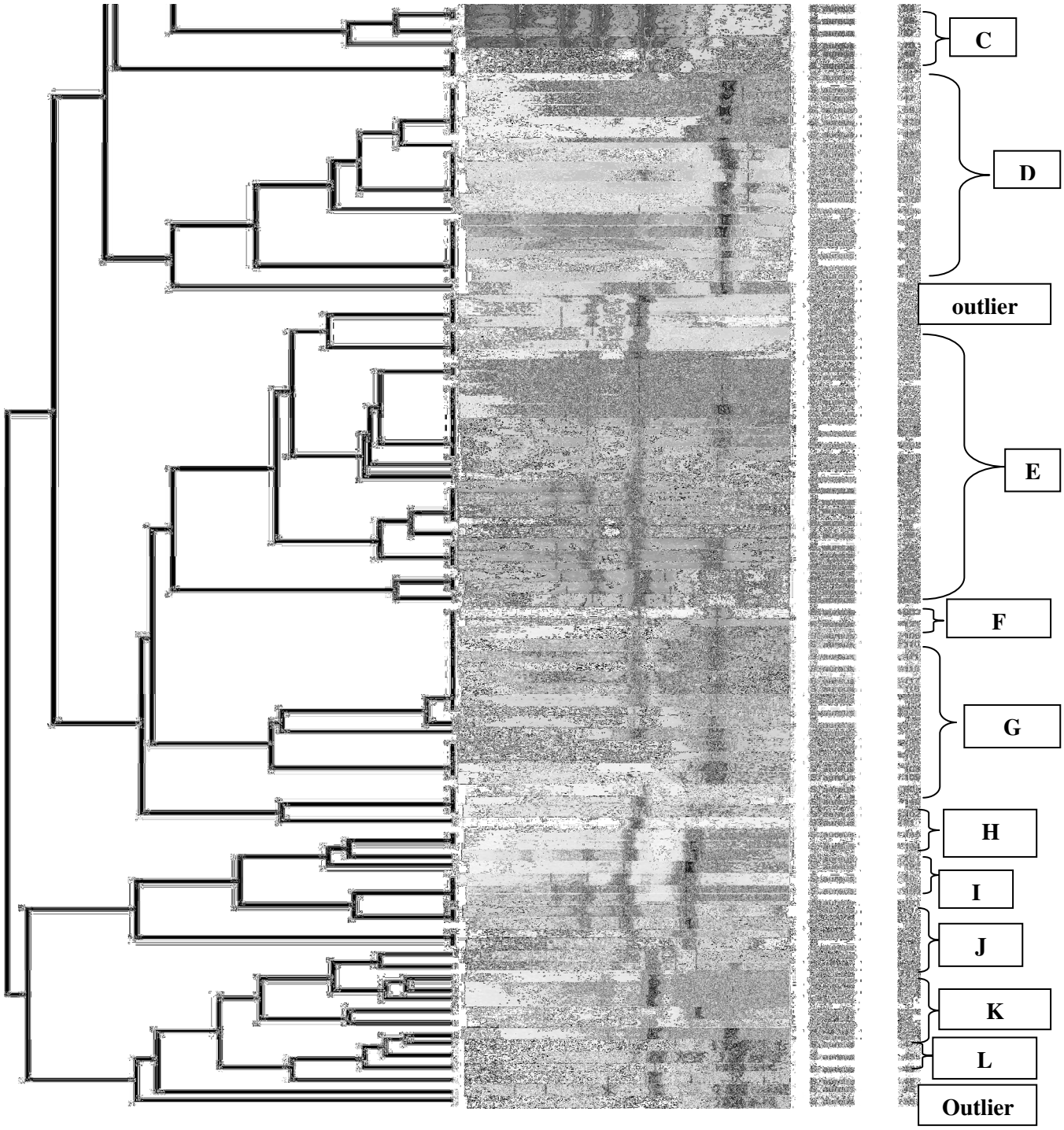

Figure 3.1: **Dendogramme obtained for *spa* typing, depicting genetic diversity of the187 typeable (Clusters A to L) clinical MRSA isolates obtained from Steve Biko Academic Hospital, Gauteng, South Africa (continued)**

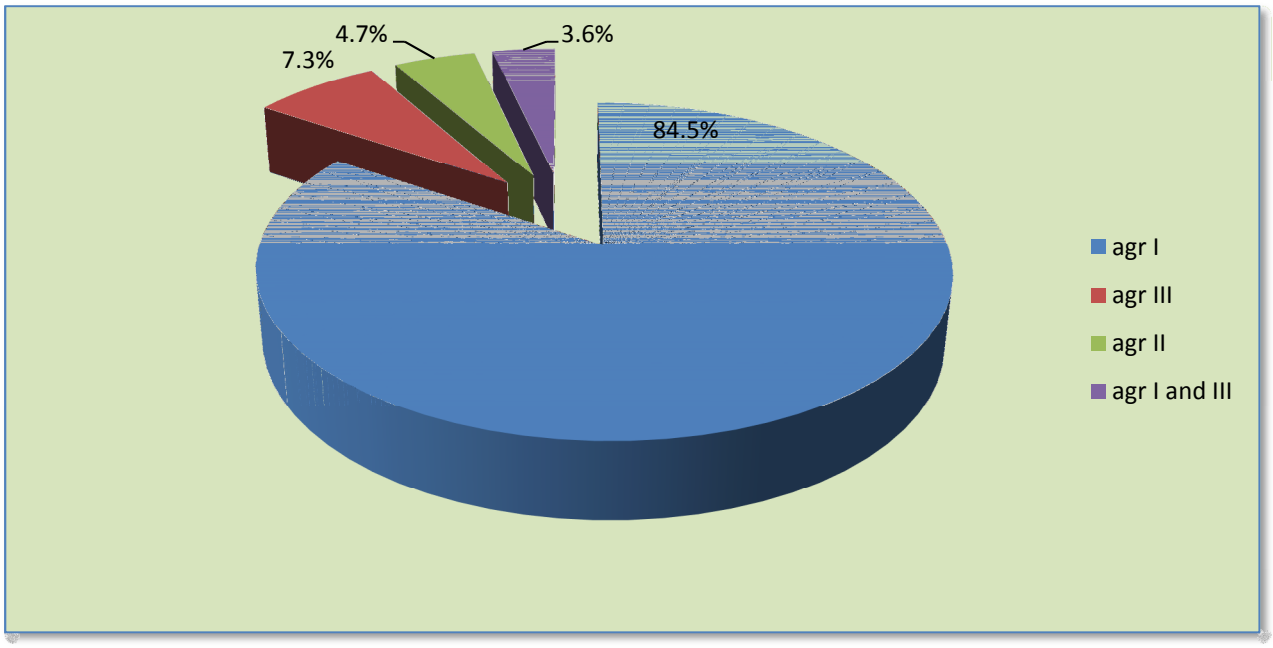

Figure 3.2: **Pie chart representing the *agr* groups detected in 193 clinical MRSA isolates analysed**

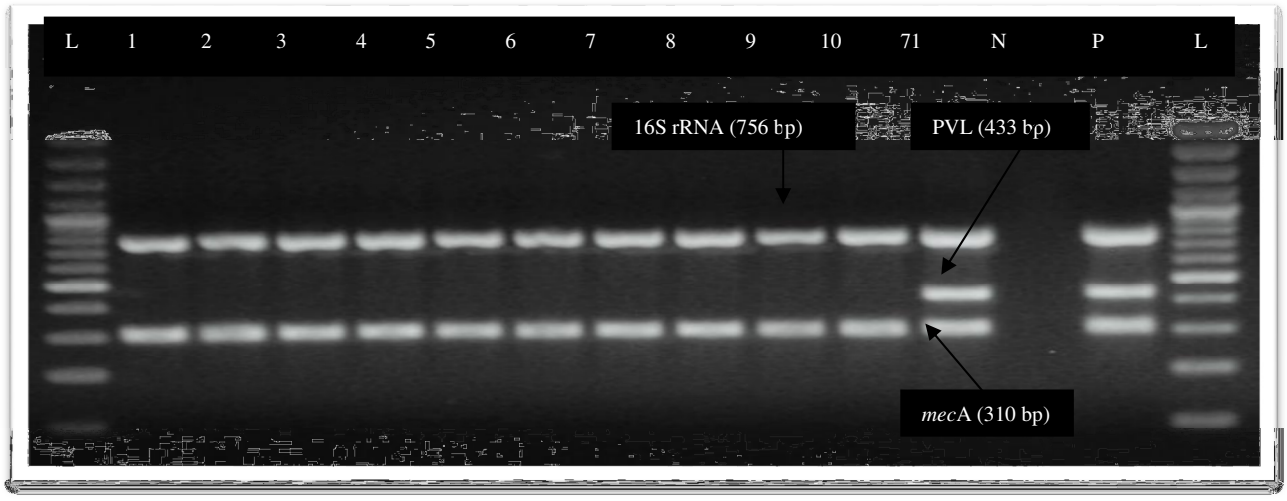

Figure 3.3: **Gel electrophoresis of the M-PCR assay for the detection of the 16S rRNA, *mec*A and PVL genes of the MRSA isolates. Lanes 1 to 10 show the 16S rRNA (756 bp) and *mec*A (310 bp). Lane 71 shows the 16S rRNA (756 bp), *mec*A (310 bp) and PVL (433 bp) genes. N= negative control, P= positive control and L = 100 bp ladder**

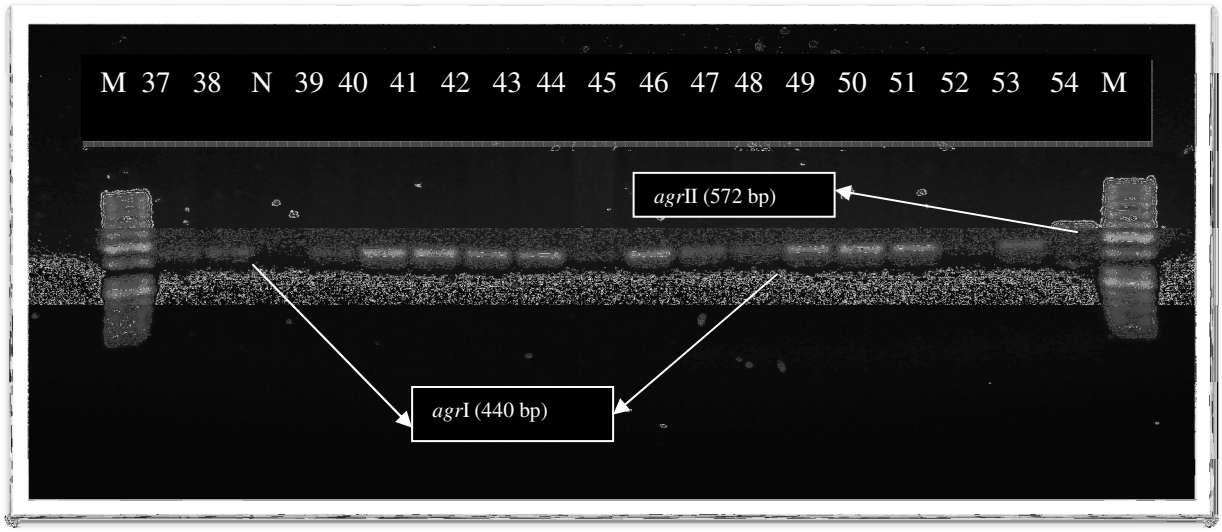

Figure 3.4: **Gel electrophoresis results of the duplex PCR assay for the detection of *agr* groups I and II. Isolates 37 to 53 belonged to *agr* group I (440 bp *agr* group I gene), while isolate 54 belonged to *agr* group II (570 bp *agr* group II gene). N= Negative control, M= 50 bp molecular marker (Thermo Scientific, USA)**

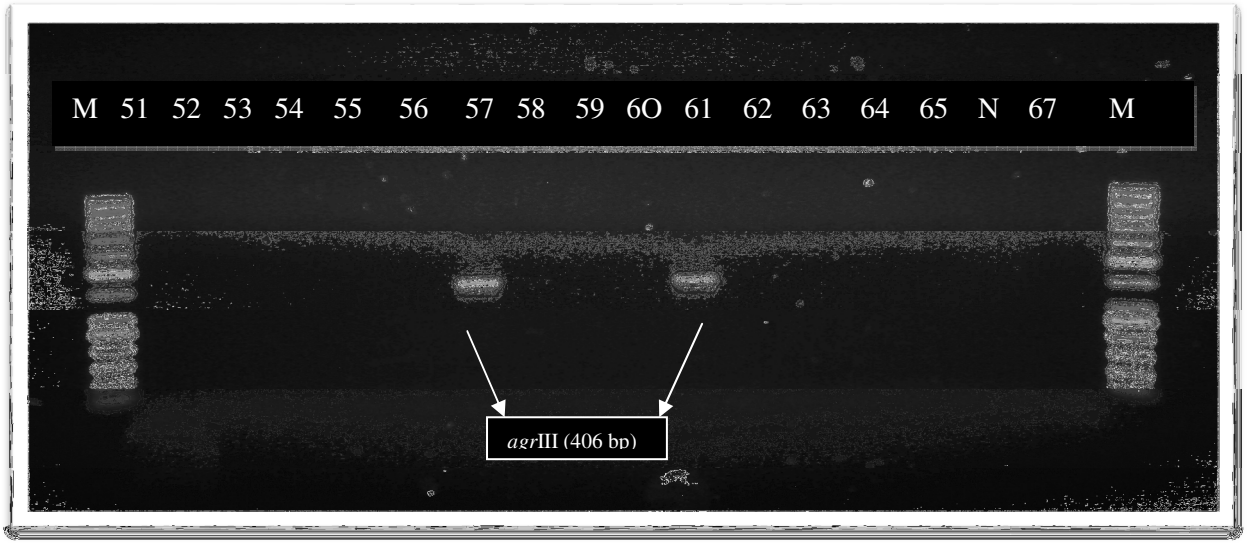

Figure 3.5: **Gel electrophoresis results of duplex PCR assay for the detection of *agr* groups III and IV. Isolates 56 and 61 belonged to *agr* group III (406 bp *agr* group III gene). None of the isolates harboured the *agr* group IV. N =Negative control, M = 50 bp molecular marker (Thermo Scientific, USA)**

CHAPTER 4

CONCLUSIONS

4.1 Concluding remarks

Globally, methicillin resistant *Staphylococcus aureus* (MRSA) is considered to be the main cause of healthcare and community-associated MRSA infections (Köck *et al.,* 2010). The bacterium is important due to the heavy burden of disease it causes, its evolution and the global spread of multi-drug resistant clones (Cookson *et al.,* 2007). Methicillin resistant *S. aureus* was first discovered in hospitals and considered as a hospital pathogen (Stefani *et al.,* 2012). The discovery of MRSA in the community (CA-MRSA) and livestock (LA-MRSA) without healthcare-associated risk factors means MRSA infections are not exclusively confined to the healthcare system (Stefani *et al.,* 2012).

Most clinical laboratories make use of different phenotypic tests for the detection of methicillin resistance in staphylococci (John *et al.,* 2009). However, the results from phenotypic tests may take 1 to 5 days to be reported, with obvious consequences for identifying MRSA carriage and infection control (Harbarth *et al.,* 2011). This could allow colonised patients up to five days to transmit infection to other patients before they are placed in isolation (Harbarth *et al.,* 2011). Molecular techniques have the advantage of a quicker turn-around time, infections can be diagnosed rapidly and patients treated accordingly (Strommenger *et al.,* 2008). Presently, there are different molecular genotyping techniques, which can be used to identify MRSA strains in the laboratories (Strommenger *et al.,* 2008). Rapid and easy molecular genotyping techniques are those based on PCR methods (Malachowa *et al.,* 2005). Though these molecular genotyping techniques are not routinely done due to the high cost involved, genotyping methods are important in cases of outbreaks to identify the specific clones.

Three different PCR-based assay namely staphylococcal protein A (*spa*) typing, accessory gene regulator (*agr*) typing and multilocus sequence typing (MLST) were utilized in this study, to detect, identify and characterise 194 MRSA clinical isolates. Pulsed-field gel electrophoresis and SCC*mec* typing were used in a previous study to determine the clonal relatedness of these same clinical MRSA isolates. The *spa* typing assay used in the current

study was easy, less tedious and showed a high discriminatory power compared to the PFGE technique. Although the PFGE typing technique was able to determine the clonal relatedness of the MRSA strains with only two isolates being untypeable (Isolates 125 and 170), the method was found to be technically demanding and labour-intensive as previously reported by Chung *et al.* (2012).

In the current study, all isolates were typeable with *agr* typing with the exception of one isolate, which was contaminated. Acessory gene regulator groups I, II and III were identified in this study. No *agr* group IV, which is common among exfoliatin producing strains was identified in this study. Different studies have reported that the absence of *agr* group IV (associated with staphylococcal scalded skin syndrome) suggest the fact that competition does not favour this MRSA strain (Shopsin *et al.,* 2003).

Groupings based on multilocus sequence typing (MLST) and *spa* sequencing is the current terminology used to describe the lineages of MRSA (Stefani *et al.,* 2012). The MLST typing and *spa* sequencing methods used for ten representative isolates in this study identified clones of MRSA, t037-ST239/CC8, t1257-ST612/CC8, t891-ST22/CC22, t012-ST36/CC30 which have been reported worldwide and in South Africa (Jansen van Rensburg *et al.,* 2011; Moodley *et al.,* 2010). The *tpi* gene for isolate 125 had a unique allele number (240) (2-3-1-1-4-240-3), which makes it a new allelic profile with a new ST yet to be identified on the MLST database (http://saureus.mlst.net/sql/multiplelocus.asp), though the allelic profile of the other six housekeeping genes (*arc*, *aro*E*, glp*F, *gmk, pta* and *yqi*L) (2-3-1-1-4-?-3) for this isolate was similar to that of ST239 (2-3-1-1-4-4-3). The allelic number for the *yqi*L gene of isolate 2 was not found on the MLST database (http://saureus.mlst.net/sql/ multiplelocus.asp), (3-3-1-1-4-88-?). The allele numbers for the *arc*, *aro*E and *pta* gene for isolates (134 and 165) were not found on the MLST database. These allele numbers could be new emerging MRSA strains and need to be further investigated using whole genome sequencing.

Different studies have reported sequence types, ST22/CC22, ST36/CC30 and ST239/CC8 as the major HA-MRSA clones found worldwide (Deurenberg and Stobberingh 2008; Otter and French, 2008). The successful dissemination of these clones worldwide and in South Africa has been reported to be due to historic emigration and the tourism between different countries (Jansen van Rensburg *et al.,* 2011). The only PVL positive isolate in this study was

identified as t891-ST22/CC22. Sequence type ST612, a member of CC8 has only been described previously in South Africa and Australia (Jansen van Rensburg *et al.,* 2011).

In this study, the MLST typing assay identified the sequence types of six out of the ten isolates, while *spa* sequencing identified the spa types of eight of the ten isolates. Staphylococcal protein A sequencing was more rapid than the MLST typing assay. This is because only one gene is targeted with *spa* sequencing whereas seven genes are targeted with MLST typing, which also makes this typing technique more expensive. Nevertheless, the three PCR based techniques used in this study [i.e *spa* typing (PCR assay and sequencing), *agr* typing and MLST typing] were rapid and less labour intensive as compared to PFGE typing. Although, MLST typing is more expensive, this typing technique allowed the identification of the sequence types circulating in this clinical setting. However, the typing of all isolates in the routine diagnostic laboratory is not feasible due to common financial restrictions. It is suggested, that where possible, clinical MRSA isolates be typed on a regular basis for large clinical settings to determine which MRSA strains are currently circulating. This will enable infection control personnel and clinicians to monitor any changes in the genetic diversity of circulating strains. In this study, *spa* typing seemed to be a suitable method in terms of cost, speed, eases of use, interpretation and standardisation as well as a complementary tool, which provided additional epidemiological value to PFGE typing (Moodley *et al.,* 2006).

4.2 Future research

The main burden of MRSA disease in South Africa continues to grow with the occurrence of identical MRSA strains across different provinces, which shows the interhospital spread and clonal nature of MRSA. Future research should be directed towards (i) detection of MRSA isolates, (ii) effective coordination of healthcare workers regarding infection control measures, (iii) immediate isolation and proper care of infected patients, (iv) the correct use of antimicrobial agents (antibiotic stewardship) and (v) the continuous surveillance of antimicrobial resistance patterns are all important for preventing the spread of MRSA (Gould *et al.,* 2010).

Further investigation on the genes and mechanisms of antimicrobial resistance of *S. aureus* is essential. This may give further insight into the pathogenesis of MRSA and current treatment

regimens can be modified accordingly to circumvent mechanisms of drug resistance. It is important to discriminate between methicillin sensitive *S. aureus* (MSSA) and MRSA for appropriate therapy and timely intervention of cross infection control (Bernardo *et al.,* 2002). The matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) has proven to be a rapid and effective technique for the rapid and simple discrimination of clonal strains of MRSA (Bernardo *et al.,* 2002). The MALDI-TOF MS might be useful for tracking epidemic outbreaks of both HA-MRSA and CA-MRSA (Bernardo *et al.,* 2002). New molecular methods, such as plasmid profiling techniques are being developed, which are expected to contribute immensely to genotyping techniques in the near future (Stefani *et al.,* 2012). The microarray-based analysis of 3 000 MRSA isolates from different parts of the world has shown potential for: (i) assigning isolates to lineages and (ii) allow for the analysis of the presence or absence of virulence and resistance genes, which are encoded on mobile genetic elements (MGEs) (Monecke *et al.,* 2011). The development of low-cost, high-throughput, bench top bacterial genome sequencing will facilitate diagnosis, resistance profiles and epidemiological typing within hours (Gray *et al.,* 2011). Whole-genome sequencing provides the necessary insights into the structure of the *S. aureus* genome and genetic predictors of virulence, compared to the conventional molecular methods such as *spa,* PFGE and MLST (Diep, 2012; Chua *et al,* 2013).

Staphylococci remain important nosocomial pathogens (Chua *et al.,* 2013). Continued future research on *Staphylococcus* genetics is, therefore, required to: (i) obtain a better understanding of bacterial evolution and (ii) to obtain insights into the various interactions between the host and the pathogen.

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APPENDIX A

Reagents and buffers used in the experimental procedures

1. Brain Heart Infusion Broth (BHI) (500 mℓ)

BHI (Merck, Darmstadt, Germany)	18.5	g
Distilled water	400	mℓ

500 mℓ/1000 X 37 g = 18.5 g in 500 mℓ

Dissolve 18.5 g of BHI broth in 400 mℓ of sterile distilled water, adjust volume to 500 mℓ. Autoclave at 121°C for 15 min

2. 50% Glycerol (1:1) solution (500 mℓ)

Glycerol (Merck, Darmstadt, Germany)	250	mℓ
Distilled water	250	mℓ

Add 250 mℓ of glycerol to 250 mℓ of sterile distilled water. Autoclave at 121°C for 15 min

3 Tris-boric EDTA (TBE) buffer 10X (pH 8.3) (500 mℓ)

Tris-base (Sigma-Aldrich, St. Louis, USA)	54	g
Boric acid (Merck, Darmstadt, Germany)	27.5	g
0.5 M EDTA (pH 8.0)	20	mℓ

Dissolve 54 g of Tris-base and 27.5 g of boric acid in 400 mℓ of sterile distilled water and add 20 mℓ of 0.5 M EDTA buffer. Adjust pH to 8.3 and bring volume to 500 mℓ. Autoclave at 121°C for 15 min

APPENDIX B

1. Confirmation of MRSA isolates and DNA extraction

a) MRSA isolates collected from the National Health Laboratory Service (NHLS) Diagnostic laboratory were subcultured on blood agar plates (Oxoid, England) to obtain single colonies for Gram-staining.
b) The blood agar plates (Oxoid, England) were incubated at 37°C for 18 h to 24 h.
c) Gram-staining was performed to confirm that the MRSA isolates were not contaminated.
d) A pure single colony was inoculated in 3 mℓ of 3.7% sterile BHI broth (Merck, Darmstadt, Germany) and incubated at 37°C for 18 h to 24 h.
e) Freeze cultures were prepared by transferring 750 µℓ of 50% sterile glycerol (Merck, Darmstadt, Germany) plus 750 µℓ of each of the broth cultures to a sterile 2 mℓ cryotube (BioExpress, Kaysville, USA).
f) The 2 mℓ cryotubes (BioExpress, Kayville, USA) were stored at -70°C (New Brunswick Scientific, England) for future use.

2. Deoxyribonucleic acid (DNA) extraction of MRSA isolates

a) Bacterial DNA was isolated with the ZR Fungal/Bacterial DNA kit (Zymo Research, USA)
b) One millilitre of the cell suspension in a BHI (Merck, Darmstadt, Germany) broth was taken after 48 h of incubation and transferred into a sterile 2 mℓ Eppendorf tube (Eppendorf AG, Hamburg, Germany) and centrifuged (Spectrafuge 24D, Labnet International, Inc., New Jersey, USA) at 4 930 *x g* for 15 min at 4°C to obtain a pellet.
c) Hundred microlitre of the pelleted bacterial cells were resuspended in 200 µℓ of phosphate buffer and placed in the ZR BashingBead^{dTM} Lysis Tube which contained 750 µℓ lysis solution was added to the tube (ZR BashingBea^{dTM} Lysis Tube).
d) The tube was vortexed (VELP Scientifica, Italy) for ± 5 min at maximum speed.
e) The ZR BashingBead Lysis Tube (Zymo Research, USA) was centrifuged in a Spectrafuge centrifuge (Labnet International, USA) at ≥10 000 *x g* for 1 min.

f) Four hundred microlitre (400 µl) of the supernatant was transferred to a Zymo-spin IV Spin Filter in a collection tube and centrifuged (Spectrafuge centrifuge, Labnet International, USA) at 4 500 *x g* for 1 min.

g) To the filtrate in the collection tube, 1 200 µl of bacterial DNA binding buffer was added.

h) A volume of 800 µl was transferred to a Zymo-Spin IIC column (Zymo Research, USA) in a collection tube and centrifuged (Spectrafuge centrifuge, Labnet International, USA) at 10 000 *x g* for 1 min.

i) The flow-through from the collection tube was discarded and the latter step repeated.

j) Two hundred microlitre of DNA Pre-Wash Buffer (Zymo Research, USA) was added to the Zymo-spin IIC column (Zymo Research, USA) in a new collection tube and centrifuged (Spectrafuge centrifuge, Labnet International, USA) at 10 000 *x g* for 1 min.

k) A volume of 500 µl of bacterial DNA wash buffer was added to the Zymo-spin IIC column (Zymo Research, USA) and centrifuged (Spectrafuge centrifuge, Labnet International, USA) at 10 000 *x g* for 1 min.

l) The Zymo-spin IIC column (Zymo Research, USA) was transferred to a clean 1.5 ml microcentrifuge tube and 100 µl DNA elution buffer (Zymo Research, USA) was added to the column matrix and centrifuged (Spectrafuge centrifuge, Labnet International, USA) at 10 000 *x g* for 30 s to elute the DNA.

m) The eluted ultra-pure DNA was stored at -20ºC until further analysis.

3. Analysis of all PCR assay amplicons

a) A 1% (M/V) Seakem® LE agarose gel (Lonza, Rockland, USA) was used for the multiplex-PCR for the 16S rRNA, *mec*A, PVL genes SCC*mec,* as well as the *agr* and single PCR for the house keeping genes for MLST analysis) and 3% (M/V) Seakem® LE agarose gel (Lonza, Rockland, USA) (*spa* typing) were prepared by dissolving 1 g and 3 g of Seakem® LE agarose powder (Lonza, Rockland, USA) respectively, in 100 mℓ of 1X TBE [45 mM Tris borate, (pH 8), 1 mM EDTA] (Sigma-Aldrich, St. Louis, USA).

b) The mixture was heated using a microwave oven (Defy, South Africa) and cooled at 50ºC in a Techne Hybridiser HB-1D oven (Techne Corporation, Cambridge, England) for 30 min.

c) Five microlitre of ethidium bromide (stock of 10 mg/ml) (Promega, Madison, USA) was added to the Seakem® LE agarose gel (Lonza, Rockland, USA) and the mixture was poured into a casting tray (Bio-Rad, California, USA) containing a 18 well comb, which was allowed to solidify for 1 h.

d) One litre of the 1X TBE buffer [45 mM Tris borate, (pH 8), 1 mM EDTA] (Sigma-Aldrich, St. Louis, USA) was poured into the electrophoresis chamber (Bio-Rad, California, USA) and the solidified gel was placed inside the electrophoresis chamber (Bio-Rad, California, USA).

e) The band sizes of the different genes obtained after PCR amplification were separated at 100 $V.cm^{-1}$ for 1 h 30 min.

f) A 50 and 100 bp molecular weight marker (Fermentas Life Sciences, Thermo Scientific, USA) was included as reference markers in the first and the last lane.

g) The gels were photographed and digitalised using a Ultra Violet light box (DigiDoc, UVP product, Upland, California).

4. Cleaning of amplicons after gel electrophoresis (Zymoclean™ Gel DNA Recovery Kit)

a) Twenty-four millilitre of 100% ethanol (Merck, Darmstadt, Germany) was added to the 6 ml of DNA wash buffer concentrate to obtain the final DNA wash buffer solution.

b) The DNA fragment was excised from the agarose gel (Lonza, Rockland, USA) using a razor blade and transferred to a 1.5 ml microcentrifuge tube (Labtech International, USA)

c) Three volumes of ADB solution (Zymo Research, USA) was added to the excised agarose gel.

d) The excised gel was incubated at 45^0C for 10 min until the gel slice was completely dissolved.

e) The dissolved agarose solution was transferred to a Zymo-Spin™ I Column in a Collection Tube.

f) The Collection Tube was centrifuged (Spectrafuge centrifuge, Labnet International, USA) at 10 000 *x g* for 60 s and the flow-through was discarded.

g) Two-hundred mililitre of the wash buffer was added to the column and centrifuged (Spectrafuge centrifuge, Labnet International, USA) at 10 000 *x g* for 30 s and the flow-through was discarded. The wash step was repeated.

h) RNase-free water (Qiagen, Germany) (6 µℓ) was added directly to the column matrix and the column was placed into a 1.5 ml tube. The column was centrifuged (Spectrafuge centrifuge, Labnet International, USA) at 10 000 *x g* for 1 min to elute the DNA.

i) The eluted ultra-pure DNA was stored at -20ºC until further analysis

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APPENDIX C: Clinical information of MRSA isolates and molecular results

Table 3.10: Combined results of the three PCR assays and (SCC*mec* and PFGE) used for the characterisation of the 194 MRSA isolates obtained from the Steve Biko Academic Hospital

MRSA isolates	Gender	Age	Clinical wards	Specimen Type	E-test	Vitek 2 System	*spa* cluster	*spa* subcluster	*spa* types (sequencing)	SCC*mec*	*agr* groups	PFGE	MLST	HA-MRSA or CA-MRSA
1	F	27 d	PS	Pus swab	NP	NP	A	A1	NA	I	II	A3	NA	HA-MRSA
2	M	35 y	Urology	BC	NP	NP	A	A1	t1257	IVd	I	E	NA	CA-MRSA
3	M	28 y	Neurosurgical	BC	NP	NP	A	A1	NA	SCC*mercury*	I	A	NA	HA-MRSA
4	M	28 y	Neurosurgical	BC	+	+	Cont	Cont	NA	II	Cont	Cont	NA	HA-MRSA
5	M	28 y	ST	BC	+	NP	A	A1	NA	SCC*mercury*	I	A	NA	HA-MRSA
6	F	38 y	ST	BC	+	NP	A	A1	NA	SCC*mercury*	I	A	NA	HA-MRSA
7	M	29 y	IM	Pus swab	+	NP	A	A1	NA	SCC*mercury*	I	A	NA	HA-MRSA
8	F	38 y	ST	BC	+	NP	A	A1	NA	SCC*mercury*	I	A	NA	HA-MRSA
9	F	38 y	ST	BC	+	NP	A	A1	NA	SCC*mercury*	I	A	NA	HA-MRSA
10	M	29 y	IM	Urine	+	NP	A	A1	NA	SCC*mercury*	I	A	NA	HA-MRSA
11	F	38 y	ST	BC	+	+	A	A1	NA	SCC*mercury*	I	A	NA	HA-MRSA
12	F	38 y	ST	BC	+	+	A	A1	NA	SCC*mercury*	I	A	NA	HA-MRSA
13	F	58 y	MP	Luki	+	+	D	D2	NA	IVd	I	A5	NA	CA-MRSA
14	F	38 y	ST	CVP tip	+	+	A	A4	NA	SCC*mercury*	I	A1	NA	HA-MRSA
15	M	4 y	Ward 60	BC	+	+	A	A1	NA	SCC*mercury*	I	A	NA	HA-MRSA
16	M	2y6m	Neurosurgical	Luki	+	NP	A	A1	NA	SCC*mercury*	I	A1	NA	HA-MRSA
17	M	63 d	Paediatric ICU	CVP tip	+	+	A	A4	NA	SCC*mercury*	I	A1	NA	HA-MRSA
18	M	2 y	Neurosurgery	CVP tip	+	NP	A	A1	NA	SCC*mercury*	I	A1	NA	HA-MRSA
19	M	65 y	OP	Tissue	+	NP	A	A4	NA	IVd	I	A5	NA	CA-MRSA
20	F	38 y	ST	BC	+	NP	E	E3	NA	SCC*mercury*	I	A	NA	HA-MRSA

BC -Blood culture; F -Female; M -Male; ND -Not detected; PS -Paediatric surgery; D -Days
ICU -Intensive care unit; m -Months; NP -Not provided; ST -Surgery and trauma; NA- Not applicable
Cont -Contaminated; IM -Internal medicine; MP -Medical pulmonology; NT -Not typeable; y -Years
CVP tip -Central venous pressure tip; Luki -Endotracheal aspirate; OP -Orthopaedic

Table 3.10: Combined results of the three PCR assays and (SCC*mec* and PFGE) used for the characterisation of the 194 MRSA isolates obtained from the Steve Biko Academic Hospital (continued)

MRSA isolates	Gender	Age	Clinical wards	Specimen Type	E-test	Vitek 2 System	*spa* cluster	*spa* subcluster	*spa* types (sequencing)	SCC*mec*	*agr* groups	PFGE	MLST	HA-MRSA or CA-MRSA
21	F	38 y	ST	CVP tip	+	NP	A	A4	NA	SCC*mercury*	I	A	NA	HA-MRSA
22	F	38 y	ST	BC	+	+	B	B4	NA	SCC*mercury*	I	A	NA	HA-MRSA
23	F	7 m	PS	BC	+	+	E	E3	NA	SCC*mercury*	I	A	NA	HA-MRSA
24	M	35 y	OP	Pus swab	+	+	E	E3	NA	IVd	I	A3	NA	CA-MRSA
25	F	4y4m	PS	BC	NP	+	E	E3	NA	SCC*mercury*	I	A3	NA	HA-MRSA
26	M	33 y	OPD	Pus swab	+	NP	E	E3	NA	IVd	I	A	NA	CA-MRSA
27	F	47 y	ST	BC	+	+	E	E3	NA	SCC*mercury*	I	A	NA	HA-MRSA
28	F	2y1m	PS	CVP tip	+	+	E	E3	NA	SCC*mercury*	I	A	NA	HA-MRSA
29	F	23 y	Nephrology	Urine	NP	NP	E	E3	NA	IVd	I	C	NA	CA-MRSA
30	M	2y6m	PS	BC	NP	+	E	E3	NA	SCC*mercury*	I	A	NA	HA-MRSA
31	M	49 y	Neurosurgery	CVP tip	+	NP	A	A4	NA	IVd	III	D	NA	CA-MRSA
32	M	45 m	HCM	BC	+	NP	B	B3	NA	II	III	A5	NA	HA-MRSA
33	F	12 d	ST	CVP tip	+	NP	A	A4	NA	SCC*mercury*	I	A	NA	HA-MRSA
34	M	22 y	OP	Tissue	+	NP	A	A1	NA	SCC*mercury*	I	A	NA	HA-MRSA
35	M	1y2m	PS	CVP tip	+	NP	E	E2	NA	I	II	A3	NA	HA-MRSA
36	F	4 m	ICU	BC	+	NP	A	A1	NA	SCC*mercury*	I	A4	NA	HA-MRSA
37	M	22 y	OP	Tissue	NP	NP	B	B4	NA	SCC*mercury*	I	A	NA	HA-MRSA
38	M	26 y	HCM	CVP tip	+	NP	D	D1	NA	IVd	I	A4	NA	CA-MRSA
39	M	92 d	Coronary ICU	BC	NP	NP	D	D2	NA	IVd	I	A4	NA	CA-MRSA
40	M	27 y	ST	CVP tip	+	+	C	C2	NA	SCC*mercury*	I	A	NA	CA-MRSA

BC -Blood culture; ICU -Intensive care unit; Cont -Contaminated; CVP tip -Central venous pressure tip; F -Female; m -Months; IM -Internal medicine; Luki -Endotracheal aspirate; M -Male; NP -Not provided; MP -Medical pulmonology; OP -Orthopaedic; ND -Not detected; ST -Surgery and trauma; NT -Not typeable; PS -Paediatric surgery; NA- Not applicable; y -Years; D -Days

Table 3.10: Combined results of the three PCR assays and (SCC*mec* and PFGE) used for the characterisation of the 194 MRSA isolates obtained from the Steve Biko Academic Hospital (continued)

MRSA isolates	Gender	Age	Clinical wards	Specimen Type	E-test	Vitek 2 System	*spa* cluster	*spa* subcluster	*spa* types (sequencing)	SCC*mec*	*agr* groups	PFGE	MLST	HA-MRSA or CA-MRSA
41	M	10 m	GP	CVP tip	+	+	D	D1	NA	IVd	I	A6	NA	CA-MRSA
42	M	2 y	PP	Sputum	NP	+	D	D2	NA	IVd	I	A4	NA	CA-MRSA
43	M	45 y	Short stay	BC	+	NP	L	L	NA	SCC*mercury*	I	A	NA	HA-MRSA
44	M	9 d	Neonatal ICU	BC	+	NP	K	K2	NA	SCC*mercury*	I	A	NA	HA-MRSA
45	M	1 d	Baby room ICU	BC	NP	NP	L	L	NA	II+SCC*mercury*	II	A	NA	HA-MRSA
46	M	17 d	Neonatal ICU	Pus swab	NP	NP	Outlier	Outlier	NA	SCC*mercury*	I	B	NA	HA-MRSA
47	M	15 y	Nephrology	BC	+	NP	L	L	NA	IVd	I	B	NA	CA-MRSA
48	M	44 y	ST	BC	+	NP	L	Outlier	NA	SCC*mercury*	I	A	NA	HA-MRSA
49	M	50 y	Neurosurgical	Luki	NP	NP	L	L	NA	IVd	I	A5	NA	CA-MRSA
50	M	25 d	Paediatric ICU	BC	+	NP	K	K2	NA	SCC*mercury*	I	A	NA	HA-MRSA
51	M	56 y	Cardiothoracic	BC	NP	+	K	K2	NA	II+SCC*mercury*	I	A	NA	HA-MRSA
52	M	22 y	Neurosurgical	CVP tip	+	+	NT	NT	NA	I	I	F	NA	HA-MRSA
53	M	38 y	ST	BC	+	+	K	K1	NA	II+SCC*mercury*	I	A	NA	HA-MRSA
54	M	1y2m	PS	CVP tip	+	NP	K	K1	NA	I	II	A3	NA	HA-MRSA
55	M	13 d	Neonatal ICU	BC	+	NP	Outlier	Outlier	NA	IVd	I	A5	NA	CA-MRSA
56	F	73 y	IM	CVP tip	+	+	C	C2	NA	II+SCC*mercury*	III	A	NA	HA-MRSA
57	M	52 y	OP	Tissue	+	+	K	Outlier	NA	II	III	A4	NA	HA-MRSA
58	M	16 d	Paediatric ICU	BC	+	NP	K	Outlier	NA	IVd	I	A1	NA	CA-MRSA
59	M	25 y	ST	BC	+	NP	D	D1	NA	SCC*mercury*	I	A2	NA	HA-MRSA
60	M	25 y	ST	BC	+	NP	H	H	NA	SCC*mercury*	I	A	NA	HA-MRSA

BC -Blood culture; F -Female; M -Male; ND -Not detected; PS -Paediatric surgery; D -Days
ICU -Intensive care unit; m -Months; NP -Not provided; ST -Surgery and trauma; NA-Not applicable
Cont -Contaminated; IM -Internal medicine; MP -Medical pulmonology; NT -Not typeable; y -Years
CVP tip -Central venous pressure tip; Luki -Endotracheal aspirate; OP -Orthopaedic

Table 3.10: Combined results of the three PCR assays and (SCC*mec* and PFGE) used for the characterisation of 194 the MRSA isolates obtained from the Steve Biko Academic Hospital (continued)

MRSA isolates	Gender	Age	Clinical wards	Specimen Type	E-test	Vitek 2 System	*spa* cluster	*spa* subcluster	*spa* types (sequencing)	SCC*mec*	*agr* groups	PFGE	MLST	HA-MRSA or CA-MRSA
61	M	27 y	ST	BC	+	+	J	J	NA	IVd	III	A4	NA	CA-MRSA
62	F	14 y	Oncology	Pus swab	+	NP	E	E3	NA	IVd	I	A	NA	CA-MRSA
63	M	57 y	HCM	CVP tip		+	F	F	NA	IVd	I	A	NA	CA-MRSA
64	M	50 y	Urology	Urine	+	NP	F	F	NA	SCC*mercury*	I	A	NA	HA-MRSA
65	M	20 y	Neurological	Urine	+	NP	F	F	NA	II+SCC*mercury*	I	A	NA	HA-MRSA
66	F	14 y	Oncology	Pus swab	+	+	D	D1	NA	II+SCC*mercury*	I	A	NA	HA-MRSA
67	M	4 d	PaediatricICU	BC	+	NP	C	C1	NA	SCC*mercury*	I	A	NA	HA-MRSA
68	M	60 y	MP	Luki	+	NP	C	C1	NA	SCC*mercury*	I	A	NA	HA-MRSA
69	M	60 y	MP	Luki	+	+	C	C1	NA	SCC*mercury*	I	A	NA	HA-MRSA
70	F	69 y	OP	Pus swab	NP	NP	C	Outlier	NA	II+SCC*mercury*	I	A	NA	HA-MRSA
71	F	20 y	OP	Pus swab	+	NP	D	D1	t891	V	I	A3	ST22	CA-MRSA
72	M	48 y	CS	Pus swab	+	NP	B	B1	NA	II+SCC*mercury*	I	A	NA	HA-MRSA
73	M	16 y	OP	Pus swab	NP	NP	E	E1	NA	IVd	I	A	NA	CA-MRSA
74	M	29 y	OP	Pus swab	NP	NP	E	E4	NA	SCC*mercury*	I	A3	NA	HA-MRSA
75	M	49 y	ICU	BC	+	NP	E	E4	NA	SCC*mercury*	I	K	NA	HA-MRSA
76	M	27 y	MP	Sputum	+	NP	E	E4	NA	IVd	I	A4	NA	HA-MRSA
77	M	49 y	ICU	BC	NP	NP	E	E4	NA	SCC*mercury*	I	A	NA	HA-MRSA
78	M	52 y	HCM	CVP tip	+	NP	E	E4	NA	SCC*mercury*	I	A	NA	HA-MRSA
79	M	10 m	GP	Tissue	NP	+	E	E4	NA	SCC*mercury*	I	A	NA	HA-MRSA
80	F	81 y	ST	Urine	+	+	J	J	NA	II	III	A5	NA	HA-MRSA

BC -Blood culture; F -Female; M -Male; ND -Not detected; PS -Paediatric surgery; D -Days
ICU -Intensive care unit; m -Months; NP -Not provided; ST -Surgery and trauma; NA-Not applicable
Cont -Contaminated; IM -Internal medicine; MP -Medical pulmonology; NT -Not typeable; y -Years
CVP tip -Central venous pressure tip; Luki -Endotracheal aspirate; OP -Orthopaediic

Table 3.10: Combined results of the three PCR assays and (SCC*mec* and PFGE) used for the characterisation of the 194 MRSA isolates obtained from the Steve Biko Academic Hospital (continued)

MRSA isolates	Gender	Age	Clinical wards	Specimen Type	E-test	Vitek 2 System	*spa* cluster	*spa* subcluster	*spa* types (sequencing)	SCC*mec*	*agr* groups	PFGE	MLST	HA-MRSA or CA-MRSA
81	F	81 y	OP	Pus swab	+	+	D	D1	NA	II+SCC*mercury*	I	A	NA	HA-MRSA
82	F	81 y	OP	Pus swab	+	NP	E	E2	NA	II+SCC*mercury*	I	A	NA	HA-MRSA
83	M	48 y	Urology	Pus swab	+	NP	E	E2	NA	SCC*mercury*	I	A5	NA	HA-MRSA
84	M	39 y	OP	Tissue	+	+	J	Outlier	NA	II	III	A1	NA	HA-MRSA
85	F	14 y	Oncology	Pus swab			Outlier	Outlier	NA	IVd	I	G	NA	CA-MRSA
86	M	67 y	NP	CVP tip	+	+	E	E1	NA	II+SCC*mercury*	I, III	A1	NA	HA-MRSA
87	F	35 d	Paediatric ICU	BC	+	+	E	E4	NA	SCC*mercury*	I	A	NA	HA-MRSA
88	M	49 y	ICU	BC	+	+	E	E4	NA	II	I, III	A	NA	HA-MRSA
89	F	26 d	Neonatal ICU	BC	+	+	E	E1	NA	SCC*mercury*	I	A	NA	HA-MRSA
90	M	31 y	NP	Pus swab	+	+	G	G1	NA	IVd	I	A6	NA	CA-MRSA
91	F	22 d	Neonatal ICU	BC	+	+	H	H	NA	II+SCC*mercury*	I	A	NA	HA-MRSA
92	M	7 d	Paediatric ICU	BC	+	+	E	E3	NA	SCC*mercury*	I	A	NA	HA-MRSA
93	F	37 y	Neurology	BC	+	+	D	D2	NA	IVd	I	A5	NA	CA-MRSA
94	M	47 y	OP	Pus swab	+	NP	A	A2	NA	II+SCC*mercury*	I	A	NA	HA-MRSA
95	F	62 y	ST	BC	+	+	A	A2	NA	IVd	I	A1	NA	CA-MRSA
96	M	29 y	Surgery burns	BC	+	NP	A	A2	NA	SCC*mercury*	I	A	NA	HA-MRSA
97	F	39 y	OP	Tissue	NP	+	D	C2	NA	SCC*mercury*	I	A	NA	HA-MRSA
98	M	40 y	CS	Luki	NP	NP	A	A5	NA	IVd	I	A4	NA	CA-MRSA
99	M	8 y	PA	Pus swab	+	+	A	A2	NA	SCC*mercury*	I	A	NA	HA-MRSA
100	M	52 y	Nephrology	BC	+	NP	A	A5	t1257	IVd	I	A6	ST612	CA-MRSA

BC -Blood culture; F -Female; M -Male; ND -Not detected; PS -Paediatric surgery; D -Days
ICU -Intensive care unit; m -Months; NP -Not provided; ST -Surgery and trauma; NA-Not applicable
Cont -Contaminated; IM -Internal medicine; MP -Medical pulmonology; NT -Not typeable; y -Years
CVP tip -Central venous pressure tip; Luki -Endotracheal aspirate; OP -Orthopaedic

Table 3.10: Combined results of the three PCR assays and (SCC*mec* and PFGE) used for the characterisation of the 194 MRSA isolates obtained from the Steve Biko Academic Hospital (continued)

MRSA isolates	Gender	Age	Clinical wards	Specimen Type	E-test	Vitek 2 System	*spa* cluster	*spa* subcluster	*spa* types (sequencing)	SCC*mec*	*agr* groups	PFGE	MLST	HA-MRSA or CA-MRSA
101	M	28 y	HCM	Pus swab	+	NP	B	A5	NA	IVd	I	B	NA	CA-MRSA
102	F	39 y	OP	Pus swab		+	H	H	NA	SCC*mercury*	I	A	NA	HA-MRSA
103	F	6 d	Neonatal ICU	BC	+	+	D	D1	NA	II+SCC*mercury*	I	A	NA	HA-MRSA
104	F	61 y	OP	BC	+	NP	D	D2	NA	IVd	I	B	NA	CA-MRSA
105	F	5 m	Neonatal ICU	BC	+	NP	D	D2	NA	IVd	I	A6	NA	CA-MRSA
106	M	7 m	Paediatric ICU	BC	NP	NP	G	G1	NA	II	I	A	NA	HA-MRSA
107	F	42 y	ST	Luki	+	+	G	G2	NA	SCC*mercury*	I	A6	NA	HA-MRSA
108	F	24 y	Neurosurgical	CVP tip	NP	NP	G	G2	NA	IVd	I	A6	NA	CA-MRSA
109	F	16 y	NP	NP	NP	NP	G	G2	NA	II+SCC*mercury*	I	A	NA	HA-MRSA
110	M	13 y	Neurosurgical	BC	+	NP	G	G1	NA	SCC*mercury*	I	A3	NA	HA-MRSA
111	F	1y1m	NP	NP	NP	NP	G	G1	NA	SCC*mercury*	I	A	NA	HA-MRSA
112	F	77 y	HCM	BC	+	NP	G	G1	NA	II+SCC*mercury*	I	A	NA	HA-MRSA
113	F	54 y	NP	NP	NP	NP	G	G1	NA	SCC*mercury*	I	A	NA	CA-MRSA
114	M	4 d	Paediatric ICU	BC	+	NP	G	Outlier	NA	SCC*mercury*	I	A	NA	HA-MRSA
115	M	31 y	ARV	Pus swab	+	+	G	G2	NA	II+SCC*mercury*	I	A	NA	HA-MRSA
116	F	24 y	GS	Pus swab	NP	+	B	B4	NA	SCC*mercury*	I	A	NA	HA-MRSA
117	M	49 y	Nephrology	Pus swab	+	NP	G	G1	NA	IVd	I	A	NA	CA-MRSA
118	M	51 y	Main casualty	Pus swab	+	NP	G	G1	NA	SCC*mercury*	I	A	NA	HA-MRSA
119	M	52 y	Surgery	BC	+	NP	G	G1	NA	II+SCC*mercury*	I	A	NA	HA-MRSA
120	M	78 y	CS	Luki	+	NP	G	G1	NA	SCC*mercury*	I	A	NA	HA-MRSA

BC -Blood culture; F -Female; M -Male; ND -Not detected; PS -Paediatric surgery; D -Days
ICU -Intensive care unit; m -Months; NP -Not provided; ST -Surgery and trauma; NA-Not applicable
Cont -Contaminated; IM -Internal medicine; MP -Medical pulmonology; NT -Not typeable; y -Years
CVP tip -Central venous pressure tip; Luki -Endotracheal aspirate; OP -Orthopaedic

Table 3.10: Combined results of the three PCR assays and (SCC*mec* and PFGE) used for the characterisation of the 194 MRSA isolates obtained from the Steve Biko Academic Hospital (continued)

MRSA isolates	Gender	Age	Clinical wards	Specimen Type	E-test	Vitek 2 System	*spa* cluster	*spa* subcluster	*spa* types (sequencing)	SCC*mec*	*agr* groups	PFGE	MLST	HA-MRSA or CA-MRSA
121	F	71 y	IM	BC	+	NP	G	G1	NA	SCC*mercury*	I	A	NA	HA-MRSA
122	M	23 d	Paediatric ICU	BC	+	NP	B	B4	NA	II+SCC*mercury*	I	A4	NA	HA-MRSA
123	F	4 y	Neonatal ICU	Briviac tip	+	+	A	A2	NA	IVd	I	A3	NA	CA-MRSA
124	F	4y3m	PP	Sputum	+	NP	B	B3	NA	IVd	I	A5	NA	CA-MRSA
125	M	17 d	Paediatric ICU	BC	+	NP	NT	NT	t037	I	I	NT	NA	HA-MRSA
126	M	6 m	Paediatric ICU	BC	+	+	B	BI	NA	II+SCC*mercury*	II	A6	NA	HA-MRSA
127	F	28 d	Neonatal ICU	Pus swab	+	NP	A	A2	NA	SCC*mercury*	I	A6	NA	HA-MRSA
128	F	65 y	OPD	Pus swab	+	NP	B	B4	NA	II+SCC*mercury*	I	A	NA	HA-MRSA
129	M	35 y	HCM	BC	+	NP	B	B4	NA	SCC*mercury*	I	A	NA	HA-MRSA
130	M	49 y	ST	Luki	NP	NP	B	B4	NA	II+SCC*mercury*	I	A4	NA	CA-MRSA
131	F	42 y	ST	NP	NP	NP	B	B4	t037	II+SCC*mercury*	II	A	ST239	HA-MRSA
132	M	60 y	IM	BC	+	NP	B	B3	NA	IVd	I	H	NA	CA-MRSA
133	M	27 y	HCM	BC	+	NP	B	B1	t037	IVa	I	A	ST239	CA-MRSA
134	F	78 y	UG	Pus swab	+	NP	NT	NT	NT	IVd	I	B2	NA	CA-MRSA
135	M	28 y	HCM	Pus swab	+	NP	B	B4	NA	II+SCC*mercury*	I	A	NA	HA-MRSA
136	M	31 y	Neurosurgical	CVP tip	+	+	B	B1	NA	SCC*mercury*	I	A1	NA	HA-MRSA
137	M	70 y	IM	CVP tip	+	NP	A	A2	NA	IVd	I	B1	NA	CA-MRSA
138	M	35 y	Main casualty	Luki		+	NT	NT	NA	IVd	I	I	NA	CA-MRSA
139	F	52 d	PS	CVP tip	+	+	NT	NT	NA	IVd	I	I	NA	CA-MRSA
140	F	40 d	Neonatal ICU	Briviac tip	+	+	H	H	NA	II+SCC*mercury*	I	A	NA	HA-MRSA

BC -Blood culture; F -Female; M -Male; ND -Not detected; PS -Paediatric surgery; D -Days
ICU -Intensive care unit; m -Months; NP -Not provided; ST -Surgery and trauma; NA-Not applicable
Cont -Contaminated; IM -Internal medicine; MP -Medical pulmonology; NT -Not typeable; y -Years
CVP tip -Central venous pressure tip; Luki -Endotracheal aspirate; OP -Orthopaedic

Table 3.10: Combined results of the three PCR assays and (SCC*mec* and PFGE) used for the characterisation of the 194 MRSA isolates obtained from the Steve Biko Academic Hospital (continued)

MRSA isolates	Gender	Age	Clinical wards	Specimen Type	E-test	Vitek 2 System	*spa* cluster	*spa* subcluster	*spa* types (sequencing)	SCC*mec*	*agr* groups	PFGE	MLST	HA-MRSA or CA-MRSA
141	F	46 y	ST	BC	NP	+	K	K1	NA	II	I	J	NA	HA-MRSA
142	M	27 y	Surgery male	Catheter tip	+	+	D	D2	NA	IVd	I, III	A3	NA	CA-MRSA
143	M	45 y	ST	Pus swab	+	+	A	A1	t1257	Iva	III	B	ST612	CA-MRSA
144	M	40 d	Neonatal ICU	BC	+	NP	A	A1	NA	II+SCC*mercy*	I	A	NA	HA-MRSA
145	F	28 y	Gynaecology	Pus swab	+	+	B	B1	NA	II+SCC*merry*	I	A3	NA	HA-MRSA
146	M	38 y	OP	Pus swab	+	+	B	Outlier	NA	IVd	I	A	NA	CA-MRSA
147	M	2y2m	ARV	Pus swab	+	+	B	B1	NA	SCC*mercury*	I	A	NA	HA-MRSA
148	F	9 d	Paediatric	BC	+	+	B	B2	NA	II+SCC*merry*	I	A	NA	HA-MRSA
149	M	39 y	OP	Tissue	NP	NP	B	B2	NA	II+SCC*merry*	I	A	NA	HA-MRSA
150	F	64 y	IM	Sputum	+	+	B	Outlier	NA	II+SCC*merry*	I	A	NA	HA-MRSA
151	F	60 y	HCM	Pus swab	+	+	B	B2	NA	II+SCC*merry*	I	A	NA	HA-MRSA
152	M	2y2m	ARV	Pus swab	+	+	B	B1	NA	II+SCC*merry*	I	A	NA	HA-MRSA
153	M	52 y	ST	CVP tip	+	+	B	B1	NA	II+SCC*merry*	I	A	NA	HA-MRSA
154	F	26 d	Neonatal ICU	BC	+	+	B	B4	NA	II+SCC*merry*	I	A	NA	HA-MRSA
155	M	7 d	PaediatricIU	BC	+	+	B	B1	NA	II+SCC*mercy*	I	A	NA	HA-MRSA
156	M	49 y	ST	Luki		+	B	B1	NA	II+SCC*merry*	I	A	NA	HA-MRSA
157	F	46 y	ST	Urine		+	B	Outlier	NA	II+SCC*merry*	I	A	NA	HA-MRSA
158	M	57 y	HCM	CVP tip	+	+	B	B4	NA	II+SCC*merry*	I	A	NA	HA-MRSA
159	M	60y	MP	Luki	+		B	B4	NA	II+SCC*merry*	I	A	NA	HA-MRSA
160	F	24y	Main casualty	Tissue	+	+	B	B4	NA	II+SCC*merry*	I	A	NA	HA-MRSA

BC -Blood culture; F -Female; M -Male; ND -Not detected; PS -Paediatric surgery; D -Days
ICU -Intensive care unit; m -Months; NP -Not provided; ST -Surgery and trauma; NA- Not applicable
Cont -Contaminated; IM -Internal medicine; MP -Medical pulmonology; NT -Not typeable; y -Years
CVP tip -Central venous pressure tip; Luki -Endotracheal aspirate; OP -Orthopaedic

Table 3.10: Combined results of the three PCR assays and (SCC*mec* and PFGE) used for the characterisation of the 194 MRSA isolates obtained from the Steve Biko Academic Hospital (continued)

MRSA isolates	Gender	Age	Clinical wards	Specimen Type	E-test	Vitek 2 System	*spa* cluster	*spa* subcluster	*spa* types (sequencing)	SCC*mec*	*agr* groups	PFGE	MLST	HA-MRSA or CA-MRSA
161	M	60 y	MP	Luki		+	B	B1	NA	I	II	A3	NA	HA-MRSA
162	F	75 y	Maxifacial	BC		+	B	B1	NA	SCC*mercury*	I	A	NA	HA-MRSA
163	M	38 y	OP	Tissue	+	+	A	A2	NA	PN	I	A	NA	NA
164	M	1y2 m	PS	CSF	+	NP	B	B4	NA	PN	II	A3	NA	NA
165	F	64 y	MP	Sputum	+	NP	NT	NT	NT	PN	I	I	NA	NA
166	M	8 y	PU	Pus swab	+	NP	B	B4	NA	II	I	J	NA	HA-MRSA
167	F	58 y	Neurosurgical	Pus swab	+	+	B	B4	NA	SCC*mercury*	I	A	NA	HA-MRSA
168	F	42 y	Surgery	Pus swab	NP	NP	B	B4	NA	SCC*mercury*	III	A	NA	HA-MRSA
169	M	32 y	HCM	BC	+	NP	B	B4	NA	SCC*mercury*	I	A	NA	HA-MRSA
170	M	16 y	OP	Pus swab	+	NP	B	B4	NA	II	I	NT	NA	HA-MRSA
171	M	16 y	OP	Pus swab	+	NP	B	B4	NA	II	I	I	NA	HA-MRSA
172	M	19 d	PS	CVP tip	+	NP	Outlier	D2	NA	II+SCC*merry*	I	A6	NA	HA-MRSA
173	F	1 y	Paediatricsuy	BC	+	NP	A	A4	NA	IV	I	A	NA	CA-MRSA
174	F	20 d	NeonatalICU	BC	+	NP	B	B4	NA	SCC*mercury*	II	A4	NA	HA-MRSA
175	M	16 y	OP	Tissue	+	NP	B	B3	NA	IV	I	B1	NA	CA-MRSA
176	F	72 y	HCM	Sputum	+	NP	D	Outlier	NA	IV	I	A5	NA	CA-MRSA
177	F	72 y	OP	Tissue	NP	NP	A	A1	NA	SCC*mercury*	I	A	NA	HA-MRSA
178	F	33 y	MP	CVP tip	+	NP	A	Outlier	NA	SCC*mercury*	I	A	NA	HA-MRSA
179	M	16 y	OP	Pus swab	NP	NP	D	D1	NA	IV	I	A	NA	CA-MRSA
180	F	69 y	OP	Tissue	+	+	A	A1	NA	SCC*mercury*	I,III	A5	NA	HA-MRSA

BC -Blood culture; F -Female; M -Male; ND -Not detected; PS -Paediatric surgery; D -Days
ICU -Intensive care unit; m -Months; NP -Not provided; ST -Surgery and trauma; NA -Not applicable
Cont -Contaminated; IM -Internal medicine; MP -Medical pulmonology; NT -Not typeable; y -Years
CVP tip -Central venous pressure tip; Luki -Endotracheal aspirate; OP -Orthopaedic

Table 3.10: Combined results of the three PCR assays and (SCC*mec* and PFGE) used for the characterisation of the 194 MRSA isolates obtained from the Steve Biko Academic Hospital (continued)

MRSA isolates	Gender	Age	Clinical wards	Specimen Type	E-test	Vitek 2 System	*spa* cluster	*spa* subcluster	*spa* types (sequencing)	SCC*mec*	*agr* groups	PFGE	MLST	HA-MRSA or CA-MRSA
181	F	30 y	HCM	BC	+	+	I	I	NA	SCC*mercury* (II+ SCC*mercury*)	III	J	NA	HA-MRSA
182	F	25 y	Neonatal ICU	BC	+	NP	A	A1	NA	SCC*mercury* (II+ SCC*mercury*)	III	A	NA	HA-MRSA
183	M	16 y	OP	Pus swab	+	NP	I	I	t012	II	I,III	J	ST36	HA-MRSA
184	F	47 y	ST	Pus swab	NP	NP	I	I	NA	II	I,III	J	NA	HA-MRSA
185	F	29 y	Main casualty	Tissue	+	NP	A	A1	NA	II	I	A	NA	HA-MRSA
186	F	29 y	Main casualty	Tissue	+	+	A	A1	NA	SCC*mercury* (II+SCC*mercury*)	I	A	NA	HA-MRSA
187	F	35 y	Main casualty	Tissue	NP	NP	A	A4	NA	SCC*mercury*	I	A	NA	HA-MRSA
188	F	69 y	OP	Tissue	+	+	A	A1	NA	SCC*mercury* (II+SCC*mercury*)	I	A	NA	HA-MRSA
189	F	38 y	Neurology	BC	+	+	D	D1	NA	IVd	I	A	NA	CA-MRSA
190	F	47 y	ST	BC	+	+	J	J	NA	II+IVc	III	J	NA	CA-MRSA
191	F	49 y	HCM	BC	+	+	J	J	NA	II+IVc	III	J	NA	CA-MRSA
192	F	47 y	ST	BC	+	+	J	J	NA	II+IVc	III	J	NA	CA-MRSA
193	F	47 y	HCM	BC	+	+	J	J	NA	II+IVc	I,III	J	NA	CA-MRSA
194	M	31 y	Main casualty	Sputum	NP	NPS	Outlier	A	NA	NT	I	A	NA	NT

BC -Blood culture; F -Female; M -Male; ND -Not detected; PS -Paediatric surgery; D -Days
ICU -Intensive care unit; m -Months; NP -Not provided; ST -Surgery and trauma; NA-Not applicable
Cont -Contaminated; IM -Internal medicine; MP -Medical pulmonology; NT -Not typeable; y -Years
CVP tip -Central venous pressure tip; Luki -Endotracheal aspirate; OP -Orthopaedic