Genetic relatedness of methicillin resistant *Staphylococcus aureus* isolates from Steve Biko hospital

TSIDISO GUGU MAPHANGA

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by

TSIDISO GUGU MAPHANGA

Submitted in partial fulfilment of the requirements for the degree

MAGISTER SCIENTIAE

MSc (Medical Microbiology)

Department of Medical Microbiology
Faculty of Health Sciences
University of Pretoria
Gauteng
South Africa

January 2013

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

Signed _____ this _____ day of _____ 2013

Research is to see what everybody else has seen and to think what nobody else has thought

Albert Szent-Györgi (US Biochemist)

DEDICATION

To my late grandmother (Mirriam Jankie Mashego): Thank you for raising me and for encouraging me to further my studies, you were such an inspiration, I will forever love and miss you

ACKNOWLEDGEMENTS

Firstly: I would like to extend my greatest gratidute to the great God without whom this MSc degree would not have been possible. My Lord, it was not easy but it was worth it.

Secondly: I would like to sincerely thank the following individuals:

Prof MM Ehlers (supervisor), Department of Medical Microbiology, University of Pretoria/NHLS, for her endless motivations, understanding and ongoing support, but most of all for making this MSc degree possible. Without your patience and guidance Prof, I would not have become the scientists I am today. God bless you.

Dr MM Kock (co-supervisor), Department of Medical Microbiology, University of Pretoria/NHLS, for her guidance, understanding, endless encouragement, ongoing support and for always believing in me when I have lost hope. I am truly blessed to have you as a co-supervisor.

Prof AA Hoosen, Department of Medical Microbiology, University of Pretoria/NHLS, for seeing the potential in me and for granting me the opportunity to further my studies.

My family: Mother (Mavis Mashego), my brothers (Mlungisi, Thabiso, Sabelo and Tumelo), my sisters (Phumla and Lesego) and my boyfriend (Sifiso Mdluli), for their constant love, prayers, endless motivation, understanding and support.

My friends: Sama, Cebo, Nolitha, Isaac, Nthutu and Ivy for their tremendous support, love and encouragement.

My extended family (colleagues): Adeola, Iqra, Janine, Ricardo, Shane and Toyin for giving me a shoulder to cry on and moral support when my MSc was getting hard.

Marisa De Jesus and Ms Olga Kgasha, I am indebted to you for your help, may the great God bless you abundantly.

Dr L Blann, Department of Infection Control, Wits University/NHLS, for assisting me with the pulsed-field gel electrophoresis (PFGE).

Medical Research Council (MRC) and National Research Foundation (NRF) for the financial support received which helped me throughout the course of my MSc degree.

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SYMBOLS

α	Alpha
β	Beta
ºC	Degree Celsius
γ	Gamma
%	Percentage

ABBREVIATIONS

ACME	Arginine catabolic mobile element
BHI	Brain-heart infusion
bla	Beta-lactamase gene
BORSA	Borderline oxacillin resistant *Staphylococcus aureus*
bp	Base pair
BURST	Based upon related sequence types
C	Cytosine
CA-MRSA	Community-associated methicillin resistant *Staphylococcus aureus*
CAP	Community-acquired pneumonia
CC	Clonal complex
ccr	Cassette chromosome recombinases
CDC	Centers for Disease Control and Prevention
CLSI	Clinical Laboratory Standards Institute
CoNS	Coagulase negative *Staphylococcus*
coa	coagulase gene
DNA	Deoxyribose nucleic acid
dNTP	Deoxynucleotide triphosphate
DRUs	Direct repeat units
EDTA	Ethylene diamine tetraacetate
ET	Exfoliative toxins
g	Gram
G	Guanine
NAG	N-acetylglucosamine
HA-MRSA	Healthcare-associated methicillin resistant *Staphylococcus aureus*
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
h	Hour
h-VISA	Heteroresistant vancomycin intermediate *Staphylococcus aureus*
HVR	Hypervariable region
ICU	Intensive care unit
IE	Infective endocarditis

IgG	Immunoglobulin G
IS	Insertion sequence
kb	Kilobase pair
kD	Kilodalton
KZN	KwaZulu-Natal
M	Molar
Mb	Megabase
MgCl	Magnesium chloride
mg	Milligram
MGEs	Mobile genetic elements
MHC	Major histocompatibility complex
MIC	Minimum inhibitory concentration
min	Minute
ml	Millilitre
MLST	Multilocus sequence typing
mM	Millimolar
MW	Molecular weight
M-PCR	Multiplex polymerase chain reaction
MRSA	Methicillin resistant *Staphylococcus aureus*
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
MSSA	Methicillin susceptible *Staphylococcus aureus*
MurNAc	N-acetylmuramic acid
NaCl	Sodium chloride
NCCLS	National Committee for Clinical Laboratory Standards
NHLS	National Health Laboratory Service
nm	Nanometre
ORF	Open reading frame
PBP2	Penicillin-binding protein 2
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
pH	Hydrogen ion activity
PMNs	Polymorphonuclear leukocytes
PRSA	Penicillin resistant *Staphylococcus aureus*
PSM	Phenol soluble modulin
PVL	Panton-Valentine leukocidin
RE	Restriction enzyme
ROS	Reactive oxygen species
s	Second
S. aureus	*Staphylococcus aureus*
SCC*mec*	Staphylococcal cassette chromosome *mec*
SBAH	Steve Biko Academic Hospital
SCV	Small colony variants
SDS	Sodium dodecyl sulfate
SE	Staphylococcal enterotoxin

*Sma*I	*Serratia marcescens* I
spa	Staphylococcal protein A
SSSS	Staphylococcal scalded skin syndrome
SSTI	Skin and soft tissue infection
ST	Sequence type
STE	Sodium chloride Tris ethylene diamine tetraacetate
TAE	Tris-acetate ethylene diamine tetraacetate
TBE	Tris-borate ethylene diamine tetraacetate
Tn	Transposon
TSST	Toxic shock syndrome toxin
µg	Microgram
µl	Microlitre
µM	Micromolar
UK	United Kingdom
UP	University of Pretoria
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
USA	United States of America
UV	Ultraviolet
VISA	Vancomycin intermediate *Staphylococcus aureus*
VRE	Vancomycin resistant enterococci
VSSA	Vancomycin susceptible *Staphylococcus aureus*
Y	Years

LIST OF PUBLICATIONS AND CONFERENCE CONTRIBUTIONS

PUBLICATION

1. **Maphanga TG, Kock MM, Becker PJ and Ehlers MM** (2012) Genetic relatedness of methicillin resistant *Staphylococcus aureus* isolates from a tertiary academic hospital in the Pretoria region, South Africa. To be submitted for publication to: *Journal of Diagnostic Microbiology and Infectious Disease*

CONFERENCE PRESENTATION

1. **Maphanga TG, Kock MM, Becker PJ, Ehlers MM** (2011) Prevalence of methicillin resistant *Staphylococcus aureus* SCC*mec* types and subtypes in the Steve Biko Academic Hospital. Faculty day, Faculty of Health Sciences, University of Pretoria, 30 August 2011. (Poster presentation)
2. **Maphanga TG, Kock MM, Becker PJ, Ehlers MM** (2011) Prevalence of methicillin resistant *Staphylococcus aureus* SCC*mec* types and subtypes in the Steve Biko Academic Hospital. Faculty day, Faculty of Health Sciences, University of Pretoria, 31 August 2011. (Oral presentation)
3. **Maphanga TG, Kock MM, Becker PJ, Ehlers MM** (2012) Prevalence of methicillin resistant *Staphylococcus aureus* SCC*mec* types and subtypes in the Steve Biko Academic Hospital. South African Societies of Biochemistry and Molecular Biology (SASBMB) conference, Drakensburg, Champagne Sport Resort, 29 January to 1 February 2012. (Poster presentation)
4. **Maphanga TG, Kock MM, Becker PJ, Ehlers MM** (2012) Prevalence of methicillin resistant *Staphylococcus aureus* SCC*mec* types and subtypes in the Steve Biko Academic Hospital. Faculty day, Faculty of Health Sciences, University of Pretoria, 25 August 2012. (Poster presentation)

Genetic relatedness of methicillin resistant *Staphylococcus aureus* isolates from Steve Biko hospital

by

Tsidiso Gugu Maphanga

PROMOTER:	**Prof Marthie M Ehlers (Department of Medical Microbiology)**
CO-PROMOTER:	**Dr Marleen M Kock (Department of Medical Microbiology)**
DEPARTMENT:	**Medical Microbiology, Faculty of Health Sciences, University of Pretoria**
DEGREE:	**MSc (Medical Microbiology)**

SUMMARY

Methicillin resistant *Staphylococcus aureus* (MRSA) causes severe infections in humans. An estimated two billion people carry *S. aureus* worldwide, of which fifty-three million harbour MRSA strains. The ability of MRSA to become multiresistant poses tremendous challenges to the healthcare system and has emerged as a major concern in the community setting worldwide. Consequently, it is important to distinguish MRSA infections because infections caused by different strains of MRSA, such as healthcare-associated MRSA (HA-MRSA) and community-associated MRSA (CA-MRSA) require different antibiotic regimens. Another concern in the hospital setting is the emergence of some of the CA-MRSA strains, which are more virulent than HA-MRSA due to the expression of the Panton-Valentine leukocidin (PVL) gene, which causes severe skin infections, sepsis and necrotising pneumonia. These current situations indicate a need for continued surveillance and infection control programmes using molecular methods to monitor MRSA strains in hospital settings so that appropriate therapy may be initiated and MRSA outbreaks may be prevented.

The relatedness of MRSA isolates, using various molecular methods, has been reported in a previous study conducted in the department. However, pulsed-field gel electrophoresis (PFGE), which is the gold standard for MRSA typing, was not included. A total of 194

clinical MRSA isolates were collected from the Steve Biko Academic Hospital in the Gauteng province (South Africa) from April 2010 to August 2011 and analysed using six multiplex-PCR (M-PCR) assays. Following identification, detection and characterisation using the M-PCR assays, PFGE was used to determine the genetic relatedness of these MRSA isolates.

The first M-PCR assay showed a prevalence of 100% for the 16S rRNA and *mec*A genes. A single isolate (0.5%) carried the PVL gene. Five staphylococcal cassette chromosome *mec* (SCC*mec*) typing M-PCR assays were used to distinguish between HA-MRSA and CA-MRSA isolates. Inconclusive results were obtained for 26.3% (51/194) of the MRSA isolates, which showed bands for either SCC*mec* type II or SCC*mercury*. The remaining 143 MRSA isolates showed a prevalence of 64.3% (92/143) for HA-MRSA and 35.7% (51/143) for CA-MRSA. The SCC*mec* types for the HA-MRSA isolates were: SCC*mercury* [74% (68/92)], SCC*mec* type II [19.5% (18/92)] and SCC*mec* type I [6.5% (6/92)]; while the SCC*mec* types and subtypes belonging to the CA-MRSA isolates were: subtypes IVd [92.1% (47/51)], IVa [3.9% (2/51)], IVb [2% (1/51)] and SCC*mec* type V [2% (1/51)]. No SCC*mec* type III or VIII was detected in the MRSA isolates. The PFGE typing method clustered the 191 MRSA isolates into eleven pulsotypes designated pulsotype A to K. Pulsotype A was the dominant pulsotype, including 66% (127/191) of the HA-MRSA and 19% (36/191) of the CA-MRSA isolates. Fifteen percent (28/191) of the MRSA isolates were unrelated to pulsotype A, which included 7% (13/191) of the HA-MRSA and 8% (15/191) of the CA-MRSA isolates.

Multiplex-PCR SCC*mec* typing assays and PFGE typing remain important tools for the characterisation of MRSA strains. A standardised SCC*mec* M-PCR assay can provide more accurate and reliable results. The results indicated that the HA-MRSA and CA-MRSA strains analysed in this study were closely related in this hospital setting, which necessitates continuous monitoring and surveillance to ensure and guide infection control policies.

CHAPTER 1

1.1 INTRODUCTION

Staphylococcus aureus (*S. aureus*) is an important bacterial pathogen and is accountable for a high proportion of cases of severe infection in hospital and outpatient units (Stürenburg, 2009; Wang *et al*., 2012). The bacterium is associated with a wide range of infections, such as superficial skin infections (carbuncles, boils, impetigo and folliculitis) and life-threatening conditions, like bacteraemia, endocarditis, pneumonia, meningitis, food poisoning and toxic shock syndrome (Kobayashi and DeLeo, 2009). The pathogenesis of the bacterium is determined by the production of several toxins, such as the 33 kD protein-α toxin (cytotoxin), Panton-Valentine leukocidin (PVL) toxin and exfoliatin toxins (A and B) (Lowy, 1998).

In 1959, methicillin was developed as a therapeutic agent for the treatment of *S. aureus* infections (Otto, 2012). Two years later, the first methicillin resistant *S. aureus* (MRSA) strains were discovered (Poulakou *et al*., 2012). The resistant *S. aureus* strains were called MRSA (Boundy *et al*., 2012). Resistance of *S. aureus* to methicillin is conferred by the penicillin binding protein (PBP2a), which encodes the *mec*A gene of the staphylococcal cassette chromosome *mec* (SCC*mec*) (Taiwo, 2009). The origin of the SCC*mec* is unknown (Tulinski *et al*., 2012). However, according to Hanssen and Ericson Sollid (2006) the *mec*A gene, prevalent in all staphylococci, may have been acquired from *S. sciuri*. *Staphylococcus sciuri* is oxidase positive, novobiocin resistant and mainly associated with animals (Severin *et al*., 2010). This bacterium carries a *mec*A gene, which is closely related to that of MRSA strains and it is believed that the MRSA *mec*A gene might have originated from the *S. sciuri mec*A gene (Tulinski *et al*., 2012).

The mechanism involved in the generation of MRSA is different from that of penicillin-resistant *S. aureus* (PRSA) (Chambers and DeLeo, 2009). In PRSA the penicillinase hydrolyses the β-lactam ring of penicillin, inactivating the antibiotic, while in MRSA, the PBP2a structure is altered resulting in the protein having lower affinity for β-lactam antibiotics, including penicillins, cephalosporins and carbapenems (Otto, 2012). The archaic COL (Colindale, UK) MRSA strain was one of the earliest clinical MRSA isolates found in the United Kingdom and Denmark in the 1960s (Otto, 2012). This strain was

isolated from a patient in Colindale, United Kingdom and is the most successful MRSA lineage in both healthcare-associated MRSA (HA-MRSA) and community-associated MRSA (CA-MRSA) strains (Chambers and DeLeo, 2009). The COL MRSA strain was found circulating in hospitals all over Europe and the United States until 1970, but by 1980 the strain was no longer isolated in European countries (Chambers and DeLeo, 2009). The reason for the COL MRSA strain's disappearance from the European countries remains unknown (Chambers and DeLeo, 2009). New COL MRSA descendant strains, such as the Iberian and Rome clones have since replaced the COL MRSA clone (Chambers and DeLeo, 2009). The Iberian and Rome MRSA strains have become pandemic worldwide causing infections in healthcare and community settings (Campanile *et al*., 2009; Chambers and DeLeo, 2009).

Community-associated MRSA strains have spread in hospitals worldwide and are increasingly identified as a cause of hospital onset infections (Chambers and DeLeo, 2009). Certain CA-MRSA strains may be more virulent than HA-MRSA due to the presence of the PVL toxin gene, which has been detected in neonatal and adult patients in intensive care units (ICU) [Cocchi *et al*., 2011; Haque *et al*., 2012]. Community-associated MRSA, which carries the PVL gene, has shown to cause chronic or recurrent soft skin tissue infection (SSTI) and necrotising pneumonia in healthy young people (Monecke *et al*., 2011). The ability of these PVL-positive CA-MRSA strains to produce virulence genes have resulted in outbreaks in the community, representing a serious problem (Monecke *et al*., 2011).

There is a high level of genetic diversity between HA-MRSA and CA-MRSA (Kennedy and DeLeo, 2009). Healthcare-associated MRSA strains are multi-drug resistant (β-lactams, tetracyclines and clindamycin), while CA-MRSA strains are susceptible to non-β-lactam antimicrobials, such as tetracyclines, sulfamethoxazole and clindamycin (Otter and French, 2011). Healthcare-associated MRSA strains carry the SCC*mec* type I, II, III and VI, while CA-MRSA strains carry SCC*mec* type IV, V, VII, VIII, IX, X and XI (Coombs *et al*., 2011a). Community-associated MRSA strains, especially SCC*mec* type IV and V have a faster growth rate than HA-MRSA strains and contain a smaller SCC*mec* size (24 kb and 27.6 kb) (Chambers and DeLeo, 2009). The attributes have been implicated in the increased mobility and transmissiblity of these genetic elements between other *S. aureus* chromosomes (Barnes and Sampson, 2011).

The increase in the possibility of transfer of the SCC*mec* elements encoding antimicrobial resistance between CA-MRSA and HA-MRSA isolates in healthcare and community settings necessitates accurate and reliable methods for the identification and detection of these strains (Song *et al*., 2011). Molecular assays, such as real-time multiplex-PCR (M-PCR) and conventional M-PCR assays have been applied for the identification and characterisation of MRSA strains (Faria *et al*., 2008; Coombs *et al*., 2011b). These M-PCR assays have shown to be rapid and robust for the detection of multiple genes, such as the *mec* and *ccr* genes found in MRSA strains as well as for typing and subtyping of the SCC*mec* elements associated with HA-MRSA and CA-MRSA strains (Chen *et al*., 2009; Makgotlho *et al*., 2009; McClure *et al*., 2010).

Genotyping methods, such as pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST) and staphylococcal protein A (*spa*) typing have been used for determining the genetic relatedness of MRSA strains (Ho *et al*., 2011). These genotyping techniques are based on the principle that related MRSA isolates share the same genetic features (McDougal *et al*., 2003). The difference between PFGE and MLST methods is that PFGE is widely used for the determination of micro-variations or short-term evolution in smaller populations, while MLST is appropriate for the determination of macro-variations or long-term evolution in large populations (Ho *et al*., 2011). Staphylococcal protein A (*spa*) typing is a single-locus typing method in which differences in highly variable tandem repeats are characterised by sequencing (Ho *et al*., 2011; Sobral *et al*., 2012). Although, many genotyping methods have been used for the typing of MRSA strains, PFGE typing has been proven to be robust enough to type MRSA strains with a high resolution and is considered the "gold standard" (McDougal *et al*., 2003). One of the disadvantages of PFGE typing technique is that, the band size may be inaccurate due to poor lane separation, which may be caused by incorrect electrophoresis voltage, gel temperature or buffer strength (Li *et al*., 2009). Furthermore, creations of the profiles are labour intensive (Schouls *et al*., 2009).

The clonal relatedness of HA-MRSA and CA-MRSA isolates has been investigated by Makgotlho *et al*. (2009) during 2006 to 2008 using conventional M-PCR assays, a real-time M-PCR assay, *spa* typing and hypervariable region (HVR) typing. No PFGE typing data regarding the clonal relatedness of HA-MRSA and CA-MRSA isolates circulating in this clinical setting is available. It is, therefore, crucial to assess the genetic relatedness of MRSA

isolates obtained from patient specimens in this hospital setting in order to initiate surveillance and infection control programmes that will prevent the spread of possible MRSA clones that are circulating in the hospital.

The aim of this study was to identify, detect and characterise 194 MRSA isolates obtained from the Steve Biko Academic Hospital (SBAH) in the Gauteng province from April 2010 to August 2011 using M-PCR assays followed by PFGE genotyping to determine the genetic relatedness of these MRSA isolates.

The objectives of this study were:

- To collect 194 MRSA isolates from the NHLS laboratory, Department of Medical Microbiology, UP/NHLS
- To determine the prevalence of the 16S rRNA, *mec*A and PVL genes in these MRSA isolates using a conventional multiplex-PCR assay
- To type and subtype SCC*mec* I to V of the MRSA isolates using a second conventional multiplex-PCR assay
- To type SCC*mec* VIII of the MRSA isolates using a third conventional multiplex-PCR assay
- To use pulsed-field gel electrophoresis to determine the genetic relatedness of the MRSA isolates and construct a dendrogramme

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Staphylococci are the most common bacteria isolated from clinical specimens worldwide (Mathews *et al*., 2010). In 1881, Sir Alexandra Ogston, a Scottish surgeon described the role of *Staphylococcus aureus* (*S. aureus*) in sepsis and abscess formation (Chakraborty *et al*., 2012). More than hundred years later, the bacterium remains a versatile and dangerous pathogen capable of causing a wide range of human infections and diseases (Gordon and Lowy, 2008). *Staphylococcus aureus* infections, particular those involving methicillin resistant strains (MRSA), are a recognized and emerging problem in both healthcare settings and in the community, worldwide (Green *et al*., 2010).

Staphylococcus aureus is a formidable pathogen because the bacterium produces extracelluar virulence factors that facilitate pathogenesis and colonisation of the human host (Grundmann *et al*., 2010). Nasal colonisation with *S. aureus* varies individually, about 20% are persistent carriers, while 30% are intermittent and 50% are never colonised (Otto, 2012). Healthcare workers are not nearly as an important reservoir as colonised or infected patients in the heathcare facility; but; the healthcare workers who are nasal carriers of *S. aureus* may serve as a source of MRSA transmission in these facilities (Grundmann *et al*., 2006). Recovery of an outbreak or endemic strain from a healthcare worker once does not provide any convincing proof that transmission of the bacterium to the patients occurred through the healthcare worker (Grundmann *et al*., 2006).

Bode *et al*. (2010) showed that the number of surgical-site infections caused by *S. aureus* acquired in the hospital setting can be reduced by rapid screening and decolonisation of nasal carriers on admission. The transmission of *S. aureus* from person to person is through skin to skin contact (direct contact) with an infected or colonised person (Miller and Diep, 2008). There are five Cs responsible for the transmission of *S. aureus* to humans, which have been listed by the Centers for Disease Control and Prevention in 2012: i) contact with an infected

or colonised person, ii) cleanliness, iii) compromised skin integrity, iv) contaminated objects and v) crowded living conditions.

Staphylococcus aureus strains were initialy susceptible to most antibiotics that were developed (Chambers and DeLeo, 2009). In the mid 1940's, a few years after the introduction of penicillin into clinical practice, penicillin resistant *S. aureus* (PRSA) strains emerged in hospitals, which spread to the community (Chambers and DeLeo, 2009). According to Kennedy and DeLeo (2009) 90% to 95% of clinical *S. aureus* strains throughout the world are resistant to penicillin. The development of multi-drug resistant *S. aureus* strains in healthcare and community settings has become a major concern because *S. aureus* is highly adaptable, easily transmissible and can be extremely virulent (Pang, 2008; DeLeo and Chambers, 2009).

In 1959, the first antistaphylococcal methicillin was developed by Beecham laboratories as a therapeutic agent for PRSA infections (Otto, 2012). Within two years of methicillin's introduction, the first MRSA strains emerged in the United Kingdom (Nastaly *et al*., 2010). Currently, MRSA is among the most important nosocomial pathogens worldwide, causing more than 50% of nosocomial infections in the United States of America (USA) (Nastaly *et al*., 2010). Methicillin resistant *S. aureus* strains has emerged in the community and are known as community-associated methicillin resistant *S. aureus* (CA-MRSA) (formerly called community-acquired methicillin resistant *S. aureus*) (Chambers and DeLeo, 2009). Community-associated MRSA strains were first discovered from the indigenous population in Western Australia in the 1990s but during the latter part of the 1990's, it became a major concern worldwide (Takano *et al*., 2008; Chambers and DeLeo, 2009). The strains have become a major cause of *S. aureus* infections in hospitals, especially in emergency departments and neonatal ICUs (Rehm and Tice, 2010). The definition of CA-MRSA is still a major problem because CA-MRSA markers, such as the Panton-Valentine leukocidin (PVL) are not carried by all CA-MRSA strains (Otter and French, 2008; Conceição *et al*., 2010).

The rapid emergence and evolution of CA-MRSA infections is one of the most surprising events in infectious diseases (DeLeo and Chambers, 2009). This is because community-associated *S. aureus* infections were initially caused by methicillin susceptible *S. aureus* (MSSA) strains, not the antibiotic resistant strains prevalent in hospitals (DeLeo and

Chambers, 2009). Thus, it remains a mystery why these antibiotic resistant bacteria emerged in a niche not obviously under the relatively high selective pressure exerted by antibiotics in the hospital setting (DeLeo and Chambers, 2009).

Phenotypic and genotypic data play an important role in the understanding of both HA-MRSA and CA-MRSA and in the evaluation of the effectiveness of infection control measures in hospitals (Shittu *et al*., 2009a). Molecular typing techniques, such as multiplex-PCR (M-PCR) assay, pulsed-field gel electrophoresis (PFGE) typing, multilocus sequence typing (MLST), Staphylococcal protein A typing (*spa*), coagulase typing (*coa*) and hypervariable region (HVR) typing have been used with great success in the identification and monitoring of the spread of unique MRSA strains that are circulating in hospitals and community settings (Strandén *et al*., 2003).

2.2 Classification of *S. aureus*

Staphylococci are prokaryotes and are grouped in the Kingdom *Eubacteria* (Ghebremedhin *et al*., 2008). Staphylococci are under the phylum *Firmicutes*, which consists of three major classes, namely *Bacilli*, *Clostridia* and *Mollicutes* (Briggs *et al*., 2012). On the basis of comparative 16S rRNA gene sequences, the genus *Staphylococcus* belongs to the Gram-positive bacteria with a low G+C DNA content (33% for *Staphylococcus aureus*) (Plata *et al*., 2009; Krismer *et al*., 2012). The highly conserved 16S rRNA is useful in the identification of staphylococci at the genus and species level (Widerström *et al*., 2012). However, the 16S rRNA lacks discriminatory power at the species level when compared to the *rpo*B (β-subunit of RNA polymerase) gene, which is able to identify staphylococci to the species and subspecies level (Widerström *et al*., 2012). Staphylococci are monophyletic and are well separated from the *Macrococcus* monophyletic genus with intergenera 16S rRNA sequence similarities of 93.4% to 95.3% (Götz *et al*., 2006; Lamers *et al*., 2012). The genus *Staphylococcus* comprises of 60 species and ten of the species have subspecies designations (Lamers *et al*., 2012; Widerström *et al*., 2012). To *et al*. (2011) reported a patient infected with catalase negative *S. aureus* subsp *aureus,* based on 16S rRNA sequencing, the isolate of this patient was found to be comparable to *S. aureus* catalase positive species (Figure 2.1).

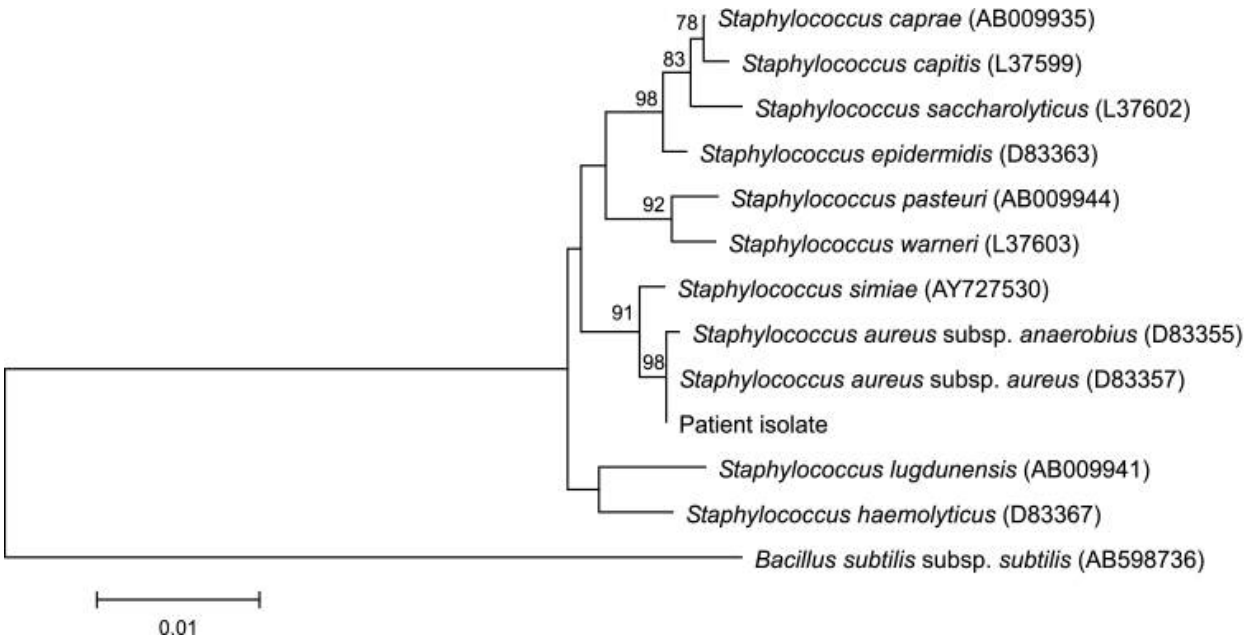

Figure 2.1: **Relationship of the patient's isolate to the related species. The tree was inferred from 16S rRNA data by the neighbour joining method and rooted using the 16S rRNA gene sequence of *Bacillus subtilis* subsp *subtilis* (To *et al*., 2011)**

The genus *Staphylococcus* includes species that are associated with humans, such as *S. aureus*, *S. capitis*, *S. caprae*, *S. epidermidis*, *S. haemolyticus*, *S. hominis*, *S. lugdunensis*, *S. saprophyticus* and *S. warneri* (Table 2.1) (Suzuki *et al*., 2012). Among the genus *Staphylococcus,* 39 coagulase negative (CoNS) species have been recognised, of which 18 are of medical importance to humans causing diseases ranging from skin infections to urinary tract infections (Piette and Verschraegen, 2009; Widerström *et al*., 2012).

Table 2.1: Scientific classification of *Staphylococcus aureus* (Yakoubou *et al*., 2010)

RANK	SCIENTIFIC NAMES
Domain	*Bacteria*
Kingdom	*Eubacteria*
Phylum	*Firmicutes*
Class	*Bacilli*
Order	*Bacillales*
Family	*Staphylococcaceae*
Genus	*Staphylococcus*
Species	*Staphylococcus aureus* *Staphylococcus epidermidis* *Staphylococcus haemolyticus* *Staphylococcus lugdunensis* *Staphylococcus saprophyticus* *Staphylococcus warneri*

The most important CoNS in staphylococci are divided into novobiocin susceptible and novobiocin resistant species (Götz *et al*., 2006). The two groups of novobiocin resistant species include *S. saprophyticus* (*S. cohnii* and *S. xylosus*) and *S. sciuri* (*S. lentus* and *S. vitulus*) (Götz *et al*., 2006; Lamers *et al*., 2012). Apart from the CoNS, two important coagulase positive and novobiocin susceptible species are *S. intermedius* (*S. delphini*) and *S. aureus* (*S. aureus* subsp *anaerobius*) (Turnidge *et al*., 2008; Lamers *et al*., 2012).

2.3 General characteristics of *S. aureus*

Staphylococcus aureus is a facultative anaerobic Gram-positive cocci, non-motile, non-spore forming, catalase and coagulase positive bacterium (Tiwari, 2009). Energy in *S. aureus* is generated through aerobic respiration and fermentation, which yields lactic acid (Plata *et al*., 2009). The bacterium is usually unencapsulated or has a thin capsule (Mims *et al*., 2004). Under a light microscope, the bacterium may be observed as single, in pairs, tetrads or having irregular grape-like clusters (Figure 2.2) (Götz *et al*., 2006). The cells of *S. aureus* are round and approximately 0.5 µm to 1.5 µm in diameter (Green *et al*., 2012). *Staphylococcus aureus* grows in a wide range of temperatures from 7°C to 48.5°C with an optimum temperature between 30°C to 37°C and at a pH of 4.2 to 9.3, with an optimum pH between 7.0 and 7.5 (Jeyasekaran *et al*., 2010).

The bacterial colonies usually form large white to golden pigmentation and appear smooth and convex (Holt *et al*., 2011). The colour of *S. aureus* colonies is caused by the presence of carotenoids (Plata *et al*., 2009). According to Plata *et al*. (2009) carotenoids are one of the virulence factors that protect *S. aureus* against oxidants that is produced by the immune system. The colonies of *S. aureus* are 6mm to 8 mm in diameter and certain *S. aureus* strains cause haemolysis on 5% sheep blood agar (Plata *et al*., 2009; Tolan *et al*., 2010). *Staphylococcus aureus* is resistant to many unfavourable environmental conditions, such as drying (Nastaly *et al*., 2010). However, the bacterium is tolerant to high concentrations of sodium chloride of up to 1.7 M and this enable the bacterium to be a temporal or permanent coloniser of the skin and mucosa (Plata *et al*., 2009; Nastaly *et al*., 2010). *Staphylococcus* species are catalase positive and this characteristic differentiates *Staphylococcus* species from *Streptococcus* species (Plata *et al*., 2009). Furthermore, *S. aureus* are oxidative negative and requires amino acids and vitamin B for growth (Plata *et al*., 2009).

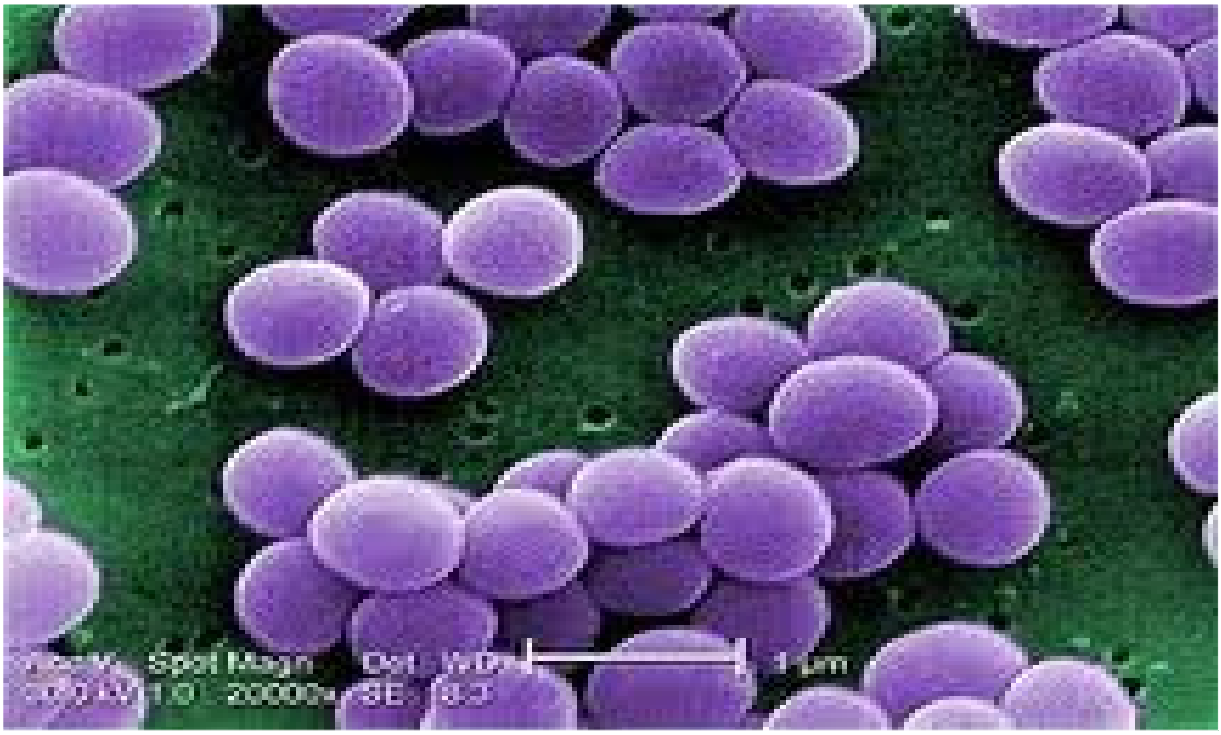

Figure 2.2: **Electron micrograph of *Staphylococcus aureus* cells showing the characteristic spherical cells arranged in grape-like purple clusters (Hung, 2011)**

The bacterium has a thick cell wall of about 20 nm to 40 nm, which consists of peptidoglycan, teichoic acid and surface proteins (Howden *et al*., 2010). The thick peptidoglycan layer of *S. aureus* is made of repeating disaccharide N-acetylglucosamine-N-acetylmuramic acid (GlcNac-MurNAc) units attached to teichoic acids (Atilano *et al*., 2010). The cell wall enables *S. aureus* to withstand dry conditions (Wasserman and Taljaard, 2011). Teichoic acids are water soluble polymers that contain repeating phosphodiester groups that are covalently linked to peptidoglycan and these groups are made of polyol (glycerol and ribitol) sugar and N-acetylamino sugars (Howden *et al*., 2010). Teichoic acid protects the bacterium to resist stressful environmental conditions, such as heat, low osmolarity, antimicrobial peptides, antimicrobial fatty acids, cationic antibiotics and lytic enzymes (eg lysozymes), which are produced by the host (Bera *et al*., 2007).

2.3.1 Genomic characteristics of *S. aureus*

Genome sequences especially for *S. aureus* have enabled scientists to explore questions of virulent strains, resistant strains, physiology and interaction of the bacterium with accuracy, which was previously not possible (Steven, 2009). The genome of *S. aureus* is about 2.8 Mb

to 2.9 Mb in size and circular (Zhang *et al*., 2009b). The chromosome of *S. aureus* encodes approximately 2 800 protein coding sequences, including structural and regulatory ribonucleic acids (McCarthy *et al*., 2012). The *S. aureus* genome consists of about a 75% conserved core genomic region, which encodes housekeeping genes (McCarthy *et al*., 2012). Ten percent are the core variable genomic region encoding surface and secreted proteins, while 15% are mobile genetic elements (MGEs) containing pathogenicity islands, bacteriophages, chromosomal cassettes, genomic islands, plasmids and transposons (Figure 2.3) (Lindsay *et al*., 2006; McCarthy *et al*., 2012).

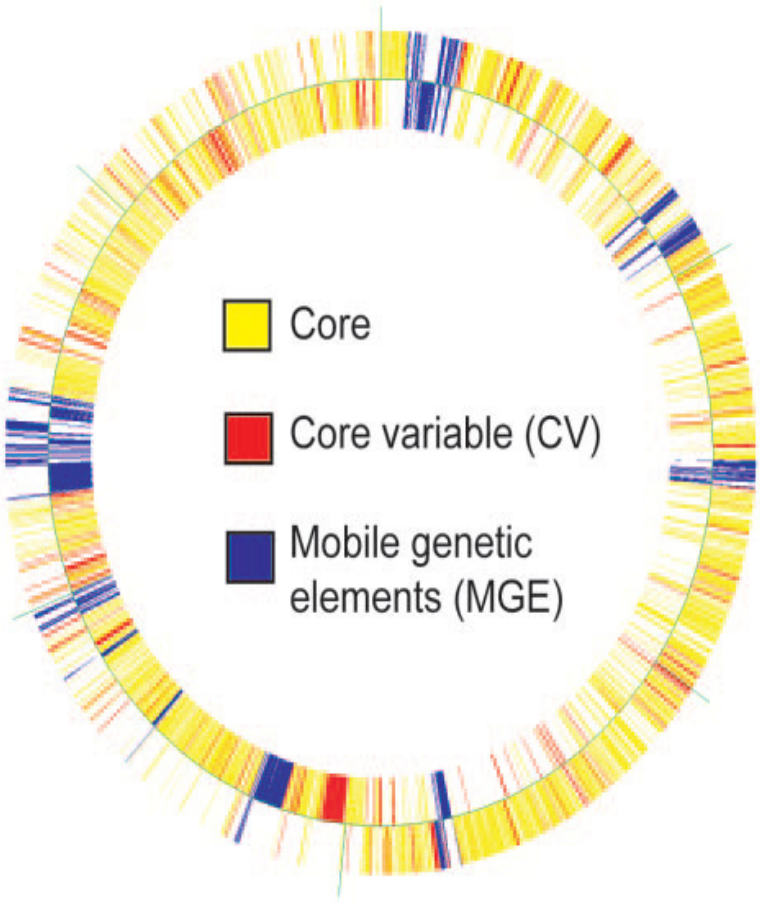

Figure 2.3: **Representation of the MRSA252 genome, with about 75% being the conserved core genomic region, 15% of the genome consists of mobile genetic elements and 10% of the genome consists of the core variable genomic region. The outer circle indicates genes on the forward coding strand and the inner circle represents genes on the complementary strand (Lindsay *et al*., 2006; McCarthy *et al*., 2012)**

Within the 75% core genomic region there are variable regions that contain genes with a high nucleotide substitution rate, such as the surface protein CapHTJK genes for *S. aureus* capsule production and global virulence regulators (*agr*, *trap* and *Sar*T), which are known to regulate

expression of the surface proteins (Lindsay *et al*., 2006; McCarthy *et al*., 2012). The surface proteins include *S. aureus* protein A (*spa*), fibronectin-binding protein A and B (Fnb A/B), collagen adhesion, *S. aureus* surface protein A to G, serine-aspartate repeat domain protein, coagulase (*coa*) and clumping factor A and B (*clf* A/B) (Steven, 2009). The accessory genomic region is assembled from MGEs that are integrated throughout the genome and carry 50% of the virulence factors (Steven, 2009). The MGEs are transferred horizontally between *S. aureus* strains and are thought to play an important role in the evolution of the bacterium's virulence and the emergence of new strains with clinical implications (Steven, 2009).

Kuroda and colleagues (2001) published the first full genome sequences of *S. aureus* strains Mu50 and N315. Up to date, more than 14 *S. aureus* genome sequences have been published and 30 await publication (Baba *et al*., 2008). The published *S. aureus* genome sequences include MW2, MRSA252 (USA200 strain), MSSA476 (USA400 strains), COL, USA300-FPR3737, USA300-HOU-MR, NCTC8325, ET3-1, JH9 and the Newman strain (Baba *et al*., 2008). The MW2 strain carrying the SCC*mec* type IVa (CA-MRSA strain) was reported to cause fatal septicaemia and septic arthritis in North Dakota (USA), while the epidemic MRSA252 (HA-MRSA) strain belonging to an epidemic MRSA-16 clone was found to be responsible for half of the MRSA infections in the UK and is one of the major clones found in the USA today (Baba *et al*., 2002; Holden *et al*., 2004).

The Mu50 and N315 clones are closely related to HA-MRSA strains and carry the SCC*mec* type II cassette and are vancomycin intermediate susceptible (VISA) (Baba *et al*., 2002; DeLeo and Chambers, 2009). The MSSA476 clone is an invasive CA-MSSA strain that contains a novel staphylococcal cassette chromosome$_{476}$ (SCC$_{476}$), which is integrated at the same site on the chromosome as the SCC*mec* element in MRSA strains and encodes a putative fusidic acid resistance protein (Holden *et al*., 2004). Phylogenetic analysis of MRSA strains has shown that COL, NCT8325, USA300 and Newman are clonally related to the N315, Mu50, MRSA252, MSSA476, JH1, JH9 and ET3-1 groups (Steven, 2009).

2.3.2 Pathogenic and virulence factors of *S. aureus*

Staphylococcus aureus has a large number of virulence factors and both structural and secreted products play a role in the pathogenesis of the infection caused by the bacterium

(Chakraborty *et al*., 2012). The bacterium's ability to cause diseases in both humans and animals is due to evasion of innate immunity, which includes resistance to killing by phagocytosis (DeLeo *et al*., 2010). Most *S. aureus* infections are caused by the production of the antiphagocytic microcapsule (type 5 or 8) and this capsule induces abscess formation (Gordon and Lowy, 2008). *Staphylococcus aureus* establishes an infection after the bacterium has been introduced into the host tissue as a result of minor abrasions, cuts or a mild injury (Gordon and Lowy, 2008; Green *et al*., 2012). Microbial surface components recognising adhesive matrix molecules (MSCRAMMs) initiate adherence to host tissues by binding molecules, such as collagen binding protein (Cna), fibronectin, fibrinogen binding protein A and B (FnbpA and FnbpB), elastin and protein A (Figure 2.4) (Gordon and Lowy, 2008; Yamamoto *et al*., 2010).

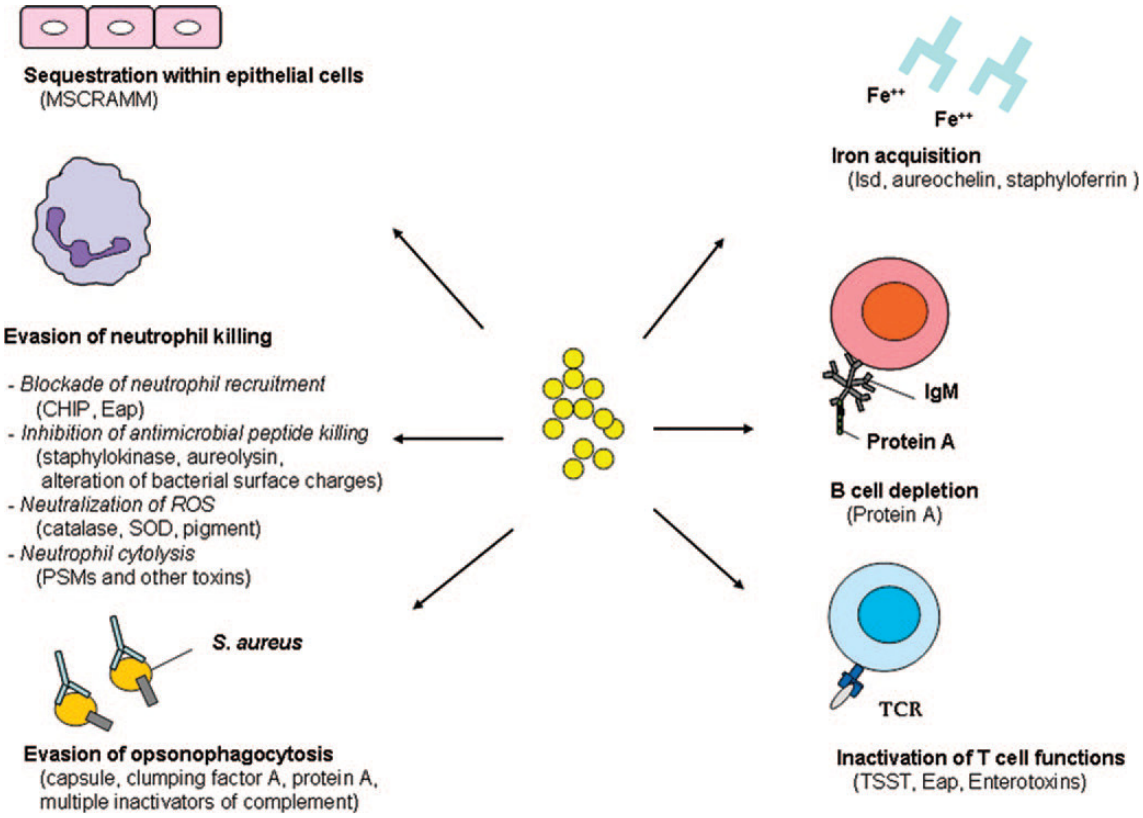

Figure 2.4: The survival strategies of *Staphylococcus aureus* during infection (Liu, 2009)

The MSCRAMM protein A binds to the Fc portion of immunoglobulins in *S. aureus*, thus preventing opsonisation (Gordon and Lowy, 2008). According to Liu (2009), *S. aureus* secretes chemotaxis inhibitory protein (CHIP) and extracellular adherence protein (Eap),

which block both neutrophil recognition to chemotactic factors and neutrophil binding to endothelial adhesion molecules. Neutrophils unleash antimicrobial substances, which include antimicrobial peptides, reactive oxygen species (ROS) and enzymes, such as proteases, lipases, lysozyme and elastases that destruct, invade and destroy the host tissue during an infection (Liu, 2009; Chakraborty *et al*., 2012). The defence mechanism against ROS is mediated by the antioxidant enzymes, such as catalase and superoxide dismutase, which neutralises the ROS and reactive nitrogen species (Liu, 2009). The antimicrobial peptides of the host are degraded by aureolysin and neutralised by staphylokinase (Liu, 2009). The ability of *S. aureus* to survive in the host cell depends on the successful acquisition of nutrients, such as iron, therefore, the bacterium secretes high affinity iron-binding compounds, such as aureocherin and staphyloferrin during iron starvation (Maresso and Schneewind, 2006; Liu, 2009).

The host adaptive immune response is induced by severe bacterial infections within 7 to 10 days to limit the infection and prevent re-infection with *S. aureus* (Liu, 2009). The mechanism of adaptive immune response evasion is poorly understood; however, enterotoxins (superantigen toxin) are believed to mediate this mechanism (Yamamoto *et al*., 2010). The toxins produced by *S. aureus* can cause various toxinoses, such as food poisoning and toxic shock syndrome (Gordon and Lowy, 2008). The superantigens cross-link major histocompatibility complex class II (MHC II) molecules, which are located on antigen-presenting cells with T-cell receptors to form a trimolecular complex (Plata *et al*., 2009). Trimolecular complex formation induces an intense T-cell proliferation in an antigen-independent manner, which results in the rapid release of cytokines especially interleukin (IL-2), interferon (IFN-γ) and tumour necrosis factor (TNF-α) (Hunault *et al*., 2012). The cytokines cause capillary leakage, epithelial damage and hypotension in the host tissue (Plata *et al*., 2009).

Three types of toxins are produced by *S. aureus*, which include cytotoxins, pyrogenic toxin and exfoliative toxins (Wu *et al*., 2011). The T cell functions are altered by the staphylococcal enterotoxins (SEs), toxic shock syndrome toxin (TSST) and Eap (a MHC class II analog) through targeting the T-cell receptor activation pathway (Liu, 2009). Moreover, TSST-1 and SEB suppress the movement of polymorphonuclear leukocytes (PNMs) through the inhibition of exoproteins' expression, allowing MRSA to invade and damage the host

tissues (Yamamoto *et al*., 2010). Some strains of *S. aureus* may produce epidermolysins or exfoliative toxins (ET) (Gordon and Lowy, 2008). The ET are produced by *S. aureus*, which digest desmoglein 1 causing exfoliation of the host epidermis cells (Mertz *et al*., 2007). The ET can be divided into ETA, ETB and ETD (Yamamoto *et al*., 2010). The ETA and ETB are commonly associated with staphylococcal scalded skin syndrome in infants and bullous impetigo in children, while ETD is associated with both bullous impetigo and deep pyoderma in adults (Nishifuji *et al*., 2008; Yamamoto *et al*., 2010).

Cytolytic toxins secreted by *S. aureus* have the ability to disrupt the host cell membrane by forming β-barrel pores in the cytoplasmic membranes resulting in the leakage of the cell's content and breakage (Plata *et al*., 2009). The different cytolytic toxins include α-haemolysin (Hlg), β-haemolysin, γ-haemolysin, leukocidin (Luk) and PVL (Prevost *et al*., 1995). The three cytolytic toxins differs such that α-haemolysin is cytolytic to human platelets and monocytes, while γ-haemolysin and leukocidin are cytolytic towards erythrocytes and leukocytes, respectively (Plata *et al*., 2009). Each leukotoxin is formed through the assembly of a Class S protein component (LukS-PVL, HlgA, HlgC, LukE) and a Class F protein component (LukF-PVL, HlgB, LukD) (Plata *et al*., 2009). The S component in PVL has the ability to bind to the host receptor tissue (polymorphonuclear leukocytes), while the F component is a toxic component that induces cell lysis (Yamamoto *et al*., 2010).

The PVL toxin gene is a bicomponent cytolysin, which contains two proteins that combine as subunits to form the leukotoxin that lyses leukocytes (Nastaly *et al*., 2010; Rehm and Tice, 2010). The two subunits are LukF-PV and LukS-PV (LukF/S-PV) proteins encoded within a prophage element called phiSLT (Figure 2.5) (Rehm and Tice, 2010). According to Löffler *et al*. (2010) the PVL gene is expressed by 2% to 3% of MSSA. Rossney *et al*. (2007) showed that less than 7% of CA-MRSA isolates in Ireland express the PVL toxin gene. In Korea and Canada, CA-MRSA strains do not carry the PVL gene (Zhang *et al*., 2008). Although PVL has long been known to be a potential virulence factor, its contribution to CA-MRSA pathogenesis, if any, still remains unclear (Kennedy and DeLeo, 2009). A study by Boyle-Vavra and Daum (2007) could not clearly show the difference between a PVL positive and PVL negative strain's ability to lyse human polymorphonuclear leukocytes.

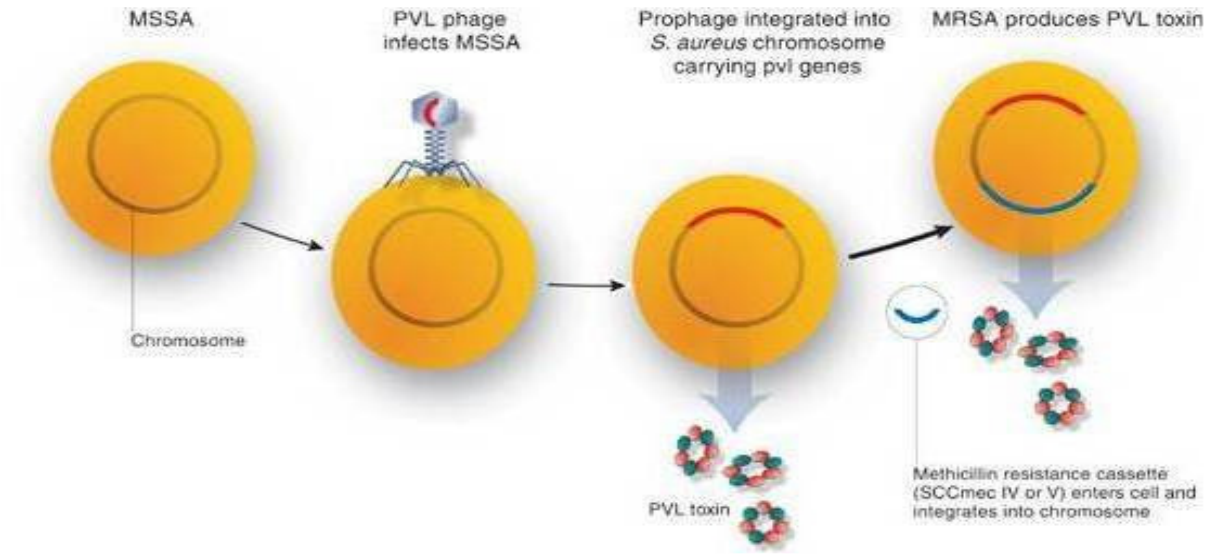

Figure 2.5: **Model for the emergence of PVL producing CA-MRSA. A MSSA strain is infected and lysogenised by a phage (phiSLT) that harbours the genes encoding the PVL. A methicillin resistant cassette (SCC*mec* IV or V) carrying the *mec*A gene is horizontally transferred into the PVL positive MSSA strain, which integrates into the genome distant from the phiSLT integration site (Rehm and Tice, 2010)**

Another virulence mechanism of MRSA includes biofilm (slime) formation, which allows HA-MRSA strains to persist on stainless steel and plastics (Smith and Hunter, 2008). The MRSA strains have retained the ability to produce biofilm using surface protein adhesins instead of polymeric *N*-acetyl-glucosamine (PNAG), which is important since the majority of bloodstream infections are associated with implanted medical devices (Schlievert *et al*., 2010; Pozzi *et al*., 2012). According to Plata *et al*. (2009), only 45% to 70% of clinical *S. aureus* strains (depending on the type of infection) are able to form biofilm. Novel cytolytic peptides called phenol-soluble modulin peptides (PSMs) are found in *S. aureus* (DeLeo *et al*., 2009). The HA-MRSA strains express a lower level of PSMs when compared to CA-MRSA strains and this indicates a possible defect in HA-MRSA virulence regulation (DeLeo *et al*., 2009).

2.4 Clinical manifestations of *S. aureus* infections

Staphylococcus aureus is the second most frequent pathogen cultured from the bloodstream of patients and is commonly found in hospitalised patients (Wasserman and Taljaard, 2011). The bacterium is able to cause local and disseminated infections, which can result in lesions in all tissues and anatomical sites (Grundmann *et al*., 2010). Primary infections caused by *S. aureus* manifest as skin and soft tissue (SST) infections and include pyogenic infections,

such as skin abscesses, carbuncles, furuncles, impetigo and atopic dermatitis (Figure 2.6) (Chakraborty *et al*., 2012; Green *et al*., 2012). The PVL positive (CA-MRSA) strains cause more severe infections compared to the PVL negative (CA-MRSA) strains (Boyle-Vavra and Daum, 2007). Primary staphylococcal infections may develop to become secondary infections through the bloodstream (Yamamoto *et al*., 2010).

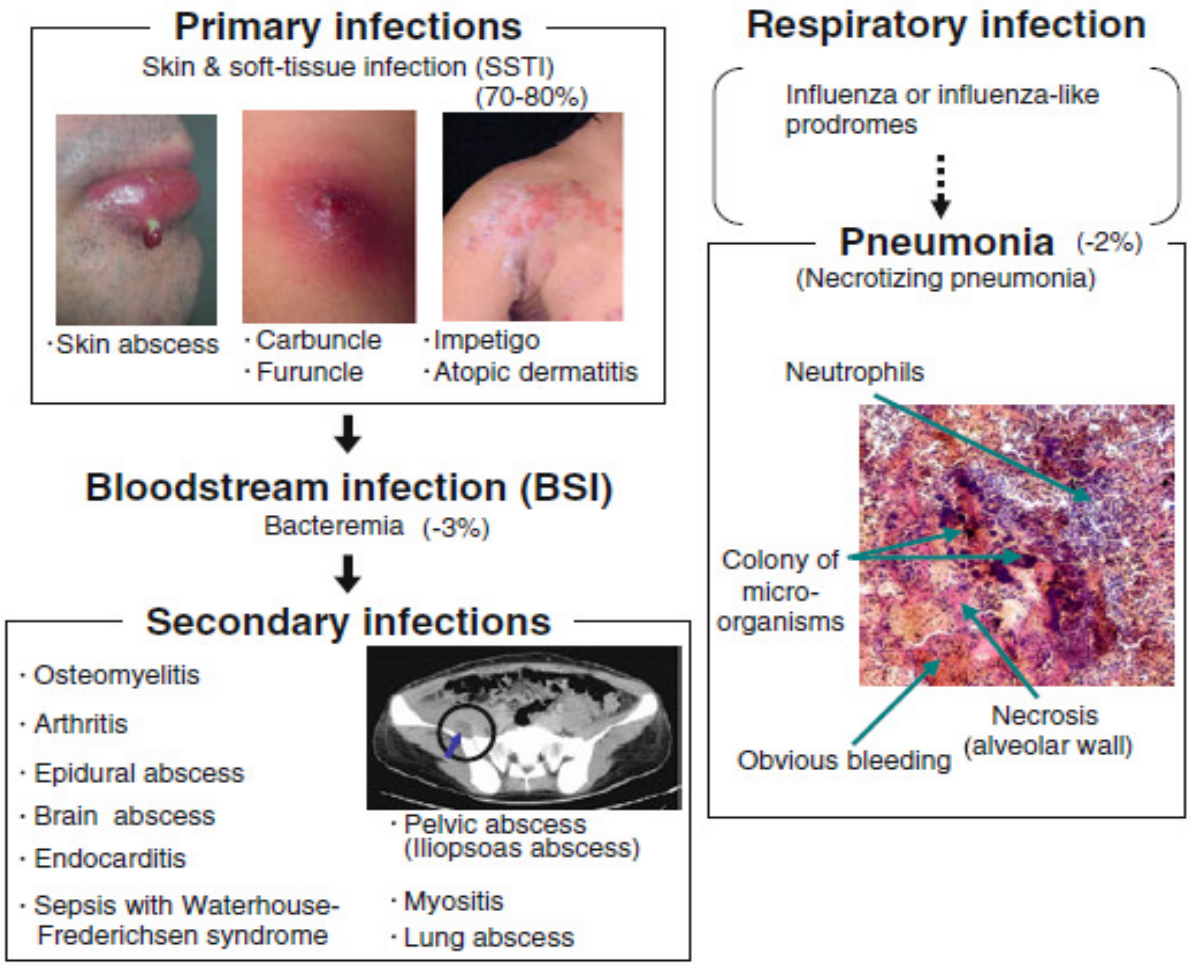

Figure 2.6: Common infections caused by *Staphylococcus aureus* and MRSA (Yamamoto *et al*., 2010)

The secondary infections include pelvic abscesses, lung abscesses, osteomyelitis, arthritis, brain abscesses, myositis and endocarditis (Yamamoto *et al*., 2010; Green *et al*., 2012). *Staphylococcus aureus* is among the most common pathogens, which cause osteomyelitis (Boucher *et al*., 2010). Osteomyelitis can affect a single portion of a bone or spread to the marrow, cortex, periosteum and surrounding soft tissue (Boucher *et al*., 2010). The symptoms and signs of osteomyelitis include an open wound exposing fractured bone, indolent draining fistula, local swelling and bone pain with no skin lesion (Boucher *et al*., 2010).

Staphylococcus aureus is an emerging cause of hospital and community-associated bacteraemia throughout the world (Plata *et al*., 2009). Mortality rates of CA-MRSA and HA-MRSA bacteraemia are 10% and 15%, respectively (Yamamoto *et al*., 2010). In the ICU, common sources of MRSA bacteraemia include implantable intravenous catheters, intravascular devices, soft tissue infections and ventilator-associated pneumonia (Boucher *et al*., 2010; Rahman *et al*., 2011). Staphylococcal pneumonia is commonly caused by CA-MRSA strains and these strains are currently called MRSA community-associated pneumonia (MRSA-CAP) (Yamamoto *et al*., 2010). The symptoms of patients with MRSA-CAP include high temperature, arterial hypotension, haemoptysis and rapid aggravation (Yamamoto *et al*., 2010). The MRSA-CAP mostly affects infants, young children and debilitated patients and can be complicated by influenza (Tolan *et al*., 2010). Apart from prolonged influenza-like prodromes (fever), PVL positive CA-MRSA can cause severe infections, such as necrotising pneumonia, necrotising fasciitis and septic shock (Orendi *et al*., 2010). In 1997 and 1998, two fatal paediatric cases, due to necrotising pneumonia of CA-MRSA (USA400), were reported in the United States of America (Yamamoto *et al*., 2010). The ability of *S. aureus* to cause infective endocarditis (IE) and other endovascular complications has led to the recommendation of cardiac imaging, such as computerised tomography for patients with *S. aureus* bacteraemia, prosthetic valves, permanent cardiac devices and cardiac conduction abnormalities (Boucher *et al*., 2010).

Other infections caused by *S. aureus* include exotoxin mediated infections, such as toxic shock syndrome (TSS), gastroenteritis (food poisoning) and staphylococcal scalded skin syndrome (SSSS) also known as Ritter von Ritterschein (in newborns) or Ritter's disease (Smith and Sandall, 2012). Symptoms of TSS include a high fever of ≥39°C, vomiting and diarrhoea, sunburn-like rash, hypotension, strawberry tongue, conjuctival hyperaemia and oedema of the palms and soles (Schlievert *et al*., 2010). The disease is most common in children under 2 years of age (Schlievert *et al*., 2010). In women, toxin TSST-1 has been shown to be associated with the use of highly absorbent tampons during menstruation (Schlievert *et al*., 2010). About 10% to 30% of women are colonised vaginally with *S. aureus* (Brosnahan and Schlievert, 2011). Only 5% of the *S. aureus* strains are capable of producing TSST-1 and this low percentage contributes to the rarity of the menstrual illness in women (Schlievert *et al*., 2007; Brosnahan and Schlievert, 2011).

Ritter von Ritterschein disease symptoms include fever, lethargy, malaise and poor feeding, followed by erythematous rash and the formation of large fragile fluid-filled blisters (Lipový *et al*., 2012). The localised form of SSSS is mainly restricted to the sites of infection and is recognised as "bullous impetigo" (Bukowski *et al*., 2010). The syndrome mainly affects newborns and childrens; however, in adults, a weak immune system and renal impairment are indicated to be risk factors (Bukowski *et al*., 2010). Food poisoning is associated with the consumption of food containing sufficient amounts of one (or more) preformed enterotoxins (Argudín *et al*., 2012). Symptoms occur rapidly, within 1 h to 6 h of ingestion and include nausea, vomiting and sometimes diarrhoea, dehydration and hypertension (Argudín *et al*., 2010). The infection does not spread to other parts of the body; however, in severe cases hospitalisation in infants, the elderly and immunocompromised people may be needed (Argudín *et al*., 2010; Drozdowski *et al*., 2010).

2.4.1 Antibiotic resistance of *S. aureus*

Most antimicrobial resistance in staphylococci emerges by the acquisition of resistance determinants found on plasmids, chromosomal cassettes, genomic islands and are associated with transposons or insertion sequences (Hanssen and Ericson Sollid, 2006; McDougal *et al*., 2010). Penicillin was highly effective for the treatment of *S. aureus* infections over the past decade (Kennedy and DeLeo, 2009). Resistance to the penicillin antibiotic is conferred by the β-lactamase enzyme, which hydrolyses the β-lactam ring, thereby inactivating the drug (Malachowa and DeLeo, 2010). The β-lactamase is encoded by the *bla*Z gene and the regulatory genes, *bla*I (repressor) and *bla*R (signal transducer) (Plata *et al*., 2009).

2.4.1.1 Methicillin resistance in *S. aureus*

Methicillin resistant *S. aureus* is generated when MSSA exogenously acquires a SCC*mec* (Tsubakishita *et al*., 2010). Isolates carrying the *mec*A gene are known as MRSA although these isolates are resistant to all β-lactam antibiotics (Plata *et al*., 2009). The *mec*A gene (2.1 kb) encodes a β-lactam insensitive cell wall that produces an abnormal penicillin-binding protein (PBP) 2a/2' (78 kD) (Oliveira and De Lencastre, 2011). The PBP2a has low affinity for all β-lactam agents and are joined to the cell membrane that speed the transpeptidation reaction (a key step for cross-linkage of peptidoglycan chains) (De Lencastre *et al*., 2007;

Chen *et al*., 2009; Marais *et al*., 2009). The PBPs of *S. aureus* is blocked by methicillin, a structural analogue of D-alanyl-D-alanine, while the new PBP2a protein acquired by MRSA strains prevents the incorporation of the β-lactam antibiotics into the cell wall (Deurenberg and Stobberingh, 2008). Therefore, the synthesis of the peptidoglycan layer is not disrupted, resulting in the growth of MRSA (Deurenberg and Stobberingh, 2008; Shariati *et al*., 2010).

The *mec*A gene is located on a large heterogeneous mobile chromosomal island or mobile genetic element, known as the SCC*mec* (Conceição *et al*., 2010; Otto, 2012). The genetic element is characterised by the presence of the *mec* complex and cassette chromosome recombinase (*ccr*) gene complex, the terminal inverted and direct repeats and the junkyard (J) regions (Japoni *et al*., 2011). The integration of SCC*mec* occurs at the 3´ end of the open reading frame X (*orf*X) at the specific site *att*B_{SCC} (bacterial chromosomal attachment site) (Berglund *et al*., 2009). The movement of the *ccr* gene complex is due to the recombinases, which mediates the integration of SCC*mec* into and out of the *S. aureus* chromosome (Ammons *et al*., 2010; Otto, 2012). To date, five classes (A to E) of the *mec* gene and three *ccr* genes complexes [*ccr*A, *ccr*B (*ccr*AB), *ccr*C] have been described (Ghaznavi-Rad *et al*., 2010). According to Kennedy and DeLeo (2009) the *ccr*C gene is only carried by SCC*mec* type III, V and VII. The SCC*mec* type III is the longest element and is composed of two cassettes, which includes SCC*mercury* (*ccr*C) and SCC*mec* type III (*ccr*AB) (Zhang *et al*., 2012).

The three major *mec* gene classes are classified as follows: class A harbouring the complete *mec*A regulon (*mec*I-*mec*R1-*mec*A-IS431); class B and class C harbouring the *mec*A regulatory genes disrupted by insertion sequences, ΨIS1272Δ*mec*R1-*mec*A-IS431 and IS431-Δ*mec*R1-*mec*A-IS431 (Oliveira and De Lencastre, 2011). The genes which are responsible for the regulation of *mec*A transcription include Δ*mec*R1 (SCC*mec* types I, IV, V, VI and VII) or *mec*R1 and *mec*I (SCC*mec* types II, III and VIII) (Milheiriço *et al*., 2007). The IS431 are found in SCC*mec* type I to VIII, while IS1272 is found in SCC*mec* type I, IV and VI (Hanssen and Ericson Sollid, 2006). The J region (J1 to J3) is used for subtyping of SCC*mec* type IV (Turlej *et al*., 2011). The J1 region is located right between the chromosome junctions to the *ccr* genes, while J2 is the region from the *ccr* genes to the *mec* complex (Deurenberg and Stobberingh, 2009). The J3 region is located from the *mec* complex to the left *orf*X of SCC*mec* (Deurenberg and Stobberingh, 2009). Up to date, the

SCC*mec* can be classified into eleven different types and eleven (SCC*mec* type IV) subtypes differing in size and combination of the *ccr* and *mec* complexes; type I (34.3 kb), II (53.0 kb), III (35.2/67 kb), IV (24.0 kb), V (27.6 kb), VI (24.0 kb), VII (41.3 kb), VIII (32.0 kb), IX (43.7 kb), X (50.8 kb) and XI (30 kb) (Figure 2.7) (Li *et al*., 2011a; Vanderhaeghen *et al*., 2012).

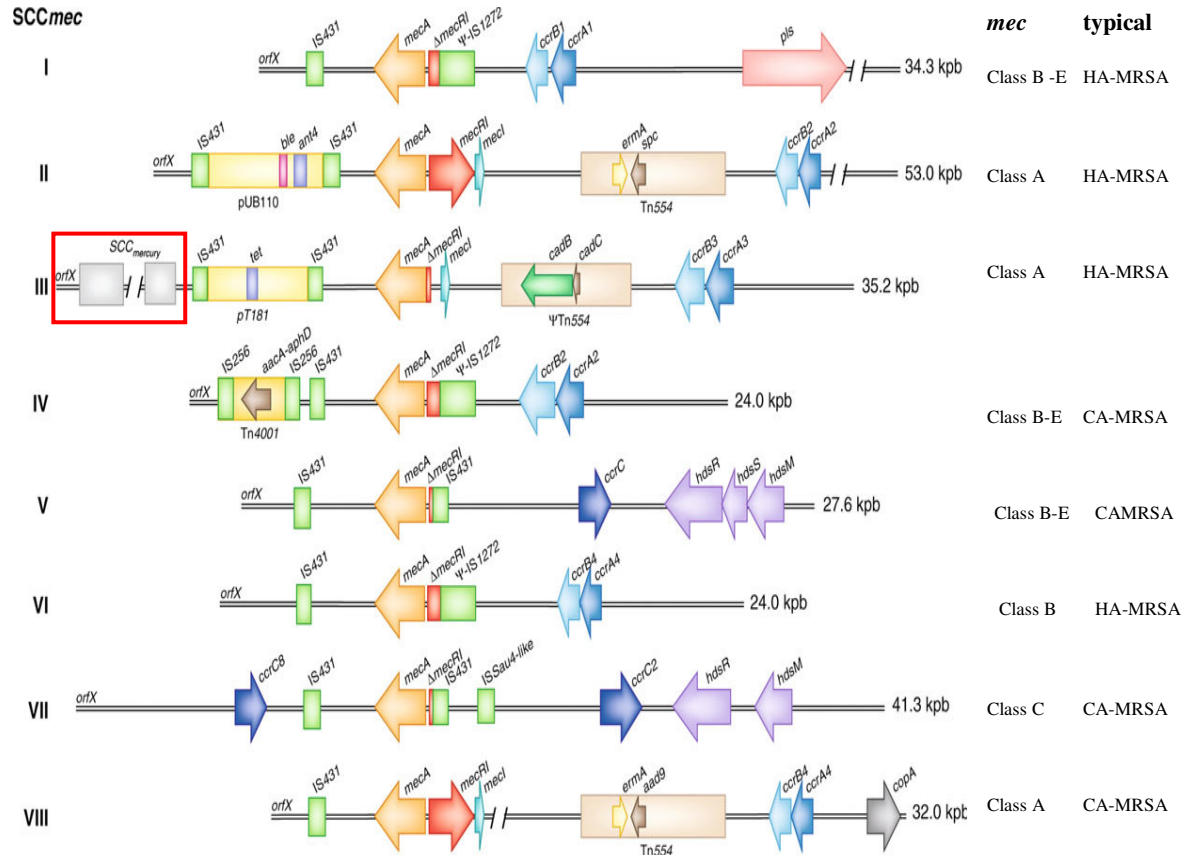

Figure 2.7: **Comparison of *S. aureus* SCC*mec* types. Class A SCC*mec* contains the complete *mec*A regulon (*mec*1-*mec*R1-*mec*A). Class B and class C SCC*mec* contain regulatory genes that are disrupted by IS1272Δ*mec*R1-*mec*A and IS431Δ*mec*R1-*mec*A, respectively. Tn554 encodes erythromycin and streptomycin/spectinomycin resistance; *cop*A encodes a putative copper-transport ATPase; *hsd*R, *hsd*M, and *hsd*S encodes a partial restriction-modification system type I; Tn4001 encodes an aminoglycoside resistance operon (*aac*A-*aph*D); plasmid pT181 encodes tetracycline resistance; WTn554 encodes cadmium resistance (*cad*B, *cad*C); plasmid pUB110 encodes bleomycin and tobramycin resistance (*ant*4') (Plata *et al*., 2009; Malachowa and DeLeo, 2010)**

The SCC*mec* type I, IV, V, VI and VII are resistant to β-lactam antibiotics only, while SCC*mec* type II, III and VIII are resistant to multiple classes of antibiotics, such as kanamycin, tobramycin, bleomycin, cadmium, tetracycline, macrolide, mercury, lincosamide

and streptogramin (Sangappa and Thiagarajan, 2012). This resistance is due to the integration of the SCC*mec* element within plasmids or transposons carrying multiple resistance genes (Deurenberg and Stobberingh, 2009). The SCC*mec* element is characterised as follows: type I-*mec* class B and *ccr*AB allotype 1(1B); type II-class A and *ccr*AB2 (2A); type III-class A and *ccr*AB3 (3A); subtypes (IVa, IVb, IVc, IVd, IVE, IVF, IVGg, IVh, IVi, IVj and IVk)-class B and *ccr*AB2 (2B) (Figure 2.8); type V-class C and *ccr*C (5C); type VI-class B and *ccr*AB4 (4B); type VII-class C and *ccr*C (5C1); type VIII-class A and *ccr*AB4 (4A), type IX-class C and *ccr*A1B1 (C2), type X-class C and *ccr*A1B6 (7C1), type XI-class E and *ccr*A1B3 (8E) (Turlej *et al*., 2011).

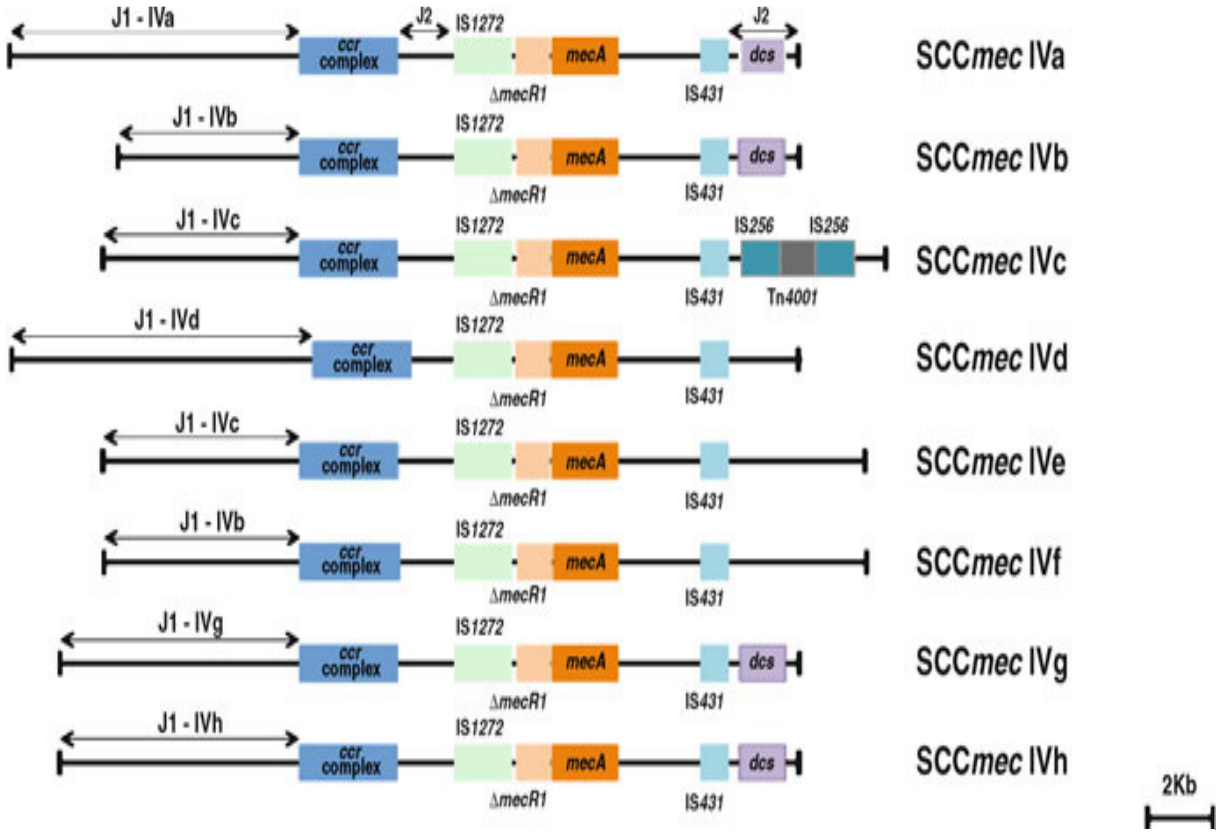

Figure 2.8: Subtypes of SCC*mec* type IV and the new SCC*mec* subtype IVh characteristic of epidemic clone MRSA-15 (Milheiriço *et al*., 2007; Oliveira *et al*., 2012)

Plasmid pUB110 carries the (*ant*4′) gene, responsible for resistance to kanamycin, tobramycin (*aad*D) and bleomycin (*ble*), while penicillin and mercury are encoded by plasmid pI258 (Deurenberg and Stobberingh, 2009). Tetracycline (*tet*K) resistance is coded by plasmid pT181, while Tn554 carries the spectinomycin (*spc*), erythromycin (*erm*A) and cadmium genes (Deurenberg and Stobberingh, 2009). Spectinomycin and *erm*A are responsible for inducible macrolide, lincosamide and streptogramin resistance (Deurenberg *et al*., 2007).

2.4.1.2 Vancomycin resistance in *S. aureus*

The glycopeptide antibiotic, vancomycin, was first released in 1958 and has since been used as a therapeutic agent for MRSA infections (Wasserman and Taljaard, 2011). Initial reports of reduced vancomycin susceptibility in clinical isolates of *S. aureus* from Japan in 1997 generated concern in the medical community (Méhes *et al*., 2012). The resistance of *S. aureus* strains to vancomycin is due to the presence of operon encoding enzymes (Courvalin, 2006). Vancomycin inhibits the last step of peptidoglycan synthesis by binding to the C-terminal dipeptide D-alanyl-D-alanine (D-Ala-D-Ala) of peptidoglycan precursors, preventing reactions catalysed by transglycosylases, transpeptidases and the D-D-carboxypeptidases (Figure 2.9) (Howden *et al*., 2010; Sangappa and Thiagarajan, 2012).

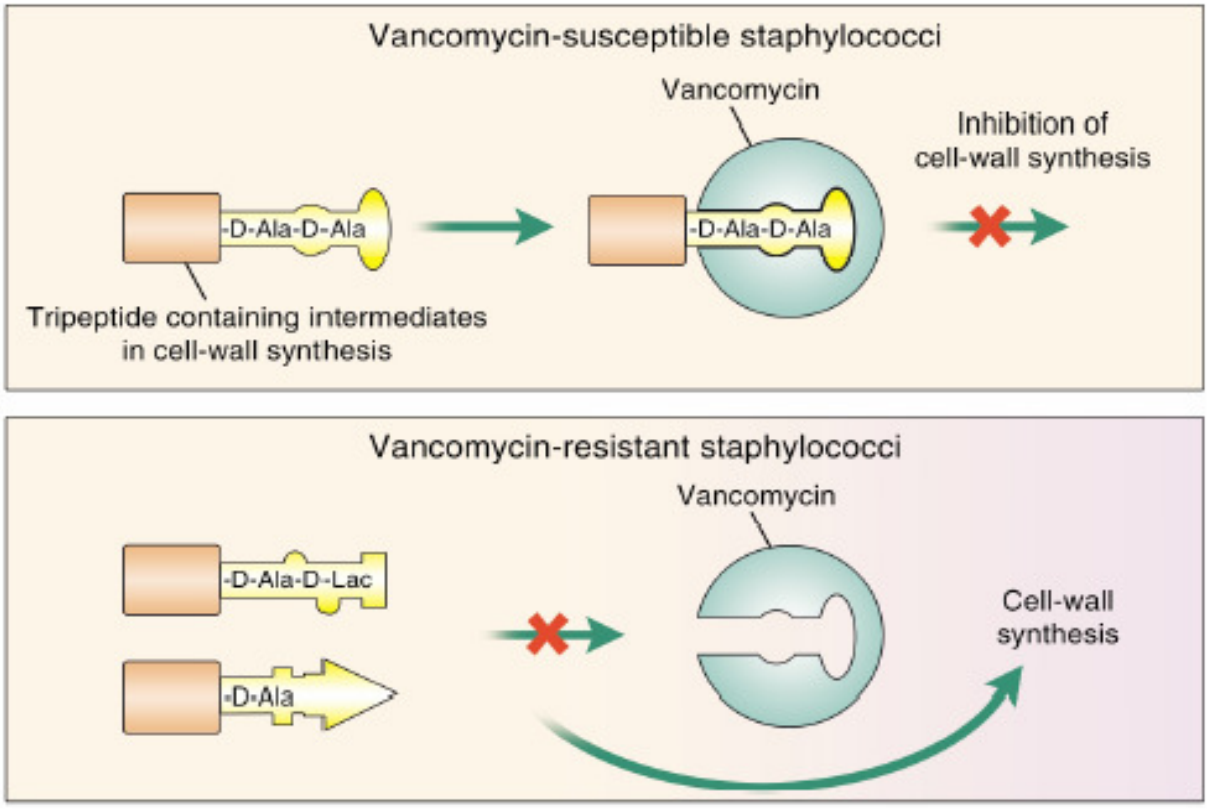

Figure 2.9: Mechanism of vancomycin resistance in VRSA strains (Sangappa and Thiagarajan, 2012)

High level of *S. aureus* resistance to vancomycin is initiated by the *van*A gene complex acquired from vancomycin resistant enterococci (VRE) (Stefani and Goglio, 2010). The *van*A gene is carried on the transposon Tn1546 in *Enterococcus* and is transferred to *S. aureus* by transconjugation (Saha *et al*., 2008). This results in the emergence of

vancomycin resistant *S. aureus* (VRSA) strains (Stefani and Goglio, 2010). *Staphylococcus aureus* strain with a minimum inhibitory concentration (MIC) of ≥16 $\mu g.m\ell^{-1}$ is called a VRSA (Kobayashi *et al*., 2012). Tiwari and Sen (2006) detected *mec*A positive VRSA strains, which were *van*A and *van*B negative by PCR, during August 2002 to July 2005 in India. To date, twelve VRSA strains have been identified in the USA that belongs to clonal complex 5 (Kobayashi *et al*., 2012).

2.4.1.3 Treatment and prevention of *S. aureus* infections

Strains of *S. aureus* are different; therefore, the first consideration for treatment should be towards the source of the infection (Wasserman and Taljaard, 2011). A localised infection, such as a deep-seated abscess requires incision and drainage rather than antibiotic use (DeLeo *et al*., 2010). Patients infected with serious HA-MRSA and CA-MRSA infections, such as endocarditis, septic shock and osteomyolitis are treated with intravenous vancomycin or daptomycin and these drugs are monitored because of their toxicity (DeLeo *et al*., 2010; Wasserman and Taljaard, 2011). The inability of vancomycin to decrease toxin production by CA-MRSA (PVL toxin) has resulted in linezolid (an oxazolidinone) being used for treatment of necrotising pneumonias, complicated skin and soft tissue (SST) infections and MRSA ventilator-associated pneumonia (Stryjewski and Corey, 2009). Clindamycin is active *in vitro* against 80% or more of CA-MRSA strains and works effectively in treating SST infections (DeLeo *et al*., 2010). The majority of MRSA isolates remain susceptible to chloramphenicol worldwide (Bouza, 2009). Six sequential multicentre national studies of *Staphylococcus* performed in Spain from 1986 to 2006 showed that the rates of chloramphenicol susceptibility ranged from 92% to 98%; however, chloramphenicol in association with vancomycin has shown antagonistic effects *in vitro* (Bouza, 2009).

Long-acting doxycycline and minocycline have greater antistaphylococcal activity than tetracycline; therefore, these antibiotics are effective in the treatment of SST infections caused by MRSA (Ruhe and Menon, 2007). Co-trimoxazole is active against 90% to 100% of SCC*mec* type IV and V (CA-MRSA) isolates, which makes this antibiotic appropriate for oral treatment of suspected CA-MRSA infections (DeLeo *et al*., 2010; Japoni *et al*., 2011). Community-associated bacterial pneumonia has been treated with tigecycline and this antibiotic appears to be unaffected by β-lactamase production or alteration in the target site

(Townsend *et al*., 2011). Patients who are allergic to β-lactam or quinolone antibiotics may benefit from the use of tigecycline (Townsend *et al*., 2011). New semisynthetic lipoglycopeptide antibiotics (dalbavancin, oritavancin, telavancin) and a cephalosporin (ceftobiprole) have been developed for the treatment of MRSA infections (Kennedy and DeLeo, 2009). *In vitro*, the three lipoglycopeptides have been shown to be effective against *S. aureus* and MRSA including the *van*B VRE (Zhanel *et al*., 2011). Dalbavancin and telavancin are effective against hetero-resistant vancomycin intermediate *S. aureus* (hVISA), vancomycin intermediate *S. aureus* (VISA) strains but not against VRSA strains (Zhanel *et al*., 2011).

Prevention methods for *S. aureus* and MRSA transmission in the healthcare and community settings include education regarding proper hand hygiene, wound coverage and disinfection (Barnes and Sampson, 2011). Other traditional methods used for prevention of multi-drug resistant *S. aureus* infections include the use of lysostaphin and antimicrobial peptides, such as mupirocin on the nasal area, tea tree oil, active and passive immunisation (Lawton *et al*., 2007; Stapleton *et al*., 2007; Schaffer and Lee, 2008). Promising vaccines, such as the capsular polysaccharide-protein conjugate vaccine and antibodies to the ligand-binding domains (clumbing factor) of several MSCRAMMs are under investigation (Daum and Spellberg, 2012).

2.5 Epidemiology of *S. aureus*

Patients who are hospitalised, immunocompromised and the elderly are commonly infected with HA-MRSA strains, while CA-MRSA strains affect young healthy people, athletes, prisoners, military personnel, intravenous drug users and homosexuals (Green *et al*., 2012). Risk factors associated with HA-MRSA infections include long-term antibiotic use, haemodialysis, ICU stay, catheters and prolonged hospitalisation (Cadena *et al*., 2012). The CA-MRSA risk factors include close physical contact, poor communal hygiene, school children and abrasion injuries (Gorwitz *et al*., 2008; Song et *al*., 2011). Researchers use various methods to characterise HA-MRSA and CA-MRSA strains into clonal groups in order to monitor outbreaks and to determine the source of infection (Otter and French, 2010). The commonly used molecular methods include PVL gene detection, SCC*mec* typing and subtyping, PFGE typing, MLST typing and *spa* typing (Table 2.2) (Yamamoto *et al*., 2010).

Table 2.2: Summary of the global distribution of the major HA-MRSA and CA-MRSA clones

Continent	Country	Strains per country/province		SCC*mec* types and subtypes	PFGE types	*spa* types	MLST	PVL Gene	References
Africa	South Africa	KZN	CA-MRSA	SCC*mec*IV	D, F, K & T	t064,	ST1173 & ST1338	-	Shittu *et al*., 2009b
			HA-MRSA	SCC*mec*III, SCC*mec*II, SCC*mec*I	F & G	t037, t045	ST239 & ST5		
		GP	CA-MRSA	SCC*mec*IV				-	Makgotlho *et al*., 2009; Moodley *et al*., 2010
			HA-MRSA	SCC*mec*III, SCC*mec*I, SCC*mec*II	D, K & T		ST239, ST612 & ST36		
		WP	CA-MRSA	SCC*mec*IV double-locus	D & E	t064, t1443 & t2196	ST612	-	Jansen van Rensburg *et al*., 2011; Stefani *et al*., 2012
			HA-MRSA	SCC*mec*III, SCC*mec*I, SCC*mec*II	A, (B to C) & F	t037, t045, t012	ST239, ST5 & ST36	-	
	Nigeria		CA-MRSA	SCC*mec*V, SCC*mec*IV		t0451, t008, t002, t064	ST8, ST94, ST5, ST8		Shittu *et al*., 2011
Asia	Japan		CA-MRSA	SCC*mec*IVx	USA300		ST8	-	Yamamoto *et al*., 2010
			HA-MRSA (NewYork/Japan clone)	SCC*mec*II	USA100		ST5, ST764	-	
	Taiwan		CA-MRSA	SCC*mec*VII/IV	USA1000, USA300		ST59 & ST30	+, +	Otter and French, 2010
			HA-MRSA (Hungarian clone)	SCC*mec*III			ST239		Yamamoto *et al*., 2010
Europe	UK		CA-MRSA (EMRSA-15 clone)	SCC*mec*IV	EUR-		ST80, ST22	+	Otter and French, 2010
			HA-MRSA (archaic clone, EMRSA-16 clone)	SCC*mec*I, SCC*mec*II		t008	ST250, ST36	-	Yamamoto *et al*., 2010
North America	USA		CA-MRSA	SCC*mec*IV	USA300, USA400		ST8, ST1	+, +	Otter and French, 2010
			HA-MRSA (New York/Japan clone)	SCC*mec*I, II	USA100		ST247, ST5	-	Yamamoto *et al*., 2010; Stefani *et al*., 2012

CA-MRSA -Community-associated methicillin resistant *Staphylococcus aureus*
EUR -Europe
GP -Gauteng
HA-MRSA -Healthcare-associated methicillin resistant *Staphylococcus aureus*
KZN -KwaZulu Natal
MLST -Multilocus sequence typing
PFGE -Pulsed-field gel electrophoresis
PVL -Panton -Valentine leukocidin
SCC*mec* -Staphylococcal chromosome cassette *mec*
spa -Protein A typing
UK -United Kingdom
USA -United States of America
WP -Western Province

The prevalence of MRSA infections varies worldwide and the variation depends on HA-MRSA and CA-MRSA characteristics (Al-Talib *et al*., 2010). Prevalence of MRSA ranges from over 50% in Portugal and Italy to below 2% in Switzerland and the Netherlands, because of infection control measures, such as active surveillance and the use of barrier precautions that were implemented (Ojulong *et al*., 2009). In Asia, the prevalence is estimated to be 50%, with extremely high rates in Hong Kong (75%) followed by Japan (72%) (Ojulong *et al*., 2009). In many African hospitals the prevalence of MRSA was estimated at 15%, with Nigeria showing a prevalence of 1.5% to 20% and South Africa with an increased prevalence of 29% to 46% (Jansen van Rensburg *et al*., 2011; Shittu *et al*., 2011).

During August 2005 to November 2006, Moodley and colleagues (2010) tested 320 MRSA isolates obtained from urine, catheter tips, tracheal aspirates, drainage fluid and CSF specimens to identify major MRSA clones that were widely spread in South Africa. The five major MRSA clones that were found to be circulating in the nine provinces are disseminated worldwide (Moodley *et al*., 2010). The major MRSA clones were PFGE type A *spa* type t045-SCC*mec*I-[sequence type (ST5)] [clonal complex (CC5)], PFGE type D *spa* type t037-SCC*mec*III-ST239 (CC8), PFGE type T *spa* type t012-SCC*mec*II-ST36 (CC30), PFGE type F *spa* type t064-SCC*mec*IV-ST612 (CC8) and PFGE type K *spa* type t012-SCC*mec*II-ST612 (CC8) (Figure 2.10) (Moodley *et al*., 2010). The MRSA clone PFGE type K *spa* type t012-SCC*mec*II-ST612 (CC8) was found to be widely distributed in all nine provinces of South Africa (Moodley *et al*., 2010). The PFGE type D *spa* type t037-SCC*mec*III-ST239 (CC8) clone was not identified in the Eastern and Western Cape provinces, while type F-*spa* CC64-SCC*mec*IV-ST612 clone was not identified in Limpopo, Gauteng and the North West provinces (Moodley *et al*., 2010).

Two different studies were done in Pretoria and KwaZulu Natal (South Africa) to identify the major clones that were circulating (Makgotlho *et al.,* 2009; Shittu *et al*., 2009b). In the Pretoria region, Makgotlho and colleagues (2009) reported a prevalence of 67% (65/97) SCC*mec* type II, 14.4% (14/97) SCC*mec* type III and 4% (4/97) SCC*mec*IVd of MRSA isolates obtained from the Steve Biko Academic hospital. Although Makgotlho *et al*. (2009) performed *spa* typing, no *spa*, MLST or PFGE types were provided. In KwaZulu Natal, Shittu *et al*. (2009b) reported three major PFGE pulsotypes, which included PFGE A t064

ST1173 SCC*mec*IV plus t064 ST1338 SCC*mec*IV [62.3% (38/61)], PFGE F t037 ST239 SCC*mec*III [16.4% (10/61)] and lastly PFGE (G) t045 ST5 SCC*mec*III [9.8% (6/61)].

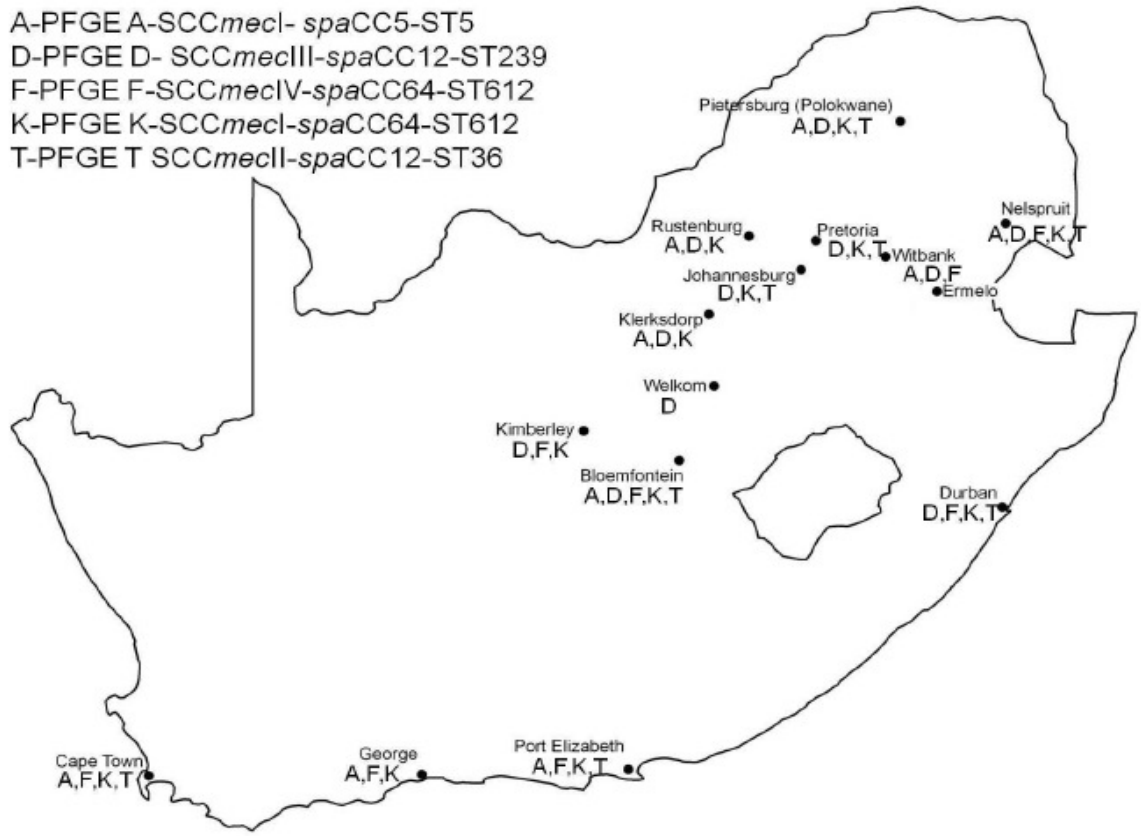

Figure 2.10: Prevalence of MRSA in South Africa demonstrated by province (Moodley *et al*., 2010)

One of the major clonal types (t037-ST239-SCC*mec*III) identified in KwaZulu Natal (South Africa) by Shittu and colleagues (2009b) were identified by Moodley and colleagues (2010) in Gauteng, South Africa. These major t037-ST239-SCC*mec*III clones were also found to be circulating in Brazilian hospitals (Yamamoto *et al*., 2010). The MRSA clones t045-ST5 identified by Moodley and colleagues (2010) carried SCC*mec* type I cassettes as opposed to the type III cassettes identified by Shittu and colleagues in 2009b. The SCC*mec* types I to V are distributed worldwide, while SCC*mec* type VI to XI are found in the countries of origin, such as Portugal, Sweden, Canada and Thailand (Turlej *et al*., 2011).

2.6 Diagnostic methods for the detection and identification of MRSA isolates

In healthcare settings, the different procedures employed for the identification and detection of MRSA from clinical specimens have variable results (Verkade *et al*., 2011). Phenotypic

methods used for the identification of MRSA in clinical samples should be accurate and have a high sensitivity and specificity (Baddour *et al*., 2007; Verkade *et al*., 2011). The use of rapid and reliable MRSA identification methods is imperative for appropriate therapy, prevention of the misuse of antibiotics and for the control of MRSA infections (Kaya *et al*., 2009). According to Schuenck *et al*. (2009) the existence of *S. aureus* isolates harbouring SCC*mec* type IV (CA-MRSA), which is associated with a MIC for methicillin near the breakpoint, could influence the detection of methicillin resistance. In addition, heterogeneous resistance occurring in oxacillin resistant staphylococci is not accurately detected when using the disk diffusion methods (Shariati *et al*., 2010). Heterogeneous resistance occurs when subpopulations of *S. aureus* (susceptible and resistant) co-exist in the same culture (Shariati *et al*., 2010).

2.6.1 Phenotypic methods for the identification and detection of MRSA isolates

Phenotypic tests are the most important diagnosis tools for staphylococcal infections (Kateete *et al*., 2010). In the clinical microbiology laboratory, the first phenotypic method to be used is culture (Reygaert, 2009a). Identification of white to yellowish colonies on blood agar plates or growth on mannitol salts or CHROMagar MRSA containing a chromogenic (blue) substrate after 24 h may indicate the growth of *S. aureus* or MRSA (Reygaert, 2009a; Lucke *et al*., 2010). A pure culture of *S. aureus* can be further confirmed by Gram-staining, catalase, DNase or tube coagulase testing (Kateete *et al*., 2010). Coagulase testing has provided false positive results, eg some staphylococci of animal origin are clumping factor negative and tube coagulase positive. These may be misidentified as *S. aureus* unless the fermentation of mannitol salt is utilised in addition (Koneman *et al*., 1997). In South Africa (KwaZulu Natal province), Shittu *et al*. (2007) reported the detection of a mannitol negative MRSA from clinical specimens.

Disk diffusion, agar dilution, microdilution and Epsilometer tests (E-tests) are different methods used for antimicrobial susceptibility testing (Shariati *et al*., 2010; Karami *et al*., 2011). Disk diffusion (Kirby-Bauer) is widely used for the detection of MRSA in routine laboratories and has a sensitivity of ≥95% and specificity of 95% to 100% (Taiwo, 2009). Unfortunately, the oxacillin disk diffusion test has been shown to be the least reliable test for the detection of MRSA (Karami *et al*., 2011). This is because borderline oxacillin resistant

S. aureus strains may hyper-produce β-lactamase and appear oxacillin resistant without possessing the genetic mechanism for the resistance (Mathews *et al*., 2010). The Clinical and Laboratory Standards Institute (CLSI, 2006) suggested the use of cefoxitin (30 µg) MIC testing as an indicator for the presence of methicillin resistance rather than oxacillin, since cefoxitin is a better inducer of the *mec*A gene (Reygaert, 2009a; Karami *et al*., 2011).

The MIC determination by either agar dilution or E-test is the gold standard for susceptibility testing (Bhateja *et al*., 2006). The E-test has shown a 100% (96/96) sensitivity and specificity for the detection of the *mec*A gene compared to the 98% (94/96) sensitivity and 99% (95/96) specificity obtained using the oxacillin agar screening method (Shariati *et al*., 2010). Other phenotypic methods include the Penicillin Binding Protein 2a Latex Agglutination (PBP2a-LA) assay, which is used for the detection of the PBP2a and has shown a sensitivity of 100% and specificity of 92.3% in identification of MRSA from blood cultures (Diab *et al*., 2008). The PBP2a-LA assay uses latex particles coated with monoclonal antibodies to PBP2a, which is extracted from the bacterial colonies and is an effective test to identify borderline MRSA, since the results are comparable to those of PCR (Rasheed and Ahmed, 2010; Yamada *et al*., 2010). The confirmation of PBP2a using the PBP2a-LA assay is fast (15 min to 30 min); however, the kit may require initial colony isolation, which might take time (Qian *et al*., 2010; Yamada *et al*., 2010).

Automated methods, such as the Vitek 2 (bioMérieux, Mary l'Etoile, France) and the MicroScan® (Siemens, Sacramento, California) can be used for combined identification and susceptibility testing and have a high sensitivity and specificity (Reygaert, 2009a). However, these methods are time consuming, since a pure culture is required for loading onto these analysers (Reygaert, 2009a). In general, phenotypic susceptibility testing methods differ between strains; therefore, the use of more than one phenotypic test is important for the accurate detection of all MRSA resistance strains (Essa *et al*., 2009). This is because MRSA isolates are affected by conditions, such as incubation time, temperature, inoculum concentration and NaCl concentration (Essa *et al*., 2009; Kaya *et al*., 2009).

2.6.2 Molecular methods for the detection and characterisation of MRSA isolates

Molecular methods have been used for the detection and typing of antibiotic resistance in *S. aureus* isolates (Afanas'ev *et al*., 2010). These techniques have the ability to distinguish between strains (or clones) of a given species allowing researchers to determine whether the causative agents of an outbreak of infection originated from a single source (Štěpán *et al*., 2004). The methods may generate either band or sequence based data of the different MRSA strains (Corrigan *et al*., 2012). Molecular methods are more advantageous in detection, typeability, discrimination and reproducibility when compared to phenotypic methods (Suhaili *et al*., 2009; Taiwo, 2009). The most widely used molecular methods for the detection and genotyping of MRSA isolates include conventional polymerase chain reaction (PCR), real-time PCR, staphylococcal cassette chromosome *mec* (SCC*mec*) typing, pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), staphylococcal proteinase A (*spa*) typing and coagulase (*coa*) typing (Oliveira *et al*., 2012).

2.6.2.1 Conventional PCR for the detection of MRSA isolates

The use of conventional PCR assays have become an important molecular tool for the detection of MRSA strains (Suhaili *et al*., 2009). Conventional PCR for the detection of the *mec*A gene is considered a gold standard for the identification of MRSA (Pramodhini *et al*., 2011). However, in order to obtain quick results, the use of a conventional multiplex-PCR (M-PCR) assay for the detection of the conserved genetic markers [16S rRNA, *nuc* (thermostable nuclease)] for species identification and resistance gene (*mec*A) detection seem to be convenient (Hendolin *et al*., 1997). The first multiplex-PCR (M-PCR) assay was developed by Chamberlain and colleagues in 1988 (Oliveira and De Lencastre, 2002). McClure *et al.* (2006) developed an M-PCR assay for the detection of the 16S rRNA, *mec*A and PVL genes. This M-PCR assay is used worldwide and is 100% accurate and reliable in the detection of the three genes (McClure *et al*., 2006). Al-Talib *et al*. (2009) used a pentaplex PCR assay to detect the 16S rRNA, *mec*A, PVL, *fem*A (involved in peptidoglycan synthesis) genes and a manufactured plasmid control. The pentaplex was found to be 97.6% sensitive and 99.3% specific in detecting the *mec*A gene of 230 clinical staphylococci isolates compared to the E-test (Al-Talib *et al*., 2009). The disadvantages of conventional M-PCR

assays include low amplification efficiency, variable efficiency on different templates, complexity and poor universality (Wen and Zhang, 2012).

2.6.2.2. Real-time PCR for the detection of MRSA isolates

Real-time PCR assays have changed the way clinical microbiology laboratories diagnose human pathogens (Espy *et al*., 2006). These assays use different fluorescents probes, such as Molecular Beacons™, TaqMan® technology and fluorescence resonance energy transfer (FRET) probes for the detection of resistance genes (Štěpán *et al*., 2004). The Becton Dickinson (BD) GeneOhm MRSA real-time PCR assay is available in the USA and has a sensitivity and specificity of 96.8% and 86.3% when compared to culture (Huh *et al*., 2011). This real-time PCR assay is used for the rapid identification of MRSA isolates from nasal swab specimens and includes primers, which target the junction between the conserved *orf*X and the SCC*mec* type I to IV (Reygaert, 2009b; Huh *et al*., 2011; Van der Zee *et al*., 2011). Amplified products are detected by fluorescent molecular beacons and the results are available within two to three hours (Tenover *et al*., 2004; Warren *et al*., 2004; Carroll, 2008). This assay is advantageous because the MRSA isolates are only ran in a batch, which is more convenient for diagnostic laboratories (Tenover *et al*., 2004; Warren *et al*., 2004; Carroll, 2008).

In 2009, Makgotlho and colleagues (2009) obtained a sensitivity of 100% (4/4) when using Taqman hydrolysis probes in the LightCycler 480 PCR assay to detect the PVL genes from MRSA isolates previously identified using a conventional M-PCR assay according to McClure *et al*. (2006). The GeneXpert MRSA assay kit from Cepheid is the latest real-time PCR assay, which detects SCC*mec* I to IV and the *orf*X gene (Reygaert, 2009b). According to Hombach *et al*. (2010) the GeneXpert MRSA real-time PCR assay has a higher sensitivity and specificity in detecting MRSA from nares and groin specimens compared to vaginal, axilla and throat specimens because of lower colonisation rates. The advantages of the GeneXpert real-time PCR assay include rapid turnaround time (75 min), extraction of DNA directly from the clinical specimens, amplification and detection of genes in separate disposable cartridges containing freeze-dried beads (Rossney *et al*., 2008). The limitation of this real-time PCR assay is that the SCC*mec* cassette are unstable where the chromocomal

*orf*X fragment merges with the *mec*A gene resulting in false-negative PCR results (Stürenburg, 2009).

2.6.2.3 Typing and subtyping of the SCC*mec* from MRSA isolates

The use of SCC*mec* typing with other typing methods, such as PFGE, allows proper clonal assignment of MRSA isolates (Deurenberg *et al*., 2007). Methicillin resistant *S. aureus* isolates can be classified using single or M-PCR assays (Chmelnitsky *et al*., 2008). The single PCR assay is the original method used to classify the HA-MRSA and CA-MRSA isolates by detecting the *ccr*, *mec* gene complexes and the J regions (Hanssen and Ericson Sollid, 2006). The discovery of more SCC*mec* elements has resulted in the development of reliable quick screening SCC*mec* typing assays, such as M-PCR assays (Zhang *et al*., 2012). The M-PCR SCC*mec* typing assay offers a rapid, simple and feasible method for typing MRSA isolates (Bouchami *et al*., 2009). In staphylococci, M-PCR assays are used for the identification of genes encoding MSCRAMMs, toxins and antibiotic resistance (Štěpán *et al*., 2004). The SCC*mec* elements can be determined using different M-PCR based methods developed over the past years, such as M-PCR assays by Ito *et al*. (2001); Oliveira and De Lencastre (2002); Zhang *et al*. (2005); McClure *et al*. (2006); Boye *et al*. (2007); Kondo *et al.* (2007); Milheiriço *et al*. (2007); Zhang *et al*. (2008); McClure *et al*. (2010) and Zhang *et al*. (2012).

Oliveira and De Lencastre (2002) developed M-PCR assay for the discrimination of SCC*mec* types I to IV from MRSA isolates. The limitation of this M-PCR assay is that, only SCC*mec* type I to IV are characterised; therefore, SCC*mec* type V (CA-MRSA) is sometimes mistyped as SCC*mercury* (Chongtrakool *et al*., 2006). Zhang *et al*. (2005) and Boye *et al*. (2007) developed M-PCR assays to study the organisation of the SCC*mec* type I to V elements. The Zhang *et al.* (2005) assay showed 100% (59/59) sensitivity and specificity for the detection of SCC*mec* type I to V and subtypes IVa to IVd. The disadvantages of this method is that SCC*mec* V is mistyped as SCC*mec* III and the primers used to identify SCC*mec* type III are specific for SCC*mercury* (Jansen *et al*., 2009). Universal primers targeting the J1 region of SCC*mec* type II and type III have been developed by Zhang *et al*. (2012) to resolve misclassification of SCC*mec* type II and III. The Boye *et al*. (2007) M-PCR assay has shown to be 98% (306/312) accurate in identifying MRSA isolates from Denmark, with 84%

(261/312) of the MRSA belonging to SCC*mec* type IV. The limitation of the Zhang *et al.* (2005) and Boye *et al.* (2007) methods is that only one locus on the majority of the SCC*mec* types is detected (Milheiriço *et al*., 2007). Kondo *et al*. (2007) developed a M-PCR assay, which uses five M-PCR reactions; however, the impracticability of the method makes it difficult for routine diagnostic laboratory usage because of the large numbers of PCR reactions required (Ghaznavi-Rad *et al*., 2010).

Milheiriço *et al*. (2007) upgraded the M-PCR assay described by Oliveira and De Lencastre (2002) by amplifying six specific loci inside the J1 region of SCC*mec* type IV and using the *ccr*AB2 gene as an internal control. The Milheiriço *et al*. (2007) assay is rapid and accurate for the detection of SCC*mec* subtype IVa to IVh. McClure *et al*. (2010) used an M-PCR assay for the detection of the SCC*mec* type VIII element, which was previously identified by Zhang *et al.* (2009a). This M-PCR assay has shown to be rapid in the discrimination of *S. aureus* strains from CoNS and MRSA from MSSA by using different primer pairs obtained from *S. aureus* and *S. epidermidis* strains (McClure *et al*., 2010). In order to avoid confusion during SCC*mec* type classification, Chongtrakool *et al*. (2006) proposed that SCC*mec* elements should be classified based on the combination of the *ccr* complex type and the *mec* gene classes. Furthermore, the subtypes should be defined based on the differences in the J region (Chongtrakool *et al*., 2006).

2.6.2.4 Pulsed-field gel electrophoresis for the typing of MRSA isolates

The PFGE method was developed by Schwartz and Cantor in 1984 (Herschleb *et al*., 2007). This method is the main discriminative MRSA typing method and provides higher resolution for MRSA outbreak investigation in hospitals compared to *spa* typing and MLST (Sangal *et al*., 2012). Bacterial genomic DNA is isolated and digested with a rare base cutter endonuclease restriction enzyme (RE) *Sma*I (recognising CCCGGG) or *Csp*I (Oliveira *et al*., 2012). The restriction generates 12 to 20 macrorestricted fragments of 48.5 kb to 582 kb when an electrical field with an alternating voltage gradient is applied in opposite directions in different time periods in the agarose gel (Oliveira *et al*., 2012). The most important factor in determining the PFGE banding pattern include the choice of RE, since the cleavage site of each RE is unique (Li *et al*., 2009). The RE that has long, not frequently occurring recognition motifs provides greater resolution in PFGE (Chen *et al*., 2005). The resulting

PFGE patterns obtained can be analysed by the GelCompar II software programme using the Dice coefficient and unweighted pair group method with arithmetic mean (UPGMA) to construct a dendrogrammes (Cerrone *et al*., 2012). The PFGE patterns can also be clustered manually according to the Tenover *et al*. (1995) criteria. The bacterial isolates that have one or two different band shifts consistent with a single genetic event are referred to as a subtype and isolates with seven or more such genetic difference are considered to be a different type (Tenover *et al*., 1995). In the USA, a national MRSA database has been established based upon the PFGE profile of genomic DNA digested with *Sma*I (McDougal *et al*., 2003). The disadvantage of PFGE is that high quality DNA is required (Hallin *et al*. 2012). Standardisation of accurate electrophoresis protocols are also needed to enable inter-laboratory comparisons (Hallin *et al*. 2012). Nonetheless, once PFGE has been established in a laboratory, the method can be used to differentiate between strains of different bacterial species (Deurenberg and Stobberingh, 2009).

2.6.2.5 Multilocus sequence typing of MRSA isolates

The multilocus sequence typing method uses DNA sequencing to uncover allelic variants in housekeeping genes (usually seven genes), such as 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) in *S. aureus* (Li *et al*., 2009). These seven genes are coding proteins of central metabolic functions (Singh and Kumar, 2001; Kuhn *et al*., 2006). An internal fragment of the housekeeping gene (about 500 bp) is amplified by each primer pair allowing accurate sequencing of the 450 bp fragments of each gene on both strands (Wisplinghoff *et al*., 2003). Each of the seven sequenced gene fragments (450 bp to 500 bp) is assigned a number, combined into an allelic profile and given a ST through the MLST database (http://saureus.mlst.net/sql/multiplelocus.asp) (Hadjihannas *et al*., 2012). Based upon the MLST method, *S. aureus* strains that are similar at five or more of the seven housekeeping genes are placed within the same CC, which is determined by a BURST (based upon related sequence types) algorithm (Kennedy and DeLeo, 2009). The MLST typing combined with PFGE typing provide more information on how to classify and differentiate isolates (eg ST8 contains both USA300 and USA500, which belongs to CC8) (Kennedy and DeLeo, 2009). However, when compared to PFGE typing, MLST typing has a lower discriminatory power,

since the method sometimes cannot differentiate bacterial strains within a species (Hall *et al*., 2010; Oliviera *et al*., 2012). The disadvantage of MLST is that sequencing of the seven housekeeping genes is expensive, time consuming and an automated DNA sequencer is required (Al Nakib *et al*., 2011).

2.6.2.6 Staphylococcal proteinase A typing of MRSA isolates

Staphylococcal protein A (*spa*) typing is used to detect the repeat region (polymorphic region X) of the protein A gene (40 kD to 60 kD) of *S. aureus* (Oliveira *et al*., 2012). Protein A is encoded by the *spa* locus (species-specific gene product) of *S. aureus,* which protects the bacteria from host defense mechanisms (Majeed *et al*., 2012). The *spa* locus is composed of about 2 150 bp and carries the IgG Fc-binding region, X-region and C-terminus (Grundmann *et al*., 2010; Majeed *et al*., 2012). The X-region of protein A consists of the variable number of tandem repeats (21 bp to 27 bp) varying in composition that results in different *spa* types (Taiwo, 2009). The *spa* locus is highly polymorphic because of the internal variable region of short tandem repeats, which vary not only in numbers but also due to nucleotide substitutions within individual repeat units (Grundmann *et al*., 2010). A public *spa* type database (http://tools.egenomics.com/) and the Ridom Spa Server (http://spaserver.ridom.de/) with differential assignment (eg *spa*1 for the former and *spa*t008 for the latter) are used for the naming of *spa* types (Yamamoto *et al*., 2010). The database of the *spa* server has more than 1 200 *spa* types that contains a combination of 100 *spa* repeats from more than 3 000 isolates typed in 36 countries around Europe (Salaam-Dreyer, 2010). Staphylococcal proteinase A typing is an appropriate method for local and short term MRSA epidemiological studies (Oliveira *et al*., 2012). This method provides portable, easy to use and biologically meaningful molecular typing data (Grundmann *et al*., 2010). Interpretation of the *spa* sequences is done using a dedicated software package known as StaphType (Ridom GMBh, Wűrzburg, Germany), which has shown 100% reproducibility between laboratories (Oliveira *et al*., 2012). However, the method has a lower discriminatory power than PFGE, since two closely related strains cannot be discriminated (Koreen *et al*., 2005; Palavecino, 2007; Petersson *et al*., 2010).

2.6.2.7 Coagulase typing of MRSA isolates

Staphylocoagulase (SC) is an extracellular protein produced by *S. aureus*, *S. intermedius*, *S. hyicus* and *S. selphini* (Hirose *et al*., 2010). This protein is a virulence factor of *S. aureus*, which causes coagulation of plasma and is used to differentiate *S. aureus* from the less virulent staphylococci, such as *S. epidermidis* (Hirose *et al*., 2010). The protein produced by *S. aureus* interacts with prothrombin in the blood by converting fibrinogen into fibrin resulting in coagulation of plasma (Plata *et al*., 2009). The coagulase gene is similar to the *spa* gene, because it has a polymorphic repeat region that is used to distinguish *S. aureus* from other staphylococci (Güler *et al*., 2005). The variable region of the *coa* gene is comprised of 81 bp tandem short sequence repeats that are variable in both number and sequence as determined by restriction fragment length polymorphism (RFLP) analysis of PCR products (Shopsin *et al*., 2000). The *coa* typing method could provide more information in epidemiological investigations, since the *coa* gene has lots of sequence variations compared to MLST and *spa* genes (Li *et al*., 2011b). The main advantage of *coa* typing is the low cost and the method only requires two sequencing reactions per isolate (Li *et al*., 2011b).

2.6.2.8 Hypervariable region typing of MRSA isolates

The hypervariable (HVR) region is found between the IS431*mec* and *mec*A gene in MRSA (Tsubakishita *et al*., 2010). This typing method is used for genotyping of MRSA strains and contains a truncated mevalonate (*mva*S) gene encoding the 3-hydroxy-3-methylglutaryl–coenzyme A (HMG-CoA) synthase and varied numbers of direct repeat units (DRUs) (40 bp each) (Schmitz *et al*., 1998; Senna *et al*., 2002; Tsubakishita *et al*., 2010). Apart from the *mva*S gene and DRUs, the HVR has a full *ugp*Q gene that encodes the glycerophosphoryl diester phosphodiesterase and a full monoamine oxidase (*mao*C) gene that encodes the acyl dehydratase (Tsubakishita *et al*., 2010). Senna and colleagues (2002) showed that the HVR technique displayed a lower discriminatory power compared to the PFGE technique. Makgotlho *et al*. (2009) showed that HVR has a greater discriminatory power than *spa* typing, when six clusters were identified compared to three clusters identified by *spa* typing (Makgotlho *et al*. 2009). The limitation of the HVR technique in the Makgotlho *et al*. (2009) study was that one of the CA-MRSA isolates could not be discriminated when compared with the *spa* typing technique. Although the *mec*-HVR PCR technique has been shown to have

low discriminatory power when compared to PFGE, this method is rapid, reproducible, inexpensive, easy to perform and capable of demonstrating the differences between MRSA strains (Senna *et al*., 2002).

2.7 Summary

The species of the genus *Staphylococcus* are subdivided into non-pathogenic and facultative pathogenic strains (Fuchs *et al*., 2012). About one-third of the general population are colonised with *S. aureus* and are at risk of acquiring infection (Springer *et al*., 2009; Guggenberger *et al*., 2012). The bacterium causes a variety of infections and secondary infections can develop after *S. aureus* disseminates from the blood into surrounding tissues resulting in death (Kokai-Kun *et al*., 2007; Edwards and Massey, 2011). The increased resistance of *S. aureus* to antibiotics is seen globally and this complicates treatment of infections (Springer *et al*., 2009). Initially, MRSA was an established hospital pathogen, but in the 1990s, the bacterium has emerged in the community causing severe infections due to the PVL gene harboured by some of the CA-MRSA isolates (Berglund *et al*., 2005; Chini *et al*., 2006; Chua *et al*., 2011). The emergence of CA-MRSA in the hospital has increased the importance of multi-drug resistant bacterium awareness (Strandén *et al*., 2009).

Different strategies have been employed to prevent the spread of MRSA worldwide (Deurenberg *et al*., 2007). These strategies include the use of rapid, accurate and reliable phenotypic and genotypic methods for the identification and detection of MRSA strains (Boye *et al*., 2007). Phenotypic methods, such as culture and catalase, DNase and coagulase tests are used as standard methods in the diagnosis of staphylococcal infections (Zurita *et al*., 2010). These phenotypic methods are used in combination with susceptibility testing, such as the E-test (Bhateja *et al*., 2006). Although, the use of phenotypic methods remains important in the diagnostic laboratories, molecular methods may be essential in the genotyping of MRSA isolates (Kennedy and DeLeo, 2009).

Molecular typing techniques are important tools in tracking the source and confirming the route of transmission during an MRSA outbreak in hospital settings (Versalovic and Lupski, 2002; Hallin *et al*., 2012). Multiplex-PCR assays are used to simultaneously detect several antibiotic resistance genes at different chromosomal loci in one single reaction (Aarts *et al*.,

2001). These assays are highly sensitive and specific in the detection of the resistant genes found in MRSA isolates (McClure *et al*., 2006). Real-time PCR assays are rapid methods that can be used for the detection of *mec*A in MRSA isolates and has been shown to improve turn-around time (Te Witt *et al*., 2010a). The use of different SCC*mec* typing and subtyping assays in laboratories has enabled the classification of MRSA strains into HA-MRSA and CA-MRSA (Iwao *et al*., 2012).

Several genotypic techniques have been used with different discriminatory powers and reproducibility, which include *spa*, MLST, PFGE, HVR typing and *coa* typing (Kwon *et al*., 2011; Moussa, 2011). Staphylococcal proteinase A typing has become the most used MRSA typing method due to the high throughput capacity and reproducibility, which enable the comparison of data globally through online Ridom StaphType software (Te Witt *et al*., 2009; Majeed *et al*., 2012). Similar to *spa* typing, MLST typing allows the definition of unequivocal strain types and the results are exchangeable between laboratories (Te Witt *et al*., 2009). Many clinical laboratories are unable to use the MLST methods due to the high cost (Te Witt *et al*., 2009). The PFGE typing using *Sma*I as restriction enzyme for the analysis of the genomic DNA has superior discriminatory power campared to *spa* typing (Nübel *et al*., 2011). Although, PFGE is time-consuming, the method has proven to be sufficiently robust to type MRSA strains with greater resolution and has been considered to be the best typing method for the investigation of healthcare-associated MRSA outbreaks and hospital-to-hospital transmission (Kwon *et al*., 2011; Lim *et al*., 2012).

Implementation of M-PCR assays and genotyping methods in clinical microbiology laboratories may provide proper diagnosis of MRSA and may contribute to the evaluation of infection control measures and surveillance programme, which may help in preventing the spread of MRSA in the particular hospital (Te Witt *et al*., 2010b; Moussa, 2011; Stegger *et al*., 2011). The aim of this study was to detect, identify and characterise 194 MRSA isolates obtained from the Steve Biko Academic Hospital in the Gauteng province from April 2010 to August 2011 using M-PCR assays followed by PFGE genotyping to determine the genetic relatedness of these MRSA isolates.

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CHAPTER 3

Genetic relatedness of methicillin resistant *Staphylococcus aureus* isolates from a tertiary academic hospital in the Pretoria region, South Africa

The editorial style of the Journal of Diagnostic Microbiology and Infectious Disease was followed in this chapter

3.1 ABSTRACT

Methicillin resistant *S. aureus* (MRSA) strains are a major cause of infections and are globally distributed in hospital and community settings. Treatment of MRSA infections is often difficult due to the resistant genes expressed by these strains. This study aimed to identify and characterise 194 clinical MRSA isolates obtained from a tertiary academic hospital in the Pretoria region during April 2010 to August 2011. The isolates were typed using six multiplex-PCR assays and grouped into clonal types using the gold standard, pulsed-field gel electrophoresis (PFGE). All 194 clinical MRSA isolates were 100% positive for the 16S rRNA and *mec*A genes. Only one (0.5%) community-associated MRSA (CA-MRSA) isolate harboured the Panton-Valentine leukocidin gene. Five staphylococcal cassette chromosome *mec* (SCC*mec*) typing multiplex-PCR assays were used to characterise healthcare-associated MRSA (HA-MRSA) and CA-MRSA isolates. Inconclusive results were obtained for 26.3% (51/194) MRSA isolates, which showed either bands for SCC*mec* type II or SCC*mercury*. The remaining 143 MRSA isolates showed a prevalence of 64.3% (92/143) for HA-MRSA and 35.7% (51/143) for CA-MRSA isolates. The dominant SCC*mec* types for the HA-MRSA isolates were: SCC*mercury* [74% (68/92)], SCC*mec* type II [19.5% (18/92)] and SCC*mec* type I [6.5% (6/92)]. The dominant SCC*mec* types and subtypes for the CA-MRSA isolates were: SCC*mec* subtypes IVd [92.1% (47/51)], IVa [3.9% (2/51)], IVb [2% (1/51)] and SCC*mec* type V [2% (1/51)]. No SCC*mec* type III or VIII was detected. Pulsed-field gel electrophoresis revealed eleven pulsotypes designated pulsotype A to K. The major pulsotype was A with subtypes A1 to A6, which included 66% (127/191) of the HA-MRSA isolates and 19% (36/191) of the CA-MRSA isolates. Pulsotypes B to K had distinct band patterns and included 7% (13/191) of the HA-MRSA isolates and 8% (15/191) of the CA-MRSA isolates. This study indicated a decrease in HA-MRSA and an increase in CA-

MRSA strains compared to the previous study conducted in this clinical setting. The majority of the HA-MRSA and CA-MRSA strains were closely related. The combined molecular methods were essential tools in the accurate characterisation and monitoring of the changes in the prevalence of the MRSA strains over time. Surveillance and control measures are necessary to monitor the circulation of MRSA strains in this clinical setting.

Keywords: CA-MRSA, HA-MRSA, *mec*A, M-PCR, MRSA, PFGE, SCC*mec*

3.2 INTRODUCTION

Staphylococcus aureus (*S. aureus*) is an important bacterial pathogen and a leading cause of morbidity and mortality worldwide (Kobayashi and DeLeo, 2009). The bacterium can cause a broad variety of infectious diseases, ranging from minor skin infections to post-operative wound infections (Deurenberg and Stobberingh, 2009). Infections caused by *S. aureus*, particularly those caused by methicillin resistant *S. aureus* (MRSA) strains are recognized as a serious problem in inpatient and outpatient settings (Ammons *et al*., 2010; Nichol *et al*., 2011). This is because MRSA strains have become resistant not only β-lactam antibiotics but to other antibiotics, such as tetracycline, rifampicin and chloramphenicol (Suhaili *et al*., 2009). Multi-drug resistant MRSA strains are of concern, since these strains represent a major challenge in both infection control and treatment strategies due to the limitation of treatment options available for these types of infections (Kolman *et al*., 2010).

These challenges are worsened by the emergence of community-associated methicillin resistant *S. aureus* (CA-MRSA) strains (Moussa, 2011). Community-associated MRSA often causes infection among healthy younger children and adults with no exposure to healthcare settings (Hudson *et al*., 2012). Patients infected with CA-MRSA strains have increasingly been noted worldwide, presenting with skin and soft tissue infections, necrotizing fasciitis, blood stream infection and septic shock (Diep *et al*., 2006; John and Schreiber, 2006; Rossney *et al*., 2007). Some CA-MRSA infections have been found to be associated with the toxic gene encoding Panton-Valentine leukocidin (PVL) (Campana *et al*., 2007). The presence of the PVL gene among certain CA-MRSA strains makes these strains more virulent (Cocchi *et al*., 2011). As a result, clinicians are faced with the emergence of CA-MRSA strains that are genetically different from HA-MRSA strains (Kader *et al*., 2011).

Up to date, eleven different SCC*mec* types (I to XI) and eleven subtypes of SCC*mec* type IV (IVa to IVk) have been described, differing in structure and size (Iwao *et al*., 2012; Rolo *et al*., 2012). Healthcare-associated methicillin resistant *S. aureus* (HA-MRSA) strains are resistant to multiple antibiotics and carry SCC*mec* types I, II, III and VI (Strandén *et al*., 2009). Community-associated MRSA strains are susceptible to most non-β-lactam antibiotics and carry the SCC*mec* types IV, V, VII, VIII, IX, X and XI (Coombs *et al*., 2011). The phenotypic and genotypic differences of HA-MRSA and CA-MRSA strains make it possible

to distinguish these two groups of MRSA (Shilo and Quach, 2011; Iwao *et al*., 2012). Outbreaks of CA-MRSA have been reported in hospital settings in the United States; with some reports stating that CA-MRSA could be replacing HA-MRSA (Joshi, 2011; Song *et al*., 2011; Hudson *et al*., 2012).

Phenotypic methods, such as the disc diffusion and microdilution tests are used in diagnostic laboratories for the identification and detection of methicillin resistance in *S. aureus* isolates (Kader *et al*., 2011). *Staphylococcus aureus* strains, which are *mec*A (methicillin determinant) positive, differ in the level of expression of resistance (Velasco *et al*., 2005; Pramodhini *et al*., 2011). The expression of the *mec*A gene maybe suppressed in some *S. aureus* strains resulting in these strains to remain undetected during phenotypic testing (Kaur *et al*., 2012). Molecular techniques are rapid and sensitive tools that can detect genes (Fluit *et al*., 2001). These techniques may provide a better understanding of multi-drug resistant strains circulating in hospital and community settings, so that monitoring the spread of resistant pathogens or resistance genes is possible (Fluit *et al*., 2001; Chen *et al*., 2009; Sibley *et al*., 2012).

Staphylococcal cassette chromosome *mec* (SCC*mec*) typing is one of the most essential molecular tools used for understanding relatedness of MRSA strains (Moussa *et al*., 2012). However, the complex nature and diverse structure of the SCC*mec* element require the use of SCC*mec* typing methods in combination with other genotyping methods, such as pulsed-field gel electrophoresis (PFGE), *spa* typing and multilocus sequence typing (MLST) to obtain more accurate and reliable results (Zhang *et al*., 2005; Frickmann *et al*., 2012; Otter and French, 2012). Pulsed-field gel electrophoresis of genomic macrorestriction fragments is by far the most widespread genotyping tool and the most discriminative MRSA typing method (Hallin *et al*., 2007; Struelens *et al*., 2009). The PFGE typing method is regarded as the gold standard for MRSA typing and has been used to identify major pandemic MRSA clones, such as the Iberian, Brazilian, Hungarian, New York/Japan and paediatric clones worldwide (Ko *et al*., 2005; Faria *et al*., 2008; Oliveira *et al*., 2012).

Defining the different characteristics of MRSA strains using SCC*mec* and PFGE assays may play an imperative role in understanding the genetic relatedness of MRSA strains and in evaluating the effectiveness of infection control in hospital settings, so that the appropriate

regimen for patients' treatment is initiated (Murchan *et al*., 2003; Hagen *et al*., 2005). In the Pretoria region, there is no information available regarding the genetic relatedness of MRSA strains using PFGE typing. The aim of this study was to detect, identify and characterise 194 clinical MRSA isolates obtained from a tertiary academic hospital in the Pretoria region, South Africa, using six M-PCR assays followed by PFGE genotyping to determine the genetic relatedness of these isolates.

3.3 MATERIALS AND METHODS

3.3.1 Study setting, collection and confirmation of MRSA isolates

This is a semi-quantitative study conducted at the University of Pretoria, Department of Medical Microbiology/National Health Laboratory Service (NHLS) and approved by the Student Ethics Committee of the University of Pretoria (protocol number S189/2010). Hundred and ninety four (194) MRSA isolates were randomly obtained from clinical specimens sent from a tertiary academic hospital in the Pretoria region for analysis to the diagnostic laboratory at the Department of Medical Microbiology during April 2010 to August 2011.

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 department, intensive care unit, anti-retroviral clinic, oncology, urology, gynaecology, nephrology and internal medicine. *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, Vitek 2 system (bioMérieux, Mary l'Etoile, France) and E-test (AB Biodisk, Solna, Sweden) methods. The MRSA isolates were recovered from blood cultures (*n*=75/194, 39%), pus swabs (*n*=44/194, 23%), central venous pressure tips (*n*=25/194, 13%), tissue (*n*=16/194, 8%), endotracheal aspirate (Luki) (*n*=12/194, 6%), sputum (*n*=7/194, 4%), unknown (*n*=6/194, 3%), urine (*n*=4/194, 2%), briviac tip (*n*=2/194, 1%), bone fragment (*n*=1/194, 0.5%), catheter tip (*n*=1/194, 0.5%) and cerebrospinal fluid (*n*=1/194, 0.5%). The MRSA isolates were subcultured on Blood agar plates (Oxoid, England) to obtain single colonies for Gram-staining. The plates were incubated (Labcon, Sepsci, South Africa) 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 °C (New Brunswick Scientific, England) for future use (Appendix B).

3.3.2 DNA extraction of MRSA isolates

Bacterial DNA extraction of the 194 MRSA isolates was performed using a phenol-chloroform based method (Sambrook and Russell, 2001; Makgotlho *et al*., 2009) with the following modifications: A volume of 10 µℓ of lysozyme (50 mg.mℓ$^{-1}$) (Roche Applied Science, Germany) was added to each of the cell suspensions and incubated (AccuBlock™, Whitehead Scientific, South Africa) at 37°C for 1 h. A volume of 50 µℓ of a 20% sodium dodecyl sulphate (SDS) solution (Promega, Madison, USA) was added to each tube, followed by 10 µℓ proteinase K (20 mg.mℓ$^{-1}$) (Roche Applied Science, Germany). Eppendorf tubes (Eppendorf AG, Hamburg, Germany) were incubated (Grant instrument, Cambridge, England) at 56°C for 1 h until the suspension was clear.

Several gentle extractions with equal volumes of mixed phenol:chloroform:isoamylalcohol (Merck, Darmstadt, Germany) (3X) in the ratio of 25:24:1 were performed. The suspension was centrifuged (Spectrafuge 24D, Labnet International, Inc., New Jersey, USA) at 4 930 *x g* for 5 min at 25°C. The supernatant was transferred to a sterile 2 mℓ Eppendorf tube (Eppendorf AG, Hamburg, Germany) and an equal volume of chloroform:isoamylalcohol (24:1) (Merck, Darmstadt, Germany) was added to the suspension. The suspension was centrifuged (Spectrafuge 24D, Labnet International, Inc., New Jersey, USA) at 4 930 *x g* for 20 min at 15°C. The supernatant (DNA-containing aqueous phase) was removed and added to a sterile 2 mℓ Eppendorf tube (Eppendorf AG, Hamburg, Germany) and the concentration of the aqueous phase was adjusted using a stock solution of 3 M sodium acetate (Merck, Darmstadt, Germany). Equal volumes of ice-cold, 100% absolute ethanol (-20°C) (Merck, Darmstadt, Germany) was added and mixed, the tubes were stored overnight at -20°C (Kelvinator, Gauteng) to precipitate the DNA. The DNA was pelleted by centrifugation (Spectrafuge 24D, Labnet International, Inc., New Jersey, USA) at 2 682 *x g* for 10 min at 4°C and the supernatant was discarded. The pellets were desalted with 400 µℓ of ice-cold, 70% absolute ethanol (-20°C) (Merck, Darmstadt, Germany) and the supernatant was discarded, the tubes were inverted to dry. The DNA pellet was resuspended in 100 µℓ of

Tris-EDTA [10 mM Tris-HCl, 1 mM EDTA (pH 8)] buffer (Sigma-Aldrich, St. Louis, USA) and stored at -20°C (Kelvinator, Gauteng) until further analysis (Appendix B).

3.3.3 Multiplex-PCR assays for the detection of MRSA genes

Six multiplex-PCR (M-PCR) assays were utilised to detect and characterise the clinical MRSA isolates. The six M-PCR assays were performed using the Qiagen Kit (Qiagen, Germany). The final concentration of each primer pair for the M-PCR assays was prepared according to the method provided in the Qiagen Multiplex PCR manual 2008 (Qiagen, Germany) (Appendix B). The DNA from the MRSA isolates was amplified using the G-storm thermocycler (Vacutec, UK). The amplicons of the six M-PCR assays were separated at 100 $V.cm^{-1}$ (voltage) using a 1% MetaPhor™ agarose gel (Lonza, Rockland, USA) containing 5 µℓ of ethidium bromide (10 $mg.mℓ^{-1}$) (Promega, Madison, USA). Visualisation of the bands was done using an Ultra Violet light box (DigiDoc, UVP product, Upland, California) (Appendix B).

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

The first M-PCR assay was 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 concentration [0.2 µM, 0.24 µM and 0.2 µM from McClure *et al*. (2006)] was modified to 2 µM for each primer. The M-PCR assay reaction compositions are shown in Table 3.2. The PCR programme for the G-storm (Vacutec, UK) thermocycler 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.

3.3.5 Multiplex-PCR assays for the typing of SCC*mec* type I to V and subtyping of SCC*mec* subtype IVa to IVd

The second and third M-PCR assays were performed to type SCC*mec* I to V and subtype SCC*mec* IVa to IVd according to the methods described by Zhang *et al*. (2005) and

Boye *et al*. (2007). The nine primer pairs (Zhang *et al*., 2005) detected SCC*mec* type I, SCC*mec* type II, SCC*mec* type III, SCC*mec* subtype IVa, SCC*mec* subtype IVb, SCC*mec* subtype IVc, SCC*mec* subtype IVd, SCC*mec* type V including an internal control for the *mec*A gene. The Boye *et al*. (2007) M-PCR assay included four primer pairs detecting the IS1272 (SCC*mec* type I), *ccr*A2-B (SCC*mec* type II), *ccr*C (SCC*mec* type III), IS1272 and *ccr*A2-B (SCC*mec* type IV), *ccr*C and *mec*A-IS*431* (SCC*mec* type V). The primer concentrations for the Zhang *et al*. (2005) method were modified according to the assay described by Makgotlho *et al*. (2009) while different primer concentrations of the third SCC*mec* M-PCR typing assay were prepared according to the method by Boye *et al*. (2007). The size of the amplicons, concentration, primer sequences and M-PCR assay reaction compositions are shown in Table 3.3 and Table 3.4. The PCR programme for the G-storm thermocycler (Vacutec, UK) was modified as follows: initial activation step at 95°C for 15 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 90 s and extension at 72°C for 90 s and a final extension step at 72°C for 10 min.

3.3.5.1 Single-PCR assay for typing of the SCC*mec* type II and III

Single-PCR assays were performed on the MRSA isolates, which showed discrepancies for SCC*mec* type II (Zhang *et al*., 2005) and SCC*mec* type III (Boye *et al*., 2007). Two primer pairs from the Boye *et al*. (2007) assay were used to target *ccr*A2-B (SCC*mec* type II) and *ccr*C (SCC*mec* type III) genes. The primer pair concentrations, amplicon size, primer sequences and M-PCR assay reaction compositions are shown in Table 3.3 and Table 3.4. The M-PCR conditions used to amplify the DNA are shown in Section 3.3.5.

3.3.6 Multiplex-PCR assay for the typing of SCC*mec* type I to III

A fourth M-PCR assay for SCC*mec* typing was performed according to the method described by Oliveira and De Lencastre (2002) on HA-MRSA isolates carrying SCC*mec* type I, SCC*mec* type II and SCC*mec* type III according to the Zhang *et al*. (2005) and Boye *et al* (2007) assays. The M-PCR assay included four primer pairs for the detection of the CIF2 (Locus A), KDP (Locus B), RIF5 (Locus F) and *mec*A (methicillin resistance) genes. Locus A is exclusive to SCC*mec* type I and amplifies a region of the *pls* gene (encoding plasmin sensitive surface protein), locus B is exclusive to SCC*mec* type II and amplifies an internal

region of the *kpd* operon (code for a high-affinity and repressible K+ transport system), locus F amplifies the *ccr*C region (site-specific recombinase) of SCC*mercury* located in the SCC*mec* type III element. The modified primer concentrations, amplicon size, primer sequences and M-PCR assay reaction compositions are shown in Table 3.5 and Table 3.6. The optimised and modified 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 94°C for 30 s, annealing at 59°C for 90 s and extension at 72°C for 90 s and a final extension step at 72°C for 10 min.

3.3.7 Multiplex-PCR assay for the typing of SCC*mec* type II and III

Confirmation of the presence of SCC*mec* type II and SCC*mec* type III among the MRSA isolates was done using a fifth M-PCR assay according to Zhang *et al*. (2012). A combination of universal primers specific for SCC*mec* type II and SCC*mec* type III + IIIA were used. The primer pair concentration, product size and primer sequences are shown in Table 3.7. The M-PCR assay reaction compositions are shown in Table 3.8 and the PCR amplification condition are as indicated in Section 3.3.5.

3.3.8 Multiplex-PCR assay for the typing of SCC*mec* type VIII

A sixth M-PCR assay was performed according to the methods described by McClure *et al*. (2010) on MRSA isolates, which were confirmed as CA-MRSA isolates by the Zhang *et al*. (2005) and Boye *et al*. (2007) assays. In this M-PCR assay, five primer pairs were used to amplify the following five genes: [*mec*I (intact regulator gene), *ccr*4 (universal chromosome cassette recombinase 4), SCC_{RP62A} (*Staphylococcus epidermidis* strain), *nuc* (thermonuclease gene) and *mec*A (methicillin resistance). The size of the product, concentration and primer sequences are shown in Table 3.9, while the M-PCR assay reaction compositions are shown in Table 3.10. Amplification of the DNA was done using the G-storm thermocycler (Vacutec, UK) and the PCR programme of McClure *et al*. (2010) was modified as follows: initial activation step at 95°C for 15 min, followed by 10 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 2 min and extension at 72°C for 2 min and another 25 cycles of 94°C for 45 s, annealing at 52°C for 45 s and extension at 72°C for 2 min and a final extension step at 72°C for 10 min.

3.3.9 Determination of the genetic relatedness of MRSA isolates using the pulsed-field gel electrophoresis genotyping technique

One of the 194 MRSA isolates was contaminated. The genetic relatedness of the 193 MRSA isolates was determined using PFGE according to the protocol described by McDougal *et al.* (2003) with the following modifications: single colonies from the Blood agar plates (Oxoid, England) were inoculated into 3 mℓ Brain Heart Infusion broth (Merck, Darmstadt, Germany) and incubated (New Brunswick Scientific co. Inc, New Jersey, USA) with vigorous shaking at 35°C for 24 h. The cell suspension was adjusted to an absorbance of 1.00 at a wavelength of 600 nm using a spectrophotometer (Jenway 6300, UK). A total of 1.5 mℓ of the cell suspension was added to a 2 mℓ Eppendorf tube (Eppendorf AG, Hamburg, Germany) and centrifuged (Eppendorf 5417C, Hamburg, Germany) at 4 293 *x g* for 2 min and the supernatant was aspirated. The pellet was resuspended in 500 µℓ of EET buffer [100 mM EDTA, 10 mM Tris-HCl (pH 8)] (Sigma-Aldrich, St. Louis, USA) and equilibrated in a heating block (Hägar designs HB2, Germany) at 37°C for 10 min. A 1.5% SeaKem Gold agarose gel (Lonza, Rockland, USA) was prepared by dissolving 0.3 g of the SeaKem Gold agarose powder (Lonza, Rockland, USA) in 20 mℓ of EET buffer [100 mM EDTA, 10 mM Tris-HCl (pH 8)] (Sigma-Aldrich, St. Louis, USA) and cooled in a 50°C waterbath (Labotec, Midrand, South Africa). Equal volumes of 500 µℓ 1.5% SeaKem Gold agarose gel (Lonza, Rockland, USA) and 500 µℓ EET buffer (containing the pellet) [100 mM EDTA, 10 mM Tris-HCl (pH 8)] (Sigma-Aldrich, St. Louis, USA) was gently mixed and 100 µℓ of the cell suspension was poured into the small PFGE plug mold (Bio-Rad, California, USA) and allowed to set for 15 min at 25°C. The plug for each MRSA isolate were removed and placed into a new 2 mℓ Eppendorf tube (Eppendorf AG, Hamburg, Germany).

Ten microlitres of lysostaphin (no. L-7386) (Sigma-Aldrich, St. Louis, USA) stock solution (1 mg.mℓ^{-1}) was added to the 2 mℓ Eppendorf tube (Eppendorf AG, Hamburg, Germany) containing EET buffer [100 mM EDTA, 10 mM Tris-HCl (pH 8)] (Sigma-Aldrich, St. Louis, USA). The plugs were incubated (New Brunswick Scientific co. Inc, New Jersey, USA) at 30°C for 5 h with gentle shaking. The plugs were removed and placed into a new 2 mℓ Eppendorf tube (Eppendorf AG, Hamburg, Germany) containing EET buffer [100 mM EDTA, 10 mM Tris-HCl (pH 8)] (Sigma-Aldrich, St. Louis, USA) with 100 µℓ Proteinase-K (20 mg. mℓ^{-1}) (Roche Applied Science, Germany) and 200 µℓ of 10% SDS (Promega,

Madison, USA). The 2 mℓ Eppendorf tube (Eppendorf AG, Hamburg, Germany) was incubated (New Brunswick Scientific co. Inc, New Jersey, USA) at 37ºC for 24 h with gentle shaking. The EET buffer [100 mM EDTA, 10 mM Tris-HCl (pH 8)] (Sigma-Aldrich, St. Louis, USA) was poured off and PFGE storage buffer [10 mM EDTA, 10 mM Tris-HCl (pH 8)] (Sigma-Aldrich, St. Louis, USA) was added. The plugs were cut into a volume of 30 µℓ (one-third) and placed into a new 2 mℓ Eppendorf tube (Eppendorf AG, Hamburg, Germany). The plugs were washed twice in 2 mℓ PFGE storage buffer [10 mM EDTA, 10 mM Tris-HCl (pH 8)] (Sigma-Aldrich, St. Louis, USA) containing 1 mM phenyl methyl sulfonyl fluoride (PMSF) (Roche Applied Science, Germany) for 45 min at 25ºC. The plugs were further washed three times in 2 mℓ of 10 mM Tris-HCl (pH 8) buffer (Sigma-Aldrich, St. Louis, USA) for 25 min at 25ºC. Excess liquid was removed and 70 µℓ solution containing 10 µℓ of a 10X reaction buffer (Fermentas Life Sciences, Thermo Scientific, USA), 10 µℓ of 0.01% bovine serum albumin (BSA) (Fermentas Life Sciences, Thermo Scientific, USA) and 30 Units *Sma*I restriction endonuclease (Fermentas Life Sciences, Thermo Scientific, USA) was added for the digestion of the DNA. The plugs were incubated (Labcon, Sepsci, South Africa) at 30ºC for 2.5 h (Appendix B).

A 1.6% SeaKem Gold agarose gel (Lonza, Rockland, USA) was prepared by dissolving 1.6 g of SeaKem Gold agarose powder (Lonza, Rockland, USA) in 160 mℓ of 0.5X TBE buffer [45 mM Tris borate, (pH 8), 1 mM EDTA] (Sigma-Aldrich, St. Louis, USA). The 1.6% SeaKem Gold agarose gel (Lonza, Rockland, USA) was poured into a casting tray (Bio-Rad, California, USA) containing a 30 well comb (Bio-Rad, California, USA) and allowed to solidify for 1 h. The 30 µℓ (one-third) plugs were loaded directly into the wells of the 1.6% SeaKem Gold agarose gel (Lonza, Rockland, USA) and sealed with 1% molten SeaKem Gold agarose gel (Lonza, Rockland, USA). A thin slice of the Lambda molecular weight markers (Biometra, Göttingen, Germany) was loaded into the first, middle and the last lanes. The 1.6% SeaKem Gold agarose gel (Lonza, Rockland, USA) was placed into the electrophoresis chamber (Bio-Rad, California, USA). Pulsed-field gel electrophoresis was performed using a CHEF-DR® III system (Bio-Rad, California, USA). The macrorestriction fragments were separated at 6 $V.cm^{-1}$ and 14°C for 20 h with an initial switch time of 5 s and a final switch time of 50 s. One strain was re-analyzed by repeating the restriction enzyme analysis and gel electrophoresis to determine the reproducibility of the method. The PFGE gel was stained with 0.5 $\mu g.m\ell^{-1}$ (10 $mg.m\ell^{-1}$ stock solution) ethidium bromide (Promega, Madison, USA) for

30 min and destained in distilled water for 10 min. The gels were photographed and digitalised using a GelDoc system (Bio-Rad, California, USA).

Interpretation of the PFGE banding patterns were done visually and the relatedness of each strain were determined according to the method described by Tenover *et al*. (1995). Strains with the same band patterns were considered to be related (represented by a capital letter A). The subtypes were defined as variants with one to three different band patterns (closely related) or variants with four to six different band patterns (possibly related) (represented by a number A1, A2 etc). Strains with distinguishable band patterns were considered unrelated (represented by a capital letter B, C, etc).

3.3.10 Statistical analysis

The sample size of 194 randomly collected MRSA isolates was analysed using percentages to determine the prevalence of HA-MRSA and CA-MRSA isolates including, 16S rRNA, *mec*A and the PVL genes. The clonal relatedness of the prevalent HA-MRSA and CA-MRSA isolates were clustered into different pulsotypes using the gold standard, the PFGE method.

3.4 RESULTS

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

The first M-PCR assay was used to determine the presence of the 16S rRNA (genus specific), *mec*A (methicillin resistance determinate) and Panton-Valentine leukocidin (PVL) genes in the MRSA isolates. All 194 MRSA isolates were 100% (194/194) positive for the 16S rRNA (756 bp) and *mec*A (310 bp) genes. One [0.5% (1/194)] CA-MRSA isolate was positive for the PVL (433 bp) gene (Figure 3.1).

3.4.2 Characterisation of the MRSA isolates using SCC*mec* typing and subtyping

One hundred and ninety four clinical MRSA isolates were tested for the presence of SCC*mec* types I to V and subtypes IVa to IVd using the Zhang *et al*. (2005) SCC*mec* typing assay. Twenty two persent (43/194) of the MRSA isolates were untypeable, but were positive for the *mec*A (internal control) gene (147 bp). Five percent (9/194) of the MRSA isolates showed

double bands for SCC*mec* type II (398 bp) and SCC*mec* subtype IVc (200 bp). The remaining 73.2% (142/194) typeable MRSA isolates were characterised as HA-MRSA and CA-MRSA. The SCC*mec* typing results for the 64.8% (92/142) HA-MRSA isolates were as follows: 1.1% (1/92) SCC*mec* type I (613 bp), 65.2% (60/92) SCC*mec* type II (398 bp), 33.7% (31/92) SCC*mercury* (280 bp) (Table 3.11). The 35.2% (50/142) CA-MRSA isolates which belonged to SCC*mec* subtype IV included: 4% (2/50) SCC*mec* subtype IVa (776 bp), 2% (1/50) SCC*mec* subtype IVb (493 bp) and 94% (47/50) SCC*mec* subtype IVd (881 bp). None of the MRSA isolates were positive for SCC*mec* subtype IVc (200 bp) or SCC*mec* type V (325 bp) (Figure 3.2).

In order to reduce the number of untypeable MRSA isolates obtained with the first SCC*mec* typing assay, the Boye *et al*. (2007) M-PCR SCC*mec* typing assay was used. All 194 MRSA isolates were typeable using this assay. Overall 73.7% (143/194) of the MRSA isolates belonged to HA-MRSA and 26.2% (51/194) belonged to CA-MRSA. The SCC*mec* typing results for the HA-MRSA isolates were as follows: 4.2% (6/143) SCC*mec* type I (415 bp), 12.6% (18/143) SCC*mec* type II (937 bp) and 83.2% (119/143) SCC*mercury* (518 bp). The CA-MRSA isolates was composed of 98% (50/51) SCC*mec* type IV (415 bp and 937 bp) and 2% (1/51) SCC*mec* type V (359 bp and 518 bp) (Figure 3.3). Single PCRs [including only the primer pairs for either SCC*mec* type II or SCC*mercury* according to Boye *et al*. (2007)] were performed on 26.3% (51/194) of the MRSA isolates that gave discrepant results between the two M-PCR assays. However, all [100% (51/51)] these MRSA isolates showed a band for both SCC*mec* type II (937 bp) and SCC*mercury* (518 bp) (Figure 3.4).

A third M-PCR assay by Oliveira and De Lencastra (2002) were performed on 143 of the HA-MRSA isolates plus one MRSA isolate, which was mistyped SCC*mec* type V according to Zhang *et al*. (2005) assay. The results were as follows: 4.2% (6/144) SCC*mec* type I (495 bp), 12.5% (18/144) SCC*mec* type II (284 bp), 55.5% (80/144) SCC*mercury* (414 bp) and 27.8% (40/144) carried double bands for SCC*mec* type II (284 bp) and SCC*mercury* (414 bp). The *mec*A gene (which was used as an internal control) was detected in 100% (144/144) of the clinical MRSA isolates (Figure 3.5).

The discrepant results (for SCC*mec* type II and SCC*mercury*) obtained using the Zhang *et al*. (2005), Boye *et al*. (2007) and Oliviera and De Lencastre (2002) assays necessitated the use

of a fourth M-PCR assay (Table 3.12). Universal primers from Zhang *et al.* (2012) were used to confirm and resolve the discrepancies of 138 (144 HA-MRSA isolates excluding the six SCC*mec* type I positive isolates resolved using the previous assay). The results showed that 50% (69/138) of these MRSA isolates were positive for SCC*mec* type II (128 bp) while the remaining 50% (69/138) of the isolates were untypeable (Figure 3.6). No SCC*mec* type III was detected according to the Zhang *et al*. (2012) assay.

All the CA-MRSA isolates [26.3% (51/194)] were also analysed to determine the presence of SCC*mec* type VIII according to the method by McClure *et al*. (2010). No SCC*mec* type VIII was detected in any of the CA-MRSA isolates. The *mec*A (112 bp) and *nuc* (279 bp) genes were detected in 98% (50/51) of the CA-MRSA isolates, while 2% (1/51) was positive for the *mec*A (112 bp), *nuc* (279 bp) and *ccr*4 (428 bp) genes (Figure 3.7).

Inconclusive results were obtained for 26.3% (51/194) MRSA isolates, which showed bands for either *SCCmec* II or III depending on the assay used. The combined results of the remaining 143 MRSA isolates according to the five SCC*mec* typing M-PCR assays performed in this study showed an overall prevalence of 64.3% (92/143) HA-MRSA and 35.7% (51/143) CA-MRSA. The results for the HA-MRSA isolates indicated that the most prevalent SCC*mec* type was SCC*mercury* [74% (68/92)] followed by SCC*mec* type II [19.5% (18/92)] and lastly SCC*mec* type I [6.5% (6/92)]. The results for the CA-MRSA isolates indicated that SCC*mec* subtype IVd [92.1% (47/51)] was the most prevalent followed by SCC*mec* subtype IVa [3.9% (2/51)] with SCC*mec* subtype IVb and SCC*mec* type V with only one isolate [2% (1/51)] each (Table 3.13).

3.4.3 Pulsed-field gel electrophoresis typing

One contaminated MRSA isolate was excluded from this study. One percent (2/193) of the 193 MRSA tested were untypeable after PFGE typing. Therefore, the 191 typeable MRSA isolates were analysed using visual comparison according to the criteria described by Tenover *et al*. (1995). Clustering of the 191 typeable MRSA isolates revealed eleven clonal patterns (Figure 3.8). Fifty seven percent (110/191) of the clinical MRSA isolates shared an indistinguishable pattern (0 band pattern differences) and were designated pulsotype A. Pulsotype A had 28% (53/191) multiple subtypes, which included subtype A1 [5% (9/191)],

A2 [0.5% (1/191)], A3 [6% (12/191)], A4 [6% (11/191)], A5 [5.8% (11/191)] and A6 [4.7% (9/191)]. A total of 15% (28/191) of the MRSA isolates yielded distinct pulsotypes (seven band pattern differences), which did not resemble any of the indistinguishable band patterns (pulsotype A) and were designated pulsotype B [2% (4/191)] with two subtypes, B1 [1.6% (3/191)] and B2 [0.5% (1/191)], C [0.5% (1/191)], D [0.5% (1/191)], E [0.5% (1/191)], F [0.5% (1/191)], G [0.5% (1/191)], H [0.5% (1/191)], I [2% (4/191)], J [5% (9/191)] and K [0.5% (1/191)]. Pulsotype A differed from subtypes A1 to A3 by one to three band patterns. Subtypes A4 to A6 differed from pulsotype A by four to six band patterns. Pulsotype B, C, D, E, F, G, H, I and K differed from pulsotype A by more than seven bands.

3.5 DISCUSSION

The current study was conducted to identify, characterise and report the clonal relatedness of clinical MRSA isolates obtained from a tertiary academic hospital. This study was a follow-up study, two years after an initial study was conducted by Makgotlho *et al*. (2009) at the same tertiary hospital. In the current study, all 194 clinical MRSA isolates were positive for the *mec*A and 16S rRNA genes, using the M-PCR assay by McClure *et al*. (2006). Makgotlho *et al*. (2009) reported that 99% (96/97) of the MRSA isolates carried the *mec*A gene, with one isolate not expressing the *mec*A gene. The low prevalence of the PVL [0.5% (1/194)] gene detected in this study was in agreement with a previous national study conducted by Moodley *et al*. (2010) during August 2005 to November 2006 in South Africa. In contrast, Makgotlho *et al*. (2009) reported a prevalence of 4% (4/97) PVL positive MRSA isolates, which was due to an outbreak during the time of isolate collection.

The MRSA isolates were grouped into HA-MRSA and CA-MRSA using five SCC*mec* typing M-PCR assays. The Zhang *et al*. (2005) SCC*mec* typing assay was able to type 73.2% (142/194) of the MRSA isolates. Healthcare-associated MRSA isolates represented 64.8% of all the isolates, with the majority of isolates being positive for SCC*mec* type II (65.2%), followed by SCC*mercury* (33.7%) and lastly SCC*mec* I (1.1%). These results were lower than the 81.4% HA-MRSA isolates [with 67% (65/97) belonging to SCC*mec* type II and 14.4% (14/97) for SCC*mec* type III] reported by Makgotlho *et al*. (2009) using the same method. One MRSA isolate was mistyped as SCC*mercury* and later confirmed to belong to SCC*mec* type V according to the method by Boye *et al*. (2007). Similar results were reported

by Jansen *et al*. (2009) from a follow-up study conducted in the Nertherlands, which revealed that four MRSA isolates were misidentified as SCC*mercury* (type III). Mistyping occurs during amplification of the 280 bp fragment found in SCC*mer* associated with SCC*mec* type III (Jansen *et al*., 2009).

Community-associated MRSA isolates represented 35.2%, with the emergence of SCC*mec* subtype IVd (94%), followed by subtype IVa (4%) and subtype IVb (2%). Contrary, Makgotlho *et al*. (2009) reported a low prevalence of 4% (4/97) CA-MRSA isolates harbouring subtype IVd. Overall, HA-MRSA isolates decreased by 16.6%, while CA-MRSA isolates increased by 31.2% in this clinical setting according to the method by Zhang *et al*. (2005). This is consistent with other studies conducted in Canada and United States, which reported an increase of CA-MRSA strains in clinical settings (Simor *et al*., 2010; Caffrey and LaPlante, 2012). Popovich *et al*. (2008) reported that CA-MRSA isolates might displace HA-MRSA in future and become the most prevalent strains in clinical settings.

Twenty two percent (43/194) of the MRSA isolates were untypeable according to the method by Zhang *et al*. (2005). The 22% untypeable MRSA was higher than the 8% (8/97) reported by Makgotlho *et al*. (2009), but slightly lower than the 23.71% (239/1 008) reported by Alon *et al*. (2011) in a similar study conducted in Israel. The remaining 5% (9/194) of the MRSA isolates showed double bands for SCC*mec* type II and IVc and were later shown to belong to SCC*mec* type II according to the methods of Oliviera and De Lencastre (2002); Boye *et al*. (2007) and Zhang *et al.* (2012). Makgotlho *et al*. (2009) reported 6% (6/97) of double bands for SCC*mec* type II and III [3% (3/97)], followed by SCC*mec* type II and IVd [2% (2/97)] and SCC*mec* type III and IVc [1% (1/97)]. However, no further SCC*mec* typing methods were performed on these MRSA isolates. The Zhang *et al*. (2005) assay was less sensitive and accurate to type the MRSA isolates in this clinical setting.

The combined results for the five SCC*mec* typing assays showed an overall prevalence of 64.3% for the HA-MRSA isolates and 35.7% for the CA-MRSA isolates. The majority of the HA-MRSA isolates were positive for SCC*mercury* (74%), followed by SCC*mec* type II (19.5%) and lastly SCC*mec* type I (6.5%). Shittu *et al*. (2009) reported a lower prevalence of SCC*mec* type III [18.0% (11/61)], SCC*mec* type IIIa [8.2% (5/61)], SCC*mec* type I [1.6% (1/61)] and SCC*mec* type II + IIIb [3.3% (2/61)] in a similar study conducted in KwaZulu

Natal (South Africa). However, the dominant CA-MRSA SCC*mec* type in the Shittu *et al.* (2009) study was SCC*mec* type IV [62.3% (38/61)], which is lower than the 98% SCC*mec* type IV and 2% SCC*mec* type V noted in this study. The increased prevalence of CA-MRSA isolates noted in this study is in agreement with other South African studies (Moodley *et al.*, 2010; Jansen van Rensburg *et al.*, 2011), which reported a prevalence of 38% (124/320) and 44% (44/100), respectively.

Inconclusive results were obtained for 26.3% (51/194) of the MRSA isolates. These MRSA isolates gave discrepant results for either *SCCmec* II or SCC*mercury* depending on the assay used. Single PCRs according to Boye *et al.* (2007) revealed that these MRSA isolates were 100% (51/51) positive for both *SCCmec* II and SCC*mercury*. The Oliviera and De Lencastre (2002) assay was the least sensitive for the detection of 11 MRSA isolates lacking the *kdp* (284 bp) band associated with SCC*mec* type II from the 51 MRSA isolates. This is in agreement with other studies, which reported MRSA isolates that lacked the *kdp* (284 bp) operon (Shore *et al.*, 2005; Romeeza *et al.*, 2010). These 11 MRSA isolates were shown to be positive for SCC*mec* type II-*kdp* according to the methods by Zhang *et al.* (2005). The MRSA isolates were further tested using the recently published Zhang *et al.* (2012) assay [which is a modification of the previous Zhang *et al.* (2005) assay] designed to correctly identify the previously misclassified SCC*mec* type II and III MRSA isolates. This assay confirmed the presence of SCC*mec* type II in 50% of the MRSA isolates. None of the MRSA isolates were positive for SCC*mec* type III according to the assay. No SCC*mec* type VIII was detected in the 26.3% (51/194) CA-MRSA isolates in this clinical setting. One CA-MRSA isolate (number 143) carried an additional *ccr*4 gene and this isolate was positive for SCC*mec* type IVa according to the methods by Zhang *et al.* (2005) and Boye *et al.* (2007). This CA-MRSA isolate was compared with another CA-MRSA isolates belonging to SCC*mec* type IVa obtained in this study and was found to be genetically unrelated after typing with the PFGE method. Multilocus sequence typing and sequencing of the SCC*mec* type IVa in this MRSA isolate may provide more information regarding its genetic make up.

The combination of the SCC*mec* typing assays with other genotyping methods, such as PFGE has been recommended to determine the genetic relatedness of HA-MRSA and CA-MRSA isolates. The 191 MRSA isolates separated into eleven pulsotypes designated A to K. The major pulsotype A [57% (110/191)] included 12% (22/191) closely related subtypes A1 to A3

and 16% (31/191) possible related subtypes A4 to A6. A total of 66% (127/191) of the pulsotype and subtypes belonged to HA-MRSA strains, while 19% (36/191) were CA-MRSA strains. A similar study by Moodley *et al*. (2010) reported three pulsotypes circulating in the Pretoria region (PFGE D-SCC*mec*III-*spa*CC12-ST239, PFGE K-SCC*mec*I-*spa*CC64-ST612 and PFGE T-SCC*mec*II-*spa*CC12-ST36). These three pulsotypes could correspond to those identified in this study; however, another study is required to compare these clones using MLST and *spa* typing.

In this study, pulsotype A band patterns corresponded to the band patterns of clone A (A1) (ST239 III *spa* type t037) identified by Jansen van Rensburg *et al*. (2011) and this clone is among the frequently described pandemic clones in South Africa. Pulsotype B to K had distinct band patterns and was considered genetically unrelated to pulsotype A and its subtypes (A1 to A6). These pulsotypes (B to K) included 7% (13/191) of the HA-MRSA and 8% (15/191) of the CA-MRSA isolates. Each pulsotype obtained in this study included MRSA strains circulating in different clinical wards, which shows that these strains could be transmitted across the hospital wards. Furthermore, the HA-MRSA and CA-MRSA isolates detected in this study shared pulsotypes, which indicated that the MRSA infections within the hospital may have been caused by the clonal expansion of a single genotype. Similarly, Shittu *et al*. (2009) has reported HA-MRSA strains and CA-MRSA strains sharing the same PFGE pulsotype and such results could be due to interhospital spread of these strains.

3.6 CONCLUSION

This study revealed a decline in the prevalence of HA-MRSA isolates and an increase of CA-MRSA isolates in this hospital over the past two years. The major shift between HA-MRSA and CA-MRSA observed in the two year period is worrisome, since different strains of CA-MRSA are being introduced in the hospital settings from the community. The smaller SCC*mec* sizes of CA-MRSA strains are more easily transferable between *S. aureus* strains than the bigger SCC*mec* size of HA-MRSA. Thus the fitness of CA-MRSA strains could make these strains to become the dominant types in this clinical setting in the future. Although a low prevalence of PVL positive CA-MRSA strain (single isolate) was observed during this study, continuous monitoring of the presence of this toxin gene in MRSA isolates is crucial in this clinical setting.

The combined SCC*mec* typing assays provided correct MRSA characterisation in this clinical setting. Combining and re-designing of the primers, which gave similar results, is necessary for the development of a standardised SCC*mec* typing assay. Although, PFGE typing was labour intensive and expensive, the method remained a powerful tool for comparing clonal relatedness among the HA-MRSA and CA-MRSA circulating in this clinical setting. This method is useful in studying clonal evolution and monitoring the trends of the major pulsotype over time, so that infection control personnel can be alerted about new strains, which may be introduced in this clinical setting. The high prevalence of closely related HA-MRSA and CA-MRSA isolates observed, warrant close monitoring of these clones, to ensure that proper surveillance and infection control programmes are in place to prevent possible outbreaks in future.

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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 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: Reagent components used in the M-PCR assay for the detection of the 16S rRNA, *mec*A and PVL genes of MRSA isolates (Qiagen, 2008)

Reagents	Volume (µℓ) X1	Final concentration
2X QIAGEN M-PCR master mix (Hotstar*Taq* DNA polymerase, M-PCR buffer and dNTP mix)	25	1X (3 mM $MgCl_2$)
10X primer mix (2 µM each primer)	5	0.2 µM
RNase-free water	15	-
Template DNA	5	<1 µg DNA/ 50 µℓ
Total volume	50	

Table 3.3: Oligonucleotide sequences of the primers used in the M-PCR for the typing of SCC*mec* type I to V and subtyping of SCC*mec* subtype IVa to IVd of MRSA isolates

Primer	Oligonucleotide sequence 5' – 3'	Target gene	Amplicon size (bp)	Conc (µM)
Zhang *et al*. (2005)				
Type I-F Type I-R	-GCTTTAAAGAGTGTCGTTACAGG- -GTTCTCTCATAGTATGACGTCC-	SCC*mec* I	613	0.2
Type II-F Type II-R	-CGTTGAAGATGATGAAGCG- -CGAAATCAATGGTTAATGGACC-	SCC*mec* II	398	0.2
Type III-F Type III-R	-CCATATTGTGTACGATGCG- -CCTTAGTTGTCGTAACAGATCG-	SCC*mec* III	280	0.2
Type IVa-F Type IVa-R	-GCCTTATTCGAAGAAACCG- -CTACTCTTCTGAAAAGCGTCG-	SCC*mec* IVa	776	0.2
Type IVb-F Type IVb-R	-TCTGGAATTACTTCAGCTGC- -AAACAATATTGCTCTCCCTC-	SCC*mec* IVb	493	0.2
Type IVc-F Type IVc-R	-ACAATATTTGTATTATCGGAGAGC- -TTGGTATGAGGTATTGCTGG-	SCC*mec* IVc	200	0.2
Type IVd-F Type IVd-R	-CTCAAAATACGGACCCCAATACA- -TGCTCCAGTAATTGCTAAAG-	SCC*mec* IVd	881	0.2
Type V-F TypeV-R	-GAACATTGTTACTTAAATGAGCG- -TGAAAGTTGTACCCTTGACACC-	SCC*mec* V	325	0.2
MecA147-F MecA147-R	-GTGAAGATATACCAAGTGATT- -ATGCGCTATAGATTGAAAGGAT-	*mec*A	147	0.2
Boye *et al*. (2007)				
B-F α3-R	-ATTGCCTTGATAATAGCCYTCT- -TAAAGGCATCAATGCACAAACACT-	*ccr*A2-B	937 bp	0.25
*ccr*CF-F *ccr*CR-R	-CGTCTATTACAAGATGTTAAGGATAAT- -CCTTTATAGACTGGATTATTCAAAATA-	*ccr*C	518 bp	0.2
1272-F1 1272-R1	-GCCACTCATAACATATGGAA- -CATCCGAGTGAAACCCAAA-	IS1272	415 bp	0.08
5R*mec*A-F 5R431-R	-TATACCAAACCCGACAACTAC- -CGGCTACAGTGATAACATCC-	*mec*A-IS431	359 bp	0.1

Table 3.4: Reagent components used in the M-PCR assay for the typing of SCC*mec* type I to V and subtyping of SCC*mec* subtype IVa to IVd of MRSA isolates (Qiagen 2008)

Reagents	Volume (µℓ) X1	Final concentration
2X QIAGEN M-PCR master mix (Hotstar*Taq* DNA polymerase, M-PCR buffer and dNTP mix)	12.5	1X (3 mM $MgCl_2$)
10X primer mix (0.08 µM to 2.5 µM each primer)	5	0.008 µM to 0.25 µM
Q-Solution	2.5	
RNase-free water	2.0	-
Template DNA	3	<1 µg DNA/ 25 µℓ
Total volume	25	

Table 3.5: Oligonucleotide sequences of the primers used in the M-PCR assay for the typing of SCC*mec* type I to III (Oliveira and De Lencastre, 2002)

Primer	Oligonucleotide sequences (5' - 3')	Locus	Amplicon size (bp)	Conc (µM)
CIF2-F2 CIF2-R2	-TTCGAGTTGCTGATGAAGAAGG- -ATTTACCACAAGGACTACCAGC-	A	495 bp	0.25
KDP-F1 KDP-R1	-AATCATCTGCCATTGGTGATGC- -CGAATGAAGTGAAAGAAAGTGG-	B	284 bp	0.25
RIF5-F10 RIF5-F13	-TTCTTAAGTACACGCTGAATCG- -GTCACAGTAATTCCATCAATGC-	F	414 bp	0.2
MECA P4 MECA P7	-TCCAGATTACAACTTCACCAGG- -CCACTTCATATCTTGTAACG-	*mec*A	162 bp	0.25

Table 3.6: Reagent components used in the M-PCR assay for the typing of SCC*mec* type I to III (Qiagen, 2008)

Reagents	Volume (µℓ) X1	Final concentration
2X QIAGEN M-PCR master mix (Hotstar*Taq* DNA polymerase, M-PCR buffer and dNTP mix)	12.5	1X (3 mM $MgCl_2$)
10X primer mix (2 µM to 2.5 µM each primer)	5	0.2 µM to 0.25 µM
Q-Solution	2.5	
RNase-free water	2.0	-
Template DNA	3	<1 µg DNA/ 25 µℓ
Total volume	25	

Table 3.7: Oligonucleotide sequences of the primers used in the M-PCR assay for the typing of SCC*mec* type II and III (Zhang *et al*., 2012)

Primer	Oligonucleotide sequences (5' - 3')	Target gene	Amplicon size (bp)	Conc (µM)
Type II-F2 Type II-R2	-TAGCTTATGGTGCTTATGCG- -GTGCATGATTTCATTTGTGGC-	SCC*mec* II,VIII	128	0.1
Type III-F5 Type III-R6	-TTCTCATTGATGCTGAAGCC- -GTGTAATTTCTTTTGAAAGATATGG-	SCC*mec* III, IIIA	257	0.2

Table 3.8: Reagent components used in the M-PCR assay for the typing of SCC*mec* type II and III (Qiagen, 2008)

Reagents	Volume (µℓ) X1	Final concentration
2X QIAGEN M-PCR master mix (Hotstar*Taq* DNA polymerase, M-PCR buffer and dNTP mix)	12.5	1X (3 mM $MgCl_2$)
10X primer mix (2 µM each primer)	5	0.2 µM
Q-Solution	2.5	
RNase-free water	2.0	-
Template DNA	3	<1 µg DNA/ 25 µℓ
Total volume	25	

Table 3.9: Oligonucleotide sequences of the primers used in the M-PCR assay for the typing of SCC*mec* type VIII of CA-MRSA isolates (McClure *et al*., 2010)

Primer	Oligonucleotide sequences (5'-3')	Target gene	Amplicon size (bp)	Conc (µM)
*mec*I-F *mec*I-R	-CCCTTTTTATACAATCTCGTT- -ATATCATCTGCAGAATGGG-	*mec*I	147	0.5
*ccr*4-Fd *ccr*4-R2	-ATCGCTCATTATGGATACYGC- -CAAAACAACCTTTTCTATAACG-	*ccr*4	428	0.24
SCC_{RP62A} SCC-CI	-CAATATTGATTTCCTTCATCGTTTACCTCC- -GAGCATCATAAGAAGCAATTTTATGTTACGC-	SCC*mec* VIII	1957	2.13
*nuc*1 *nuc*2	-GCGATTGATGGTGATACGGTT- -AGCCAAGCCTTGACGAACTAAAGC-	*nuc*	279	0.04
*mec*A147-F *mec*A112-R	-GTGAAGATATACCAAGTGATT- -ATCAGTATTTCACCTTGTCCG-	*mec*A	112	0.4

Table 3.10: Reagent components used in the M-PCR assay for the typing of SCC*mec* type VIII of CA-MRSA isolates (Qiagen, 2008)

Reagents	Volume (µℓ) X1	Final concentration
2X QIAGEN M-PCR master mix (Hotstar*Taq* DNA polymerase, M-PCR buffer and dNTP mix)	12.5	1X (3 mM $MgCl_2$)
10X primer mix (2 µM each primer)	5	0.2 µM
Q-Solution	2.5	
Rnase-free water	2.0	-
Template DNA	3	<1 µg DNA/ 25 µℓ
Total volume	25	

Table 3.11: Summary of the prevalence rates of HA-MRSA and CA-MRSA isolates circulating in the Pretoria region during April 2010 to August 2011 according to the five M-PCR assays

SCC*mec* types and subtypes	**Prevalence of the SCC*mec* types and subtypes %**							
	SCC*mec* typing assays							
	Zhang *et al.* (2005)		**Boye *et al.* (2007)**		**Single Boye *et al.* (2007)**	**Oliveira and De Lencastre (2002)**	**Zhang *et al.* (2012)**	**McClure *et al.* (2010)**
Not typeable	22% (43/194)		ND		ND	ND	50% (69/138)	ND
SCC*mec* type II+IVc	5% (9/194)		ND		ND	ND	ND	PN
HA-MRSA or CA-MRSA	**HA-MRSA** 64.8% (92/142)	**CA-MRSA** 35.2% (50/142)	**HA-MRSA** 73.7% (143/194)	**CA-MRSA** 26.3% (51/194)	**HA-MRSA** 26.3% (51/194)	**HA-MRSA** 74.2% (144/194)	**HA-MRSA** 71.1% (138/194)	**CA-MRSA** 26.3% (51/194)
SCC*mec* type I	1.1% (1/92)		4.2% (6/143)			4.2% (6/144)	PN	PN
SCC*mec* type II	65.2% (60/92)		12.6% (18/143)			12.5% (18/144)	50% (69/138)	PN
SCC*mercury*	33.7% (31/92)		83.2% (119/143)			55.5% (80/144)	ND	PN
SCC*mec* type IV		PN		98% (50/51)		PN	PN	PN
SCC*mec* type IVa		4% (2/50)		PN		PN	PN	PN
SCC*mec* type IVb		2% (1/50)		PN		PN	PN	PN
SCC*mec* type IVc		ND		PN		PN	PN	PN
SCC*mec* type IVd		94% (47/50)		PN		PN	PN	PN
SCC*mec* type V		ND		2% (1/51)		PN	PN	PN
SCC*mec* type VIII		PN		PN		PN	PN	ND
SCC*mec* type II+SCC*mercury*	ND		ND		100% (51/51)	27.8% (40/144)	ND	PN

CA-MRSA -Community-associated methicillin resistant *Staphylococcus aureus*
HA-MRSA -Healthcare-associated methicillin resistant *Staphylococcus aureus*
PN -Primer not included
SCC*mec* -Staphylococcal cassette chromosome *mec*

Table 3.12: Summary of the discrepancies of HA-MRSA and CA-MRSA isolates obtained using different SCC*mec* typing assays

SCC*mec* types and subtypes	**Discrepancies of the SCC*mec* types and subtypes** **SCC*mec* typing assays**				**PVL gene carriage**
Zhang *et al.* (2005)	SCC*mec* type I 1.1% (1/92)	SCC*mec* type II 55.4% (51/92)	SCC*mec* type II+IVc 5% (9/194)	Not typeable 22% (43/194)	SCC*mercury* 0.5% (1/194)
Boye *et al.* (2007)	SCC*mec* type I 4.2 % (6/143)	SCC*mercury* 35.7% (51/143)	SCC*mec* type II 6% (9/143)	ND	SCC*mec* type V 0.5% (1/194)
Oliveira and De Lencastre (2002)	SCC*mec* type I 4% (6/144)	SCC*mec* type II + SCC*mercury* 27.8% (40/144)	SCC*mec* type II 6.3% (9/144)	ND	SCC*mercury* 0.7% (1/144)
Zhang *et al.* (2012)	PN	SCC*mec* type II 37% (51/138)	SCC*mec* type II 6.2% (9/138)	Not typeable 50% (69/143)	-

ND -Not detected **PVL** -Panton-Valentine Leukocidin
PN -Primers not included **SCC*mec*** -Staphylococcal cassette chromosome *mec*

Table 3.13: Overall prevalence of the HA-MRSA and CA-MRSA isolates according to the five combined SCC*mec* typing assays and PFGE typing

SCC*mec* types and subtypes	**Overall prevalence %**		**PFGE group**	
Inconclusive SCC*mec* type II+SCC*mercury*	26.3% (51/194)		A, A1, A4 to A6	
HA-MRSA or CA-MRSA	**HA-MRSA** 64.3% (92/143)	**CA-MRSA** 35.7% (51/143)	**Related HA-MRSA** [66% (127/191)] **CA-MRSA** [19% (36/191)]	**Unrelated HA-MRSA** [7% (13/191)] **CA-MRSA** [8% (15/191)]
SCC*mec* type I	6.5% (6/92)		A3, F	
SCC*mec* type II	19.5% (18/92)		A, A1, A4, A5, I, J	
SCC*mec* type III	ND			
SCC*mercury*	74% (68/92)		A to A6, B, K	
SCC*mec* type Iva		3.9% (2/51)	A, B	
SCC*mec* type IVb		2% (1/51)	I	
SCC*mec* type IVc		ND		
SCC*mec* type IVd		92.1% (47/51)	A to A6, B, B1, B2, C, D, E, G, H, I	
SCC*mec* type V		2% (1/51)	A3	
SCC*mec* type VIII		ND		

CA-MRSA -Community-associated methicillin resistant *Staphylococcus aureus*
HA-MRSA -Healthcare-associated methicillin resistant *Staphylococcus aureus*
ND -Not detected
PFGE -Pulsed-field gel electrophoresis
SCC*mec* -Staphylococcal cassette chromosome *mec*

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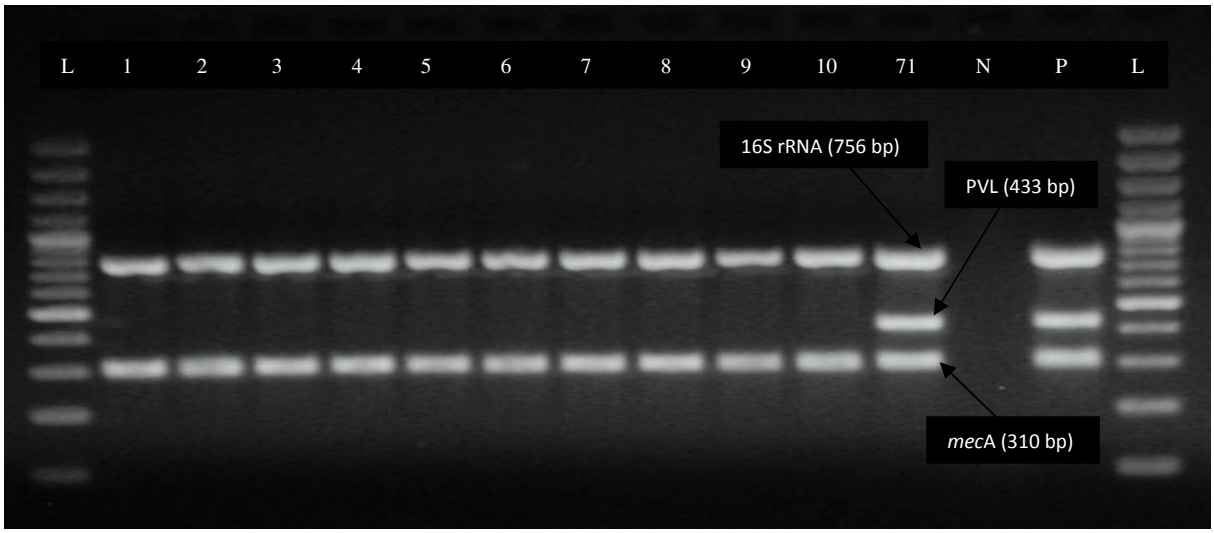

Figure 3.1: **Gel electrophoresis of the M-PCR assay for the detection of the 16S rRNA, *mec*A and PVL genes of the MRSA isolates. Lanes 1 to 10 show the 16S rRNA (756 bp) and *mec*A (310 bp). Lane 71 shows the 16S rRNA (756 bp), *mec*A (310 bp) and PVL (433 bp) genes. N= negative control, P= positive control and L = 100 bp ladder**

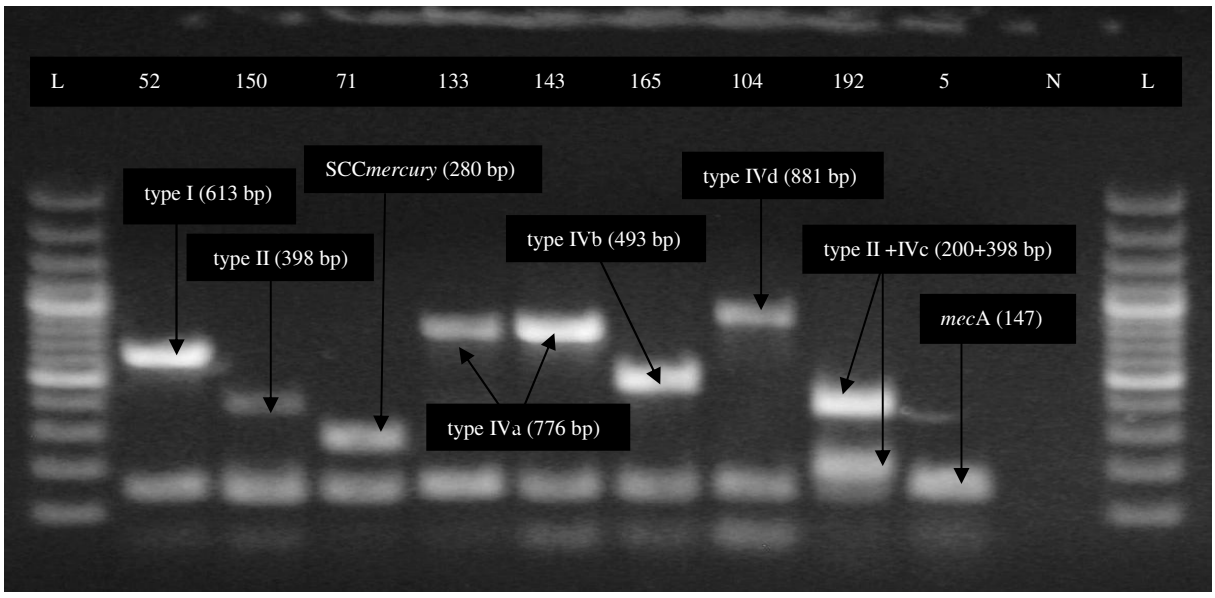

Figure 3.2: **Gel electrophoresis of the M-PCR assay for the characterisation of SCC*mec* types and subtypes (I, II, III, IVa to IVd) and simultaneous detection of the *mec*A gene of the clinical MRSA isolates. Lane 52 shows SCC*mec* type I (613 bp). Lane 150 shows SCC*mec* type II (398 bp). Lane 71 shows SCC*mercury* (280 bp). Lanes 133 and 143 show SCC*mec* subtype IVa (776 bp). Lane 165 shows SCC*mec* type IVb (493 bp). Lane 104 shows SCC*mec* type IVd (881 bp). Lane 192 shows double bands of SCC*mec* type II (398 bp) and IVc (200 bp). Lane 5 shows *mec*A (147 bp) untypeable MRSA isolate. N= negative control, L= 100 bp ladder**

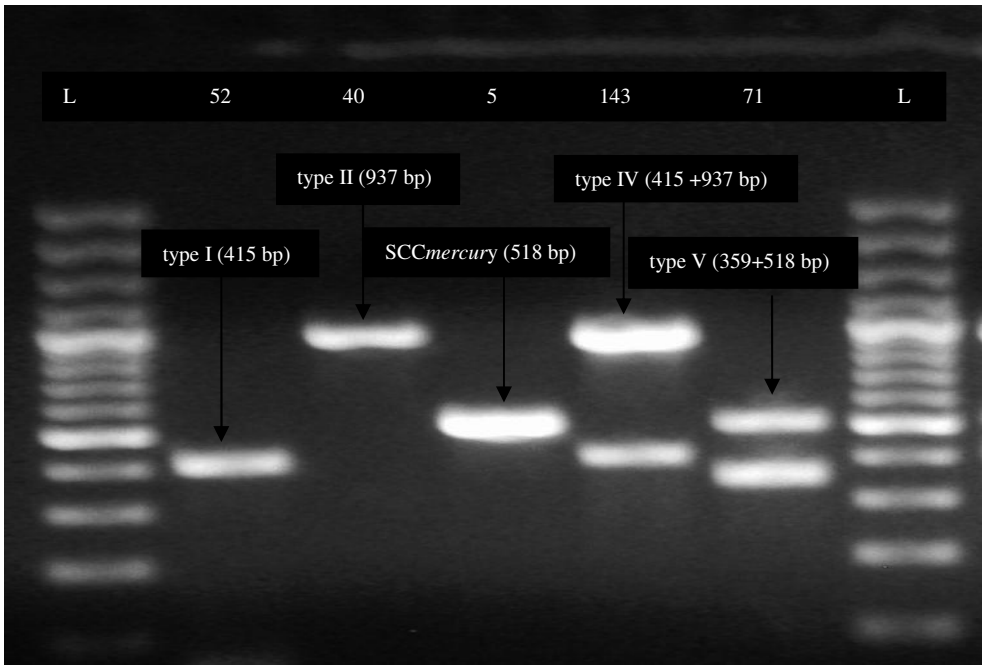

Figure 3.3: **Gel electrophoresis results of the M-PCR assay for the characterisation of SCC*mec* types I to V of the clinical MRSA isolates. Lane 52 shows SCC*mec* type I (415 bp), Lane 40 shows SCC*mec* type II (937 bp), Lane 5 shows SCC*mercury* (518 bp), Lane 143 shows SCC*mec* type IV (415 bp + 937 bp), Lane 71 shows SCC*mec* type V (359 bp + 518 bp). L= 100 bp ladder**

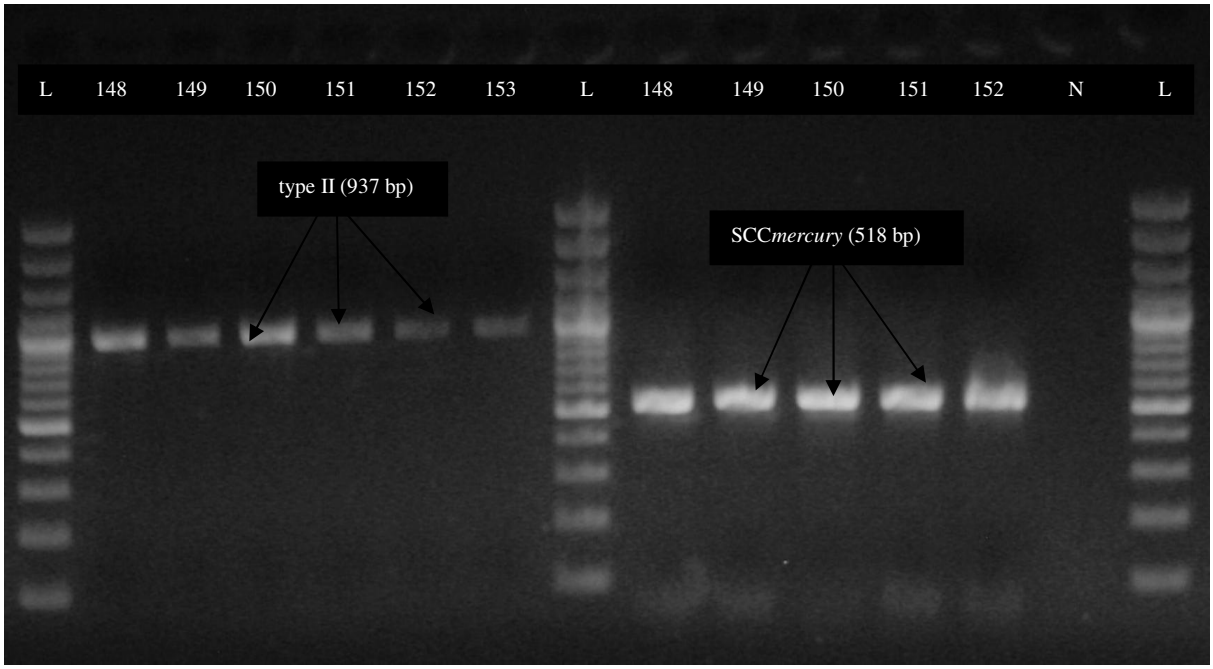

Figure 3.4: **Gel electrophoresis results of the single-PCR assay for the detection of SCC*mec* types II and III of the clinical MRSA isolates. Lanes 148 to 153 show SCC*mec* type II (937 bp). Lanes 148 to 152 show SCC*mercury* (518 bp). L = 100 bp ladder, N = negative control**

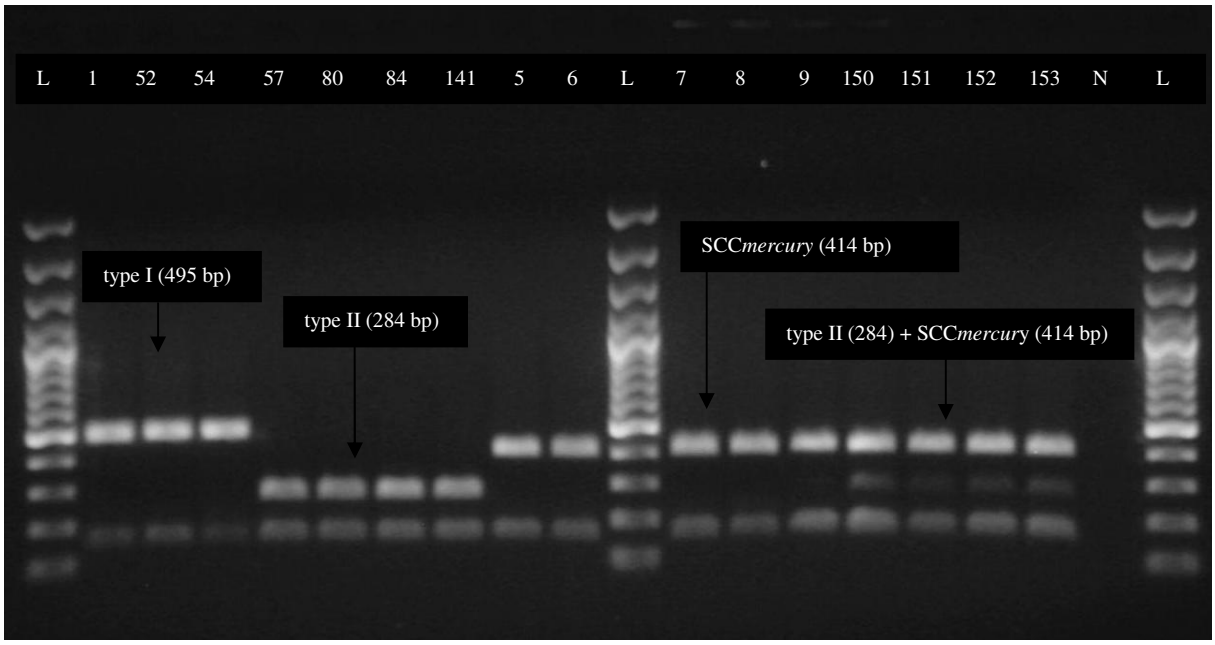

Figure 3.5: **Gel electrophoresis results for the characterisation of the SCC*mec* types I to III and simultaneous detection of the *mec*A gene of the clinical MRSA isolates. Lanes 1, 52 and 54 show SCC*mec* type I (495 bp). Lanes 57, 80, 84 and 141 show SCC*mec* type II (284 bp). Lanes 5 to 9 show SCC*mercury* (414 bp). Lanes 150 to 153 show double bands of SCC*mec* type II (284) and SCC*mercury* (414 bp). L= 100 bp ladder, N= negative control**

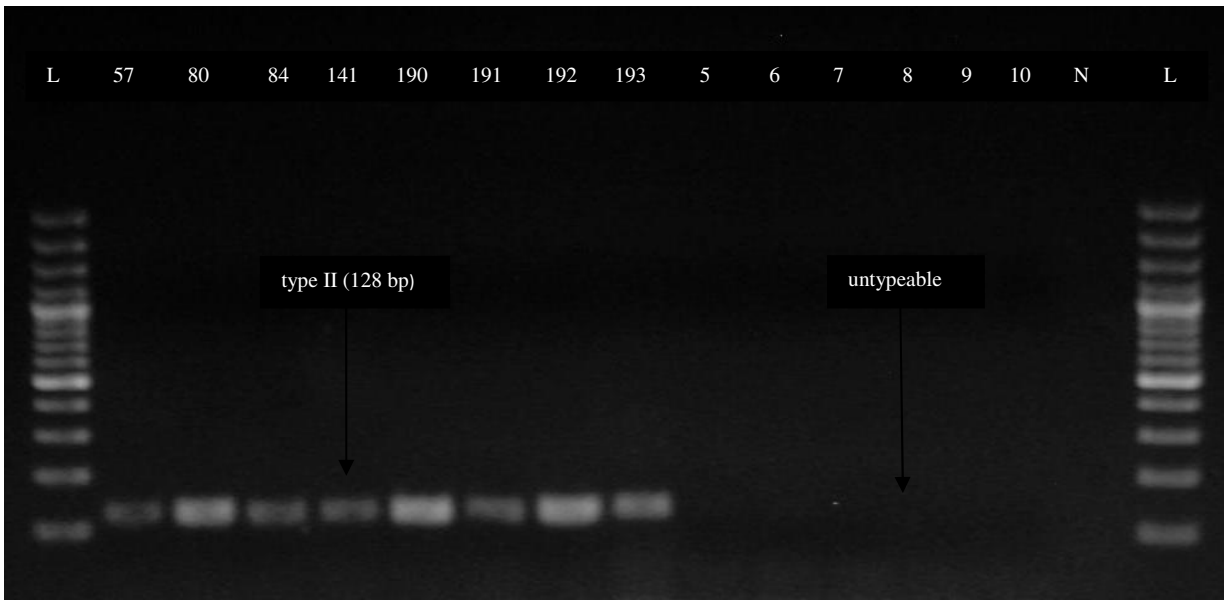

Figure 3.6: **Gel electrophoresis results for the detection of SCC*mec* types II and III of the clinical MRSA isolates. Lanes 57, 80, 84, 141, 190 to 193 show SCC*mec* type II (128 bp). Lanes 5 to 10 show untypeable isolates. L= 100 bp ladder, N= negative control**

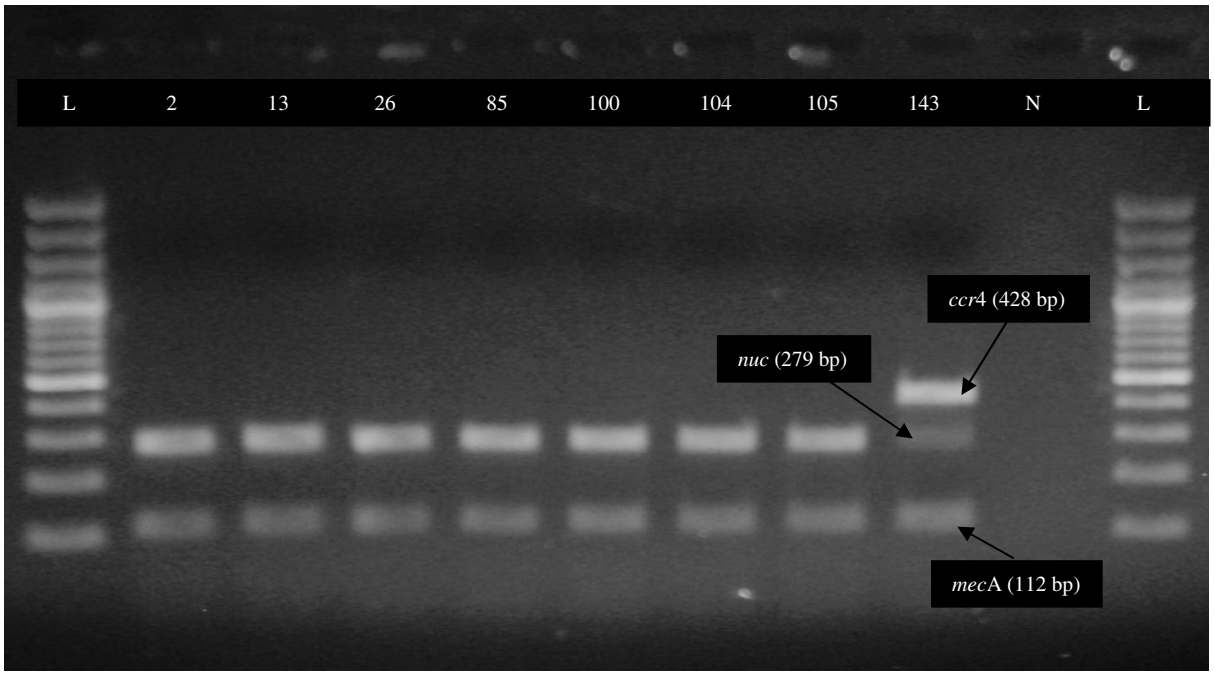

Figure 3.7: **Gel electrophoresis results of the M-PCR assay for the characterisation of SCC*mec* type VIII of the CA-MRSA isolates. Lanes 2, 13, 26, 85, 100, 104, 105 show the *mec*A (112 bp) and *nuc* (279 bp) genes. Lane 143 shows the *mec*A (112 bp), *nuc* (279 bp) and *ccr*4 (428) genes. L= 100 bp ladder, N= negative control**

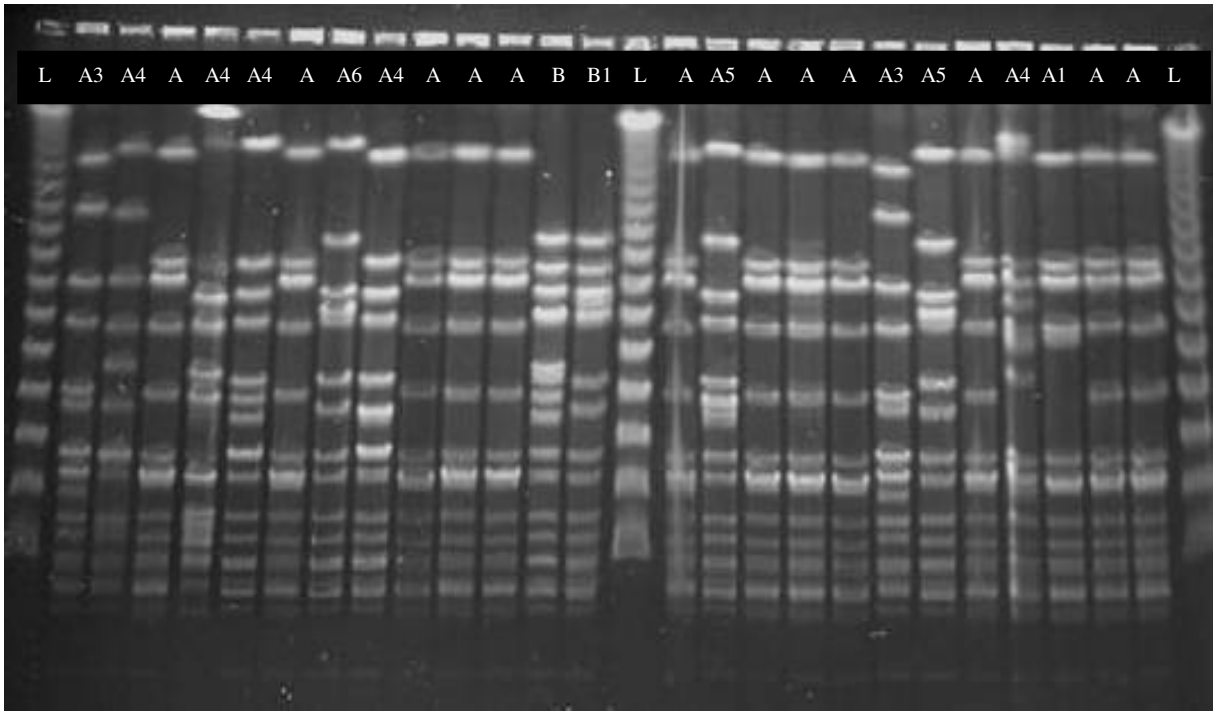

Figure 3.8: **Pulsed-field gel electrophoresis pulsotypes showing the separation of restriction fragments of the MRSA genome digested with the *Sma*I enzyme. A indicates major pulsotype A. A1 to A3 indicate MRSA strains, which are closely related to pulsotype A. A3 to A5 indicate MRSA strains, which are possibly related to pulsotype A. B and B1 indicate MRSA strains, which are unrelated to pulsotype A. L= Lambda ladder**

CHAPTER 4

CONCLUSION

4.1 Concluding remarks

Over the past decades, *Staphylococcus aureus* (*S. aureus*) has been the most virulent species of the *Staphylococcus* genus (Mathanraj *et al*, 2009). This bacterium is the most prevalent pathogen isolated from hospital settings and the second most common bacterium isolated in the community setting (Oliveira and De Lencastre, 2011). Globally, there is great concern in the public health sector regarding antibiotic resistance and virulence genes in *S. aureus*, particularly methicillin resistant *S. aureus* (MRSA) strains (De Lencastre *et al*., 2007; Plata *et al*., 2009; Ahmed, 2011). About 29% to 46% of *S. aureus* isolates are resistant to methicillin in South Africa and some have emerged as a common cause of community-associated infections (Jansen van Rensburg *et al*. 2011).

Standardised phenotypic methods, such as the cefoxitin disc diffusion, have been used for the identification of MRSA isolates (Brown *et al*., 2005). Although these methods are useful in the identification of MRSA isolates, detection of the *mec*A gene is considered the gold standard for MRSA diagnosis and confirmation (Anand *et al*., 2009). Fast, easy and reliable molecular identification methods for MRSA detection and characterisation are important, not only for determining the appropriate antibiotic treatment for patients, but also for the control of MRSA transmission within the hospital setting (Pramodhini *et al*., 2011). Six different M-PCR assays were utilised in this study, to detect, identify and characterise 194 MRSA clinical isolates. The gold standard pulsed-field gel electrophoresis (PFGE) was used to investigate the clonal relatedness of these MRSA isolates. The hyphothesis of this study was that HA-MRSA and CA-MRSA isolates obtained from clinical specimens from the Pretoria Hospital were genetically distinct.

The McClure *et al*. (2006) M-PCR assay was easy and accurate in detecting the 16S rRNA, *mec*A and the PVL genes in this study. This assay could be easily implemented in the diagnostic laboratory for the rapid identification of MRSA isolates. The PVL gene was only detected in one MRSA isolate. These results are consistent with other South Africa studies,

which reported a low prevalence of PVL positive (0% to 4%) MRSA isolates in clinical settings (Makgotlho *et al*., 2009; Shittu *et al*., 2009; Moodley *et al*. 2010; Jansen van Rensburg *et al*., 2011). The low prevalence indicates that the PVL gene cannot be used as a sole marker to distinguish CA-MRSA from HA-MRSA isolates in this clinical setting.

The five SCC*mec* M-PCR typing assays (Oliviera and De Lencastra, 2002; Zhang *et al*., 2005; Boye *et al*., 2007; McClure *et al*., 2010; Zhang *et al*., 2012) showed an overall prevalence rate of 64.3% (92/143) for the HA-MRSA isolates and 35.7% (51/143) for the CA-MRSA isolates. The increased prevalence of the CA-MRSA in this clinical setting could be because the hospital is a referral hospital; therefore, MRSA could be easily introduced by infected patients or healthcare workers who are transferred from other hospitals. The other reason could be due to factors, such as overcrowding in the big cities compared to rural areas and the lack of cleanliness (Chambers and DeLeo, 2009; Samie and Shivambu, 2011). Thus, continuation of proper infection control measures may help to stabilise the rising prevalence rates of CA-MRSA in this clinical setting.

In the current study, the five SCC*mec* typing assays gave different results and these results were compared to obtain overall reliable results. The Zhang *et al*. (2005) assay proved to be a useful tool for subtyping of SCC*mec* type IV [IVa (4%), IVb (2%) and IVd (94%)] of the CA-MRSA clinical isolates in comparison to the Boye *et al*. (2007) assay. However, the use of nine primer pairs could make this assay unfeasible for diagnostic laboratories, particularly when a large volume of isolates have to be analysed. Furthermore, identification of the SCC*mec* type is solely based on the detection of a specific fragment within the J1 region and neither the *mec* classes nor *ccr* types are used (Milheiriço *et al*., 2007). The Boye *et al*. (2007) SCC*mec* typing assay, which was used for the confirmation of the SCC*mec* types, could be useful in the screening of large numbers of MRSA isolates for the detection of SCC*mec* type I, IV and V (Turlej *et al*., 2011). The assay can also be useful in reducing the number of untypeable MRSA isolates and correctly type SCC*mec* type V, which is known to be mistyped according to the Zhang *et al*. (2005) and Oliviera and De Lencastre (2002) assays (Turlej *et al*., 2011). However, the lower sensitivity of the primer pairs in the detection of SCC*mec* type II and mistyping of SCC*mercury* as SCC*mec* type III makes the Boye *et al*. (2007) assay inadequate for the characterisation of SCC*mec* type II and type III in this clinical setting (Turlej *et al*., 2011). In cases of inconclusive SCC*mec* types, such as the MRSA

isolates having double bands of SCC*mec* type II and IVc (5%), the Boye *et al*. (2007) assay could be advantageous in resolving the SCC*mec* types.

Although, the M-PCR assay for SCC*mec* typing by Oliveira and De Lencastre (2002) was useful in the confirmation of the discrepant results, several limitations were noted using this assay. Firstly, the assay could not detect the *kdp* gene from 11 MRSA isolates; secondly, the *ccr*C primers were specific for SCC*mercury* instead of SCC*mec* type III and lastly, SCC*mec* type V was misclassified as SCC*mec* type III. This observation was similar to the results reported by Shore *et al*. (2005); Chongtrakool *et al*. (2006) and Jansen *et al*. (2009). A fourth SCC*mec* typing assay according to Zhang *et al*. (2012) was used to resolve the discrepancies between SCC*mec* type II and III. This assay was sensitive in detecting SCC*mec* type II (100%), not SCC*mec* type III. According to Zhang *et al*. (2012), the universal primers are designed to correct the misclassification of SCC*mercury* as SCC*mec* type III obtained using the Zhang *et al*. (2005) assay. Overall all the five SCC*mec* typing assays gave inconclusive results for SCC*mec* type II and SCC*mercury* (type III) and the presence of SCC*mec* type III or VIII was not observed from the 194 MRSA isolates.

The genetic similarities denoted by PFGE typing revealed 11 pulsotypes among the 193 MRSA isolates analysed. Although, the PFGE method was able to determine the clonal relatedness of the MRSA strains in this hospital setting, the technique was found to be labour intensive, technically demanding and time consuming as previously reported by Chung *et al*. (2012). A high level of technical expertise was required to perform this technique (Al-Zahrani *et al*. 2011). Comparison of the PFGE patterns with other studies done in South Africa was difficult. Nonetheless, the PFGE typing showed that the majority (85%) of the MRSA isolates circulating in this clinical were closely related to one another, opposing the hypothesis made in the beginning of this study. The pulsotypes were determined manually because not all of the banding patterns were of the same intensity to use the GelCompar II software to construct the dendrogrammes. Pulsed-field gel electrophoresis remained an essential tool for determining and discriminating the clonally related MRSA strains in this clinical setting. The results obtained in this study will serve as a database for future comparison with other studies over time in this clinical setting.

The use of combined genotyping methods, such as SCC*mec* typing, PFGE, MLST and *spa* typing for the characterisation of MRSA isolates could provide reliable and accurate results (Shore *et al*. 2005; Kim, 2009). These assays may also be important tools in the early recognition of the dominant MRSA clones and can be used as guidance in the selection of proper empiric antibiotic treatment (Shore *et al*., 2011). Each assay has its own limitations, eg there is no universal SCC*mec* typing assay for the detection of SCC*mec* types; using different SCC*mec* M-PCR assays does not always provide similar results (Kim, 2009; Ghaznavi-Rad *et al*., 2010). However, a SCC*mec* typing assay may work better in one laboratory compared to another; therefore, optimisation of the M-PCR assays in different laboratories is required in order to obtain accurate and acceptable results (Zhang *et al*., 2012). Nonetheless, once the M-PCR assay for SCC*mec* typing is optimised and validated, the assay becomes a valuable tool, which facilitates the understanding of evolutionary relationships among MRSA isolates (Deurenberg and Stobberingh, 2008; Chen *et al*., 2009). Moreover, multiple genes can be detected in one M-PCR reaction, which makes characterisation of HA-MRSA and CA-MRSA strains rapid and possible for correct treatment of the infections caused by these strains (Turlej *et al*., 2011).

Periodical typing of MRSA isolates using combined genotyping methods is recommended to ensure that the trends of these isolates are monitored adequately to prevent the spread of these strains in this clinical setting (Shore *et al*., 2005; Otter and French, 2012). Furthermore, proper hygiene, disinfection, patient isolation, screening and decolonisation may decrease the MRSA infections in this clinical setting (Cookson *et al*., 2011; Skov *et al*., 2012).

4.2 Future research

Up to date, susceptibility testing, such as disk diffusion and agar screening methods are the only standardised methods for identifying MRSA in the diagnostic laboratories (Rallapalli *et al*., 2008). The drawback of these tests is that the inoculum size, incubation time, temperature, salt concentration of the medium, pH of the medium and exposure of the bacterium to β-lactam antibiotics influence the phenotypic expression of resistant genes (Rallapalli *et al*., 2008). Further investigations are required to determine the mechanism in which the SCC*mer* (SCC*mercury*) positive strains acquire antibiotic resistance in this clinical setting. Moreover, future studies should be based on determining the antibiotic resistance of

other *Staphylococcus* (CoNS) species, such as *S. epidermidis*. This is because, *S. epidermidis* may serve as reservoirs of the *mec*A and PVL toxin genes for *S. aureus* strains, making these strains more virulent (Turlej *et al*., 2011). The role of antibiotic resistance and virulence genes present in zoonotic *Staphylococcus* strains should also be investigated to determine the cross-transmission of these strains between animals and human contacts in the community and the environment. It is important to determine the potential health implications associated with these zoonotic strains and the effect it may have on CA-MRSA as well as HA-MRSA infections in the future.

Future studies are required to combine, optimise and re-design the primer pairs, which showed similar results including primers for the detection of SCC*mec* types (VI and VII) in order to obtain a standardised SCC*mec* typing for this clinical setting. A follow-up study is required to type the 194 MRSA isolates using more rapid genotyping methods with high discriminatory power, such as MLST, *spa* typing, *agr* typing and matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF) to obtain complete characterisation of these isolates. Future research should be directed towards the development of strict infection control policies and surveillance programmes in the hospital so that MRSA outbreaks may be prevented. Studies should focus on the continuation of educating healthcare workers regarding infection control measures, such as isolation of patients who are infected with MRSA and monitoring of patients antibiotic usage, so that the emergence and spread of multidrug resistant MRSA strains can be limited.

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APPENDIX A

Reagents and electrophoresis 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, bring volume to 500 mℓ. Autoclave at 121°C for 15 min

2. DNA extraction reagents (Sambrook and Russell, 2001)

a) Ethylene diamine tetra-acetate (EDTA) (pH 8.0) (500 mℓ)

EDTA, Disodium salt (dihydrate) (Merck, Darmstadt, Germany)	93.5	g
Sodium hydroxide (NaOH) pellet (Merck, Darmstadt, Germany)		
Distilled water	400	mℓ

m=cmv

374.2 X 0.5 X 500 mℓ= 93.5 g in 500 mℓ

Dissolve 93.5 g of 0.5 M EDTA in 400 mℓ of sterile distilled water and use the NaOH pellet to adjust pH to 8.0 then bring volume to 500 mℓ. Autoclave at 121°C for 15 min

b) Sodium Tris EDTA (STE) (pH 8) (500 mℓ)

10 mM NaCl (Merck, Darmstadt, Germany)	0.292	g
10 mM Tris-HCl (Sigma-Aldrich, St. Louis, USA)	1.575	g
1 mM EDTA (Sigma-Aldrich, St. Louis, USA)	1	mℓ
Distilled water	450	mℓ

Dissolve 0.292 g of NaCl, 1.575 g of Tris-HCl and 1 mℓ of EDTA in 450 mℓ of sterile distilled water and adjust pH to 8, then bring volume to 500 mℓ. Autoclave at 121°C for 15 min

c) Lysozyme (50 mg.mℓ$^{-1}$) (1 mℓ)

Lyophilised lysozyme (Roche Applied Science, Germany)	0.05	g
Distilled water	500	µℓ

Dissolve 0.05 g of lyophilised lysozyme in 500 µℓ of distilled water then bring volume to 1 mℓ

The lysozyme was used to disrupt *S. aureus* cell wall by digesting the peptidoglycan, which is responsible for the rigidity of the cell

d) Sodium dodecyl sulphate (SDS) 20% solution (pH 7.2) (500 mℓ)

SDS (Promega, Madisa, USA)	100	g
Distilled water	480	mℓ

Dissolve 100 g of electrophoresis grade SDS in 480 mℓ of sterile distilled water and heat at 68°C. Stir with a magnetic stirrer to dissolve the SDS and adjust pH to 7.2 by adding a few drops of HCl (Merck, Darmstadt, Germany), then bring volume to 500 mℓ. Autoclave at 121°C for 15 min

Sodium dodecyl sulphate is a detergent, which dissolves the cell membrane made of lipid and is also used to denature protein once the cell has been lysed

e) Proteinase K (20 mg.mℓ$^{-1}$) (1 mℓ)

Proteinase K (Roche Applied Science, Germany)	0.02	g
Distilled water	500	µℓ

Weigh 0.02 g of proteinase K and dissolve in 500 µℓ of sterile distilled water then bring volume to 1 mℓ

Proteinase K is an enzyme that is used to digest protein and removes contaminant during DNA isolation. Adding proteinase K to nucleic acid preparation rapidly inactivates nucleases that might otherwise degrade the DNA or RNA during purification

f) Phenol:chloroform:isoamylalcohol (25:24:1)

Phenol:chloroform:isoamylalcohol (25:24:1) solution (Merck, Darmstadt, Germany)

The solution is used to purify the DNA and also to separate proteins and inactivate DNase enzymes

g) Chloroform:isoamylalcohol (24:1) (500 mℓ)

Chloroform alcohol (Merck, Darmstadt, Germany)	480	mℓ
Isoamylalcohol (Merck, Darmstadt, Germany)	20	mℓ

500 X 24/25 = 480 mℓ

500 X 1/25 = 20 mℓ

Take 480 mℓ of Chloroform and add to 20 mℓ of isoamylalcohol

Chloroform:isoamylalcohol is used to separate the phenol layer from the aqueous layer containing the DNA

h) Sodium acetate (NaOAc) 3 M (pH 5.2) (500 mℓ)

Sodium acetate (Merck, Darmstadt, Germany)	204.2	g
Distilled water	400	mℓ

Dissolve 204.2 g of NaOAc in 400 mℓ of sterile distilled water and adjust pH to 5.2 using glacial acetic acid (Merck, Darmstadt, Germany) and bring volume to 500 mℓ. Autoclave at 121°C for 15 min

Sodium acetate regulates the salt concentration of the DNA strands, allowing more adhesion of the strands to one another

i) 70% ethanol (500 mℓ)

Ethanol (Merck, Darmstadt, Germany)	350	mℓ
Distilled water	150	mℓ

Add 150 mℓ of sterile distilled water to 350 mℓ ethanol

j) Tris EDTA (TE) buffer (pH 8) (500 mℓ)

10 mM Tris-HCl (Sigma-Aldrich, St. Louis, USA)	1.58	g
1 mM EDTA (Merck, Darmstadt, Germany)	1	mℓ
Distilled water	400	mℓ

Dissolve 1.58 g of Tris-HCl in 400 mℓ of sterile distilled water and add 1 mℓ of EDTA buffer. Adjust pH to 8 and bring volume to 500 mℓ. Autoclave at 121°C for 15 min

TE buffer assists in dissolving the DNA pellet and to re-suspend DNA

k) 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. Pulsed-field gel electrophoresis reagents (McDougal *et al*., 2003)

3.1 Stock solutions of 0.5 M EDTA (pH 8) (500 mℓ)

EDTA (Merck, Darmstadt, Germany)	93.5	g
Distilled water	450	mℓ

m=cmv

374.2 g X 500 mℓ X 500 mℓ =93.5 g in 500 mℓ

Weigh 93.5 g of EDTA and add 450 mℓ of sterile distilled water. Adjust pH to 8 and bring volume to 500 mℓ. Autoclave at 121°C for 15 min

3.2 Stock solutions of 1 M Tris-HCl (pH 8) (500 mℓ)

Tris-HCl (Sigma-Aldrich, St. Louis, USA)	78.8	g
Distilled water	450	mℓ

Weigh 78.8 g of Tris-HCl and add 450 mℓ of sterile distilled water. Adjust pH to 8 and bring volume to 500 mℓ. Autoclave at 121°C for 15 min

3.3 The following reagents were prepared using the stock solutions above:

a) EET buffer (pH 8) (500 mℓ)

100 mM EDTA (Merck, Darmstadt, Germany)	100	mℓ
10 mM Tris-HCl (pH 8) (Sigma-Aldrich, St. Louis, USA)	5	mℓ
Distilled water	395	mℓ

Add 100 mℓ of 0.5 M EDTA (stock solution) and 5 mℓ of 1 M Tris-HCl (stock solution) to 395 mℓ of sterile distilled water. Autoclave at 121°C for 15 min

b) Tris-EDTA (TE) buffer (storage buffer) (pH 8) (500 mℓ)

10 mM EDTA (Merck, Darmstadt, Germany)	10	mℓ
10 mM Tris-HCl (pH 8) (Sigma-Aldrich, St. Louis, USA)	5	mℓ
Distilled water	485	mℓ

Add 10 mℓ of 0.5 M EDTA (stock solution) and 5 mℓ of 1 M Tris-HCl (stock solution) to 485 mℓ of sterile distilled water. Autoclave at 121°C for 15 min

c) Sodium dodecyl sulphate 10% solution (500 mℓ)

SDS (Promega, Madisa, USA)	50	g
Distilled water	480	mℓ

Dissolve 50 g of electrophoresis grade SDS in 480 mℓ of sterile distilled water and heat at 68°C and stir with a magnetic stirrer to dissolve the SDS, then bring volume to 500 mℓ. Autoclave at 121°C for 15 min

d) Lysostaphin (5 mg.mℓ$^{-1}$) (5 mℓ)

Lysostaphin (Sigma-Aldrich, St. Louis, USA)	0.25	g
Distilled water	4.5	mℓ

0.005g X 5 mℓ =0.25 g in 5 mℓ

Weigh 0.25 g of lysostaphin and add 4.5 mℓ of sterile distilled water then bring volume to 5 mℓ

e) Phenyl methyl sulfonyl fluoride (PMSF) 400 mM

PMSF (Roche, Molecular Diagnostics, Germany)	0.7	g
Ethanol (Merck, Wadeville, Gauteng)	10	mℓ

Dissolve 0.7 g of PMSF in 10 mℓ of Ethanol and store at -20°C

f) Tris-HCl (pH 8.0) (500 mℓ)

10 mM Tris-HCl (Sigma-Aldrich, St. Louis, USA)	5	mℓ
Distilled water	495	mℓ

Add 5 mℓ of 1 M Tris-HCl (stock solution) in 495 mℓ of sterile distilled water. Autoclave at 121°C for 15 min

g) 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 EDTA buffer. Adjust pH to 8.3 and bring volume to 500 mℓ. Autoclave at 121°C for 15 min

h) *Sma*I digestion enzyme 0.3 U/µl

*Sma*I enzyme (Fermentas Life Sciences, Thermo Scientific, USA)	3	µℓ
10X Reaction buffer (Fermentas Life Sciences, Thermo Scientific, USA)	10	µℓ
Distilled water	57	µℓ

Add 3 µℓ of *Sma*I enzyme and 10 µℓ of reaction buffer containing 0.1% bovin serum albumin (BSA) (Fermentas Life Sciences, Thermo Scientific, USA), bring the volume to 70 µℓ using distilled water

References

McDougal LK, Steward CD, Killgore GE, Chaitram JM, McAllister SK and Tenover FC (2003) Pulsed-field gel electrophoresis typing of oxacillin resistant *Staphylococcus aureus* isolates from the United States: Establishing a national database. *Journal of Clinical Microbiology* **41**:5113-5120

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APPENDIX B

1. Confirmation of MRSA isolates

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 (Labcon, Sepsci, South Africa) at 37°C for 18 h to 24 h.

c) Gram-staining was perfomed 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 (Labcon, Sepsci, South Africa) at 37°C for 18 h to 24 h.

e) Freeze cultures were prepared by transferring 750 µℓ of a 50% sterile glycerol (Merck, Darmstadt, Germany) plus 750 µℓ of the broth culture to a sterile 2 mℓ cryotubes (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) One millilitre of the cell suspension in a BHI (Merck, Darmstadt, Germany) was taken after 48 h of incubation (Labcon, Sepsci, South Africa) and inoculated 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.

b) The supernatant was discarded and the pellets were placed on ice.

c) Pellets were re-suspended in 1 mℓ sodium STE buffer [0.1 M NaCl (Merck, Darmstadt, Germany), 1 mM EDTA (pH 8.0) (Merck, Darmstadt, Germany) and 10 mM Tris-HCl (pH 7.5) (Sigma-Aldrich, St. Louis, USA)] by careful up and down aspiration with a pipette.

d) Ten microlitres of lysozyme (50 mg.mℓ$^{-1}$) (Roche Applied Science, Germany) was added to each of the cell suspensions and incubated (AccuBlock^{TM}, WhiteScientific, South Africa) at 37°C for 1 h.

e) A volume of 50 µℓ of a 20% SDS solution (Promega, Madison, USA) was added to each tube, followed by 10 µℓ proteinase K (20 mg.mℓ$^{-1}$) (Roche Applied Science, Germany).

f) The Eppendorf tubes (Eppendorf AG, Hamburg, Germany) were incubated (Grant instrument, Cambridge, England) at 56°C for 1 h or until the suspension was clear.

g) Several gentle extractions were performed thrice with equal volumes of mixed phenol:chloroform:isoamylalcohol (Merck, Darmstadt, Germany) in the ratio of 25:24:1, respectively.

h) The suspensions was centrifuged (Spectrafuge 24D, Labnet International, Inc., New Jersey, USA) at 4 930 *x g* for 5 min at 25°C.

i) The supernatant was transferred to a sterile 2 mℓ Eppendorf tube (Eppendorf AG, Hamburg, Germany) and an equal volume of chloroform:isoamylalcohol (24:1) (Merck, Darmstadt, Germany) was added to the suspension.

j) The suspension was centrifuged (Spectrafuge 24D, Labnet International, Inc., New Jersey, USA) at 4 930 *x g* for 20 min at 15°C to remove any traces of phenol from the DNA suspension.

k) The supernatant (DNA-containing aqueous phase) was removed and added to a sterile 2 mℓ Eppendorf tube (Eppendorf AG, Hamburg, Germany) and the concentration of the aqueous phase was adjusted using a stock solution of 3 M sodium acetate (Merck, Darmstadt, Germany).

l) Equal volumes of ice-cold, 100% absolute ethanol (-20°C) (Merck, Darmstadt, Germany) was added and mixed, the tubes were stored overnight at -20°C (Kelvinator, Gauteng) to precipitate the DNA.

m) The DNA was pelleted by centrifugation (Spectrafuge 24D, Labnet International, Inc., New Jersey, USA) at 2 682 *x g* for 10 min at 4°C and the supernatant was discarded.

n) The pellets were desalted with 400 µℓ of ice-cold, 70% absolute ethanol (-20°C) (Merck, Darmstadt, Germany) and the supernatant was discarded, the tubes were inverted to dry.

o) The DNA pellet was resuspended in 100 µℓ of TE buffer [10 mM Tris HCl, 1 mM EDTA (pH 8)] (Sigma-Aldrich, St. Louis, USA) and stored at -20°C (Kelvinator, Gauteng) until further analysis.

3. Determination of the genetic relatedness of MRSA isolates using the pulsed-field gel electrophoresis genotyping technique

a) Determination of the clonal relatedness of the 193 MRSA isolates was performed according to the method described by McDougal *et al*. (2003).

b) Single colonies from the Blood agar plates (Oxoid, England) were inoculated into 3 mℓ BHI (Merck, Darmstadt, Germany) and incubated (New Brunswick Scientific co. Inc, New Jersey, USA) with vigorous shaking at 35°C for 24 h.

c) The cell suspension was adjusted to an absorbance of 1.00 at a wavelength of 600 nm using a spectrophotometer (Jenway 6300, UK).

d) A total of 1.5 mℓ of the cell suspension was added to a 2 mℓ Eppendorf tube (Eppendorf AG, Hamburg, Germany) and centrifuged (Eppendorf 5417C, Hamburg, Germany) at 4 293 *x g* for 2 min and the supernatant was aspirated.

e) The pellet was resuspended in 500 µℓ of EET buffer [100 mM EDTA, 10 mM Tris-HCl (pH 8)] (Sigma-Aldrich, St. Louis, USA) and equilibrated in a heating block (Hägar designs HB2, Germany) at 37°C for 10 min.

f) The wells of the PFGE plugs (Bio-Rad, California, USA) were labelled with the appropriate isolate numbers.

g) A 1.5% of SeaKem Gold agarose gel (Lonza, Rockland, USA) was prepared by dissolving 0.3 g of the SeaKem Gold agarose powder (Lonza, Rockland, USA) in 20 mℓ of EET buffer [100 mM EDTA, 10 mM Tris-HCl (pH 8)] (Sigma-Aldrich, St. Louis, USA) and cooled in a 50°C waterbath (Labotec, Midrand, South Africa).

h) Equal volumes of 500 µℓ 1.5% SeaKem Gold agarose gel (Lonza, Rockland, USA) and 500 µℓ EET buffer (containing the pellet) [100 mM EDTA, 10 mM Tris-HCl (pH 8)] (Sigma-Aldrich, St. Louis, USA) was gently mixed and 100 µℓ of the cell suspension was poured into the small PFGE plug mold (Bio-Rad, California, USA) and allowed to set for 15 min at 25°C.

i) The plug for each MRSA isolate was removed and placed into a new 2 mℓ Eppendorf tube (Eppendorf AG, Hamburg, Germany).

j) Ten microlitres of lysostaphin (no. L-7386) (Sigma-Aldrich, St. Louis, USA) stock solution (1 mg.mℓ$^{-1}$) was added to the 2 mℓ Eppendorf tube (Eppendorf AG, Hamburg, Germany) containing EET buffer [100 mM EDTA, 10 mM Tris-HCl (pH 8)] (Sigma-Aldrich, St. Louis, USA).

k) The plugs were incubated (New Brunswick Scientific co. Inc, New Jersey, USA) at 30°C for 5 h with gentle shaking.

l) The plugs were removed and placed into a new 2 mℓ Eppendorf tube (Eppendorf AG, Hamburg, Germany) containing EET buffer [100 mM EDTA, 10 mM Tris-HCl (pH 8)] (Sigma-Aldrich, St. Louis, USA) with 100 µℓ Proteinase-K (20 mg.mℓ$^{-1}$) (Roche Applied Science, Germany) and 200 µℓ of 10% SDS (Promega, Madison, USA).

m) The 2 mℓ Eppendorf tube (Eppendorf AG, Hamburg, Germany) was incubated (New Brunswick Scientific co. Inc, New Jersey, USA) at 37ºC for 24 h with gentle shaking.

n) The EET buffer (100 mM EDTA, 10 mM Tris-HCl [pH 8]) (Sigma-Aldrich, St. Louis, USA) was poured off and PFGE storage buffer [10 mM EDTA, 10 mM Tris-HCl (pH 8)] (Sigma-Aldrich, St. Louis, USA) was added.

o) The plugs were stored at 4ºC until further analysis.

4. *Sma*I restriction endonuclease digestion

a) The plug slices were cut into a volume of 30 µℓ (one-third) and placed into a new 2 mℓ Eppendorf tube (Eppendorf AG, Hamburg, Germany).

b) The plugs were washed twice in 2 mℓ PFGE storage buffer [10mM EDTA, 10mM Tris-HCl (pH 8)] (Sigma-Aldrich, St. Louis, USA) containing 1 mM phenyl methyl sulfonyl fluoride (PMSF) (Roche Applied Science, Germany) for 45 min at 25ºC.

c) The plugs were further washed thrice in 2 mℓ 10 mM Tris-HCl (pH 8) buffer (Sigma-Aldrich, St. Louis, USA) for 25 min at 25ºC.

d) Excess liquid was removed and 70 µℓ solution containing 10 µℓ of a 10X reaction buffer (Fermentas Life Sciences, Thermo Scientific, USA), 10 µℓ of 0.01% BSA

(Fermentas Life Sciences, Thermo Scientific, USA) and 30 Units *Sma*I restriction endonuclease (Fermentas Life Sciences, Thermo Scientific, USA) was added for the digestion of the DNA.

e) The plugs were incubated (Labcon, Sepsci, South Africa) at 30°C for 2.5 h.

5. Pulsed-field gel eletrophoresis running parameters and conditions

a) A 1.6% SeaKem Gold agarose gel (Lonza, Rockland, USA) was prepared by dissolving 1.6 g of SeaKem Gold agarose powder (Lonza, Rockland, USA) in 160 mℓ of 0.5X TBE buffer [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 to 50ºC in a waterbath (Labotec, Midrand, South Africa) for 30 min.

c) The 1.6% SeaKem Gold agarose gel (Lonza, Rockland, USA) was poured into a casting tray (Bio-Rad, California, USA) containing a 30 well comb (Bio-Rad, California, USA) and allowed to solidify for 1 h.

d) A 2 ℓ of 0.5X TBE [45 mM Tris borate, (pH 8), 1 mM EDTA] (Sigma-Aldrich, St. Louis, USA) buffer was poured into the electrophoresis chamber (Bio-Rad, California, USA) and the peristaltic pump was switched on, followed by the cooling unit set to 14ºC to allow the 0.5X TBE buffer to reach 14ºC.

e) The 30 µℓ (one-third) plugs were loaded directly into the wells of the 1.6% SeaKem Gold agarose gel (Lonza, Rockland, USA) and the wells were sealed with 1% molten SeaKem Gold agarose gel (Lonza, Rockland, USA).

f) A thin slice of the Lambda molecular weight markers (Biometra, Göttingen, Germany) was loaded into the first, middle and the last lanes.

g) The 1.6% SeaKem Gold agarose gel (Lonza, Rockland, USA) was placed into the electrophoresis chamber (Bio-Rad, California, USA).

h) Pulsed-field gel electrophoresis was performed using a contour-clamped homogeneous electric field CHEF-DR® III system (Bio-Rad, California, USA).

i) Running parameters was as follows: 6 $V.cm^{-1}$ at 14°C for 20 h, with an initial switch time of 5 s and a final switch time of 50 s

6. Analysis of the M-PCR assays amplicons and PFGE products

6.1 Multiplex-PCR assays amplicons analysis

a) A 1% MetaPhor™ agarose gel (Lonza, Rockland, USA) was prepared by dissolving 1 g of MetaPhor™ agarose powder (Lonza, Rockland, USA) 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 µℓ of ethidium bromide (stock of 10 mg^{-1}) (Promega, Madison, USA) was added to the MetaPhor™ agarose gel (Lonza, Rockland, USA) and the mixture was poured into a casting tray (Bio-Rad, California, USA) containing a 15 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 M-PCR amplification were separated at 100 $V.cm^{-1}$ for 1 h 30 min.

f) The MetaPhor™ agarose gel (Lonza, Rockland, USA) was used to easily distinquish between similarly sized amplicons differing by less than 20 bp.

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

h) A negative control was included in all six M-PCR assays, which consisted of RNase free water (Promega, Madison, USA).

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

6.2 Pulsed-field gel electrophoresis products analysis

a) The PFGE gels was stained with 0.5 µg.mℓ$^{-1}$ (stock solution 10 mg.mℓ$^{-1}$) ethidium bromide (Promega, Madison, USA) for 30 min and destained in distilled water for 10 min.

b) The band patterns were viewed, photographed and digitalised using a GelDoc (Bio-Rad, California, USA).

c) Band patterns were analysed using Tenover *et al*. (1995) recommendations

References

McDougal LK, Steward CD, Killgore GE, Chaitram JM, McAllister SK and Tenover FC (2003) Pulsed-field gel electrophoresis typing of oxacillin resistant *Staphylococcus aureus* isolates from the United States: Establishing a national database. *Journal of Clinical Microbiology* **41**:5113-5120

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APPENDIX C: Clinical information of MRSA isolates and molecular results

Table 3.14: Overall results of the six M-PCR assays 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	First M-PCR (McClure *et al.*, 2006)		SCC*mec* M-PCR typing assays						PFGE group
							16S rRNA & *mec*A	PVL	Type I to V & subtype IVa to IVd (Zhang *et al.*, 2005)	Type I to V (Boye *et al.*, 2007)	Type I to III (Oliveira and De Lencastre, 2002)	Type II & III (Zhang *et al.*, 2012)	Type VIII (McClure *et al.*, 2010)	Overall results of the five M-PCR SCC*mec* typing assays	
1	F	27 d	PS	Pus swab	NP	NP	+	-	NT	I	I	PN	PN	I	A3
2	M	35 y	Urology	BC	NP	NP	+	-	IVd	IV	PN	PN	ND	IVd	E
3	M	28 y	Neurosurgical	BC	NP	NP	+	-	SCC*mercury*	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
4	M	28 y	Neurosurgical	BC	+	+	+	-	II	II	II	II	PN	II	Cont
5	M	28 y	ST	BC	+	NP	+	-	SCC*mercury*	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
6	F	38 y	ST	BC	+	NP	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
7	M	29 y	IM	Pus swab	+	NP	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
8	F	38 y	ST	BC	+	NP	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
9	F	38 y	ST	BC	+	NP	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
10	M	29 y	IM	Urine	+	NP	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
11	F	38 y	ST	BC	+	+	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
12	F	38 y	ST	BC	+	+	+	-	SCC*mercury*	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
13	F	58 y	MP	Luki	+	+	+	-	IVd	IV	PN	PN	ND	IVd	A5
14	F	38 y	ST	CVP tip	+	+	+	-	SCC*mercury*	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A1
15	M	4 y	Ward 60	BC	+	+	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
16	M	2 y6m	Neurosurgical	Luki	+	NP	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A1
17	M	63 d	Paediatric ICU	CVP tip	+	+	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A1
18	M	2 y	Neurosurgery	CVP tip	+	NP	+	-	SCC*mercury*	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A1
19	M	65 y	OP	Tissue	+	NP	+	-	IVd	IV	PN	PN	ND	IVd	A5
19	M	65 y	OP	Tissue	+	NP	+	-	IVd	IV	PN	PN	ND	IVd	A5
20	F	38 y	ST	BC	+	NP	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
21	F	38 y	ST	CVP tip	+	NP	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
22	F	38 y	ST	BC	+	+	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A

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

Table 3.14: Overall results of the six M-PCR assays and PFGE used for the characterisation of the 194 MRSA isolates obtained from the Steve Biko Academic Hospital (cont)

MRSA isolates	Gender	Age	Clinical wards	Specimen Type	E-test	Vitek 2 System	First M-PCR (McClure *et al.*, 2006)		SCC*mec* M-PCR typing assays						PFGE group
							16S rRNA & *mec*A	PVL	Type I to V & subtype IVa to IVd (Zhang *et al.*, 2005)	Type I to V (Boye *et al.*, 2007)	Type I to III (Oliveira and De Lencastre, 2002)	Type II & III (Zhang *et al.*, 2012)	Type VIII (McClure *et al.*, 2010)	Overall results of the five M-PCR SCC*mec* typing assays	
23	F	7 m	PS	BC	+	+	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
24	M	35 y	OP	Pus swab	+	+	+	-	IVd	IV	PN	PN	ND	IVd	A3
25	F	4y4 m	PS	BC	NP	+	+	-	SCC*mercury*	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A3
26	M	33 y	OPD	Pus swab	+	NP	+	-	IVd	IV	PN	PN	ND	IVd	A
27	F	47 y	ST	BC	+	+	+	-	SCC*mercury*	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
28	F	2y1 m	PS	CVP tip	+	+	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
29	F	23 y	Nephrology	Urine	NP	NP	+	-	IVd	IV	PN	PN	ND	IVd	C
30	M	2y6 m	PS	BC	NP	+	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
31	M	49 y	Neurosurgery	CVP tip	+	NP	+	-	IVd	IV	PN	PN	ND	IVd	D
32	M	45 m	HCM	BC	+	NP	+	-	II	II	II	II	PN	II	A5
33	F	12 d	ST	CVP tip	+	NP	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
34	M	22 y	OP	Tissue	+	NP	+	-	SCC*mercury*	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
35	M	1y2 m	PS	CVP tip	+	NP	+	-	NT	I	I	PN	PN	I	A3
36	F	4 m	ICU	BC	+	NP	+	-	SCC*mercury*	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A4
37	M	22 y	OP	Tissue	NP	NP	+	-	SCC*mercury*	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
38	M	26 y	HCM	CVP tip	+	NP	+	-	IVd	IV	PN	PN	ND	IVd	A4
39	M	92 d	Coronary ICU	BC	NP	NP	+	-	IVd	IV	PN	PN	ND	IVd	A4
40	M	27 y	ST	CVP tip	+	+	+	-	SCC*mercury*	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
41	M	10 m	GP	CVP tip	+	+	+	-	IVd	IV	PN	PN	ND	IVd	A6
42	M	2 y	PP	Sputum	NP	+	+	-	IVd	IV	PN	PN	ND	IVd	A4
43	M	45 y	Short stay	BC	+	NP	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
44	M	9 d	Neonatal ICU	BC	+	NP	+	-	SCC*mercury*	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
45	M	1 d	Baby room ICU	BC	NP	NP	+	-	II	SCC*mercury* (II+SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A
46	M	17d	Neonatal ICU	Pus swab	NP	NP	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	B

BC	-Blood culture	GP	-General paediatric	M	-Male	NT	-Not typeable	PP	-Paediatric pulmonology
CVP tip	-Central venous pressure tip	HCM	-High care medical	M	-Months	OP	-Orthopaediatric	PS	-Paediatric surgery
D	-Days	ICU	-Intensive care unit	ND	-Not detected	OPD	-Outpatient department	ST	-Surgery
F	-Female	Luki	-Endotracheal aspirate	NP	-Not provided	PN	-Primer not included	Y	-Years

Table 3.14: Overall results of the six M-PCR assays and PFGE used for the characterisation of the 194 MRSA isolates obtained from the Steve Biko Academic Hospital (Cont)

MRSA isolates	Gender	Age	Clinical wards	Specimen Type	E-test	Vitek 2 System	First M-PCR (McClure *et al.*, 2006)		SCC*mec* M-PCR typing assays						PFGE group
							16S rRNA & *mec*A	PVL	Type I to V & subtype IVa to IVd (Zhang *et al.*, 2005)	Type I to V (Boye *et al.*, 2007)	Type I to III (Oliveira and De Lencastre, 2002)	Type II & III (Zhang *et al.*, 2012)	Type VIII (McClure *et al.*, 2010)	Overall results of the five M-PCR SCC*mec* typing assays	
47	M	15 y	Nephrology	BC	+	NP	+	-	IVd	IV	PN	PN	ND	IVd	B
48	M	44 y	ST	BC	+	NP	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
49	M	50 y	Neurosurgical	Luki	NP	NP	+	-	IVd	IV	PN	PN	ND	IVd	A5
50	M	25 d	Paediatric ICU	BC	+	NP	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
51	M	56 y	Cardiothoracic	BC	NP	+	+	-	II	SCC*mercury* (II+SCC*mercury*)	SCC*mercury*	II	PN	II+ SCC*mercury*	A
52	M	22 y	Neurosurgical	CVP tip	+	+	+	-	I	I	I	PN	PN	I	F
53	M	38 y	ST	BC	+	+	+	-	II	SCC*mercury* (II+SCC*mercury*)	SCC*mercury*	II	PN	II+ SCC*mercury*	A
54	M	1y2 m	PS	CVP tip	+	NP	+	-	NT	I	I	PN	PN	I	A3
55	M	13 d	Neonatal ICU	BC	+	NP	+	-	IVd	IV	PN	PN	ND	IVd	A5
56	F	73 y	IM	CVP tip	+	+	+	-	II	SCC*mercury* (II+SCC*mercury*)	II+SCC*mercury*	II	PN	II+ SCC*mercury*	A
57	M	52 y	OP	Tissue	+	+	+	-	II+IVc	II	II	II	PN	II	A4
58	M	16 d	Paediatric ICU	BC	+	NP	+	-	IVd	IV	PN	PN	ND	IVd	A1
59	M	25 y	ST	BC	+	NP	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A2
60	M	25 y	ST	BC	+	NP	+	-	SCC*mercury*	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
61	M	27 y	ST	BC	+	+	+	-	IVd	IV	PN	PN	ND	IVd	A4
62	F	14 y	Oncology	Pus swab	+	NP	+	-	IVd	IV	PN	PN	ND	IVd	A
63	M	57 y	HCM	CVP tip		+	+	-	IVd	IV	PN	PN	ND	IVd	A
64	M	50 y	Urology	Urine	+	NP	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
65	M	20 y	Neurological	Urine	+	NP	+	-	II	SCC*mercury* (II+SCC*mercury*)	SCC*mercury*	II	PN	II+ SCC*mercury*	A

BC -Blood culture; CVP tip -Central venous pressure tip; D -Days; F -Female; HCM -High care medical; ICU -Intensive care unit; IM -Internal medicine; Luki -Endotracheal aspirate; M -Male; M -Months; ND -Not detected; NP -Not provided; NT -Not typeable; PN -Primer not included; OP -Orthopaedic; PS -Paediatric surgery; ST -Surgery and trauma; Y -Years

Table 3.14: Overall results of the six M-PCR assays and PFGE used for the characterisation of the 194 MRSA isolates obtained from the Steve Biko Academic Hospital (Cont)

MRSA isolates	Gender	Age	Clinical wards	Specimen Type	E-test	Vitek 2 System	First M-PCR (McClure *et al.*, 2006)		SCC*mec* M-PCR typing assays						PFGE group
							16S rRNA & *mec*A	**PVL**	**Type I to V & subtype IVa to IVd** (Zhang *et al.*, 2005)	**Type I to V** (Boye *et al.*, 2007)	**Type I to III** (Oliveira and De Lencastre, 2002)	**Type II & III** (Zhang *et al.*, 2012)	**Type VIII** (McClure *et al.*, 2010)	**Overall results of the five M-PCR SCC*mec* typing assays**	
66	F	14 y	Oncology	Pus swab	+	+	+	-	II	SCC*mercury* (II+SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A
67	M	4 d	Paediatric ICU	BC	+	NP	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
68	M	60 y	MP	Luki	+	NP	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
69	M	60 y	MP	Luki	+	+	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
70	F	69 y	OP	Pus swab	NP	NP	+	-	II	SCC*mercury* (II+SCC*mercury*)	SCC*mercury*	II	PN	II+ SCC*mercury*	A
71	F	20 y	OP	Pus swab	+	NP	+	+	SCC*mercury*	V	SCC*mercury*	NT	PN	V	A3
72	M	48 y	CS	Pus swab	+	NP	+	-	II	SCC*mercury* (II+SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A
73	M	16 y	OP	Pus swab	NP	NP	+	-	IVd	IV	PN	PN	ND	IVd	A
74	M	29 y	OP	Pus swab	NP	NP	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A3
75	M	49 y	ICU	BC	+	NP	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	K
76	M	27 y	MP	Sputum	+	NP	+	-	IVd	IV	PN	PN	ND	IVd	A4
77	M	49 y	ICU	BC	NP	NP	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
78	M	52 y	HCM	CVP tip	+	NP	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
79	M	10m	GP	Tissue	NP	+	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
80	F	81 y	ST	Urine	+	+	+	-	II	II	II	II	PN	II	A5
81	F	81 y	OP	Pus swab	+	+	+	-	II	SCC*mercury* (II+SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A
82	F	81 y	OP	Pus swab	+	NP	+	-	II	SCC*mercury* (II+SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A
83	M	48 y	Urology	Pus swab	+	NP	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A5
84	M	39 y	OP	Tissue	+	+	+	-	II+IVc	II	II	II	PN	II	A1
85	F	14 y	Oncology	Pus swab			+	-	IVd	IV	PN	PN	ND	IVd	G

BC -Blood culture; CVP tip -Central venous pressure tip; CS -Cardiothoracic surgery; D -Days; F -Female; GP -General paediatric; HCM -High care medical; ICU -Intensive care unit; Luki -Endotracheal aspirate; M -Male; M -Months; MP -Medical pulmonology; ND -Not detected; NP -Not provided; NT -Not typeable; OP -Orthopaediatric; PN -Primer not included; ST -Surgery and trauma; Y-Years

Table 3.14: Overall results of the six M-PCR assays and PFGE used for the characterisation of the 194 MRSA isolates obtained from the Steve Biko Academic Hospital (Cont)

MRSA isolates	Gender	Age	Clinical wards	Specimen Type	E-test	Vitek 2 System	First M-PCR (McClure *et al*., 2006)		SCC*mec* M-PCR typing assays						PFGE group
							16S rRNA & *mec*A	PVL	Type I to V & subtype IVa to IVd (Zhang *et al*., 2005)	Type I to V (Boye *et al*., 2007)	Type I to III (Oliveira and De Lencastre, 2002)	Type II & III (Zhang *et al*., 2012)	Type VIII (McClure *et al*., 2010)	Overall results of the five M-PCR SCC*mec* typing assays	
86	M	67 y	NP	CVP tip	+	+	+	-	II	SCC*mercury* (II+SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A1
87	F	35 d	Paediatric ICU	BC	+	+	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
88	M	49 y	ICU	BC	+	+	+	-	II	II	II	II	PN	II	A
89	F	26 d	Neonatal ICU	BC	+	+	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
90	M	31 y	NP	Pus swab	+	+	+	-	IVd	IV	PN	PN	ND	IVd	A6
91	F	22 d	Neonatal ICU	BC	+	+	+	-	II	SCC*mercury* (II+ SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A
92	M	7 d	Paediatric ICU	BC	+	+	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
93	F	37 y	Neurology	BC	+	+	+	-	IVd	IV	PN	PN	ND	IVd	A5
94	M	47 y	OP	Pus swab	+	NP	+	-	II	SCC*mercury* (II+ SCC*mercury*)	SCC*mercury*	II	PN	II+ SCC*mercury*	A
95	F	62 y	ST	BC	+	+	+	-	IVd	IV	PN	PN	ND	IVd	A1
96	M	29 y	Surgery burns	BC	+	NP	+	-	SCC*mercury*	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
97	F	39 y	OP	Tissue	NP	+	+	-	SCC*mercury*	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
98	M	40 y	CS	Luki	NP	NP	+	-	IVd	IV	PN	PN	ND	IVd	A4
99	M	8 y	PA	Pus swab	+	+	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
100	M	52 y	Nephrology	BC	+	NP	+	-	IVd	IV	PN	PN	ND	IVd	A6
101	M	28 y	HCM	Pus swab	+	NP	+	-	IVd	IV	PN	PN	ND	IVd	B
102	F	39 y	OP	Pus swab		+	+	-	SCC*mercury*	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
103	F	6 d	Neonatal ICU	BC	+	+	+	-	II	SCC*mercury* (II+ SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A
104	F	61 y	OP	BC	+	NP	+	-	IVd	IV	PN	PN	ND	IVd	B
105	F	5 m	Neonatal ICU	BC	+	NP	+	-	IVd	IV	PN	PN	ND	IVd	A6

BC -Blood culture; CS -Cardiothoracic surgery; CVP tip -Central venous pressure tip; D -Days; F -Female; HCM -High care medical; ICU -Intensive care unit; Luki -Endotracheal aspirate; M -Months; ND -Not detected; NP -Not provided; NT -Not typeable; OP -Orthopaediatric; PA -Pulmonary adult; PN -Primer not included; ST -Surgery and trauma; Y -Years

Table 3.14: Overall results of the six M-PCR assays and PFGE used for the characterisation of the 194 MRSA isolates obtained from the Steve Biko Academic Hospital (Cont)

MRSA isolates	Gender	Age	Clinical wards	Specimen Type	E-test	Vitek 2 System	First M-PCR (McClure *et al.*, 2006)		SCC*mec* M-PCR typing assays						PFGE group
							16S rRNA & *mec*A	**PVL**	**Type I to V & subtype IVa to IVd** (Zhang *et al.*, 2005)	**Type I to V** (Boye *et al.*, 2007)	**Type I to III** (Oliveira and De Lencastre, 2002)	**Type II & III** (Zhang *et al.*, 2012)	**Type VIII** (McClure *et al.*, 2010)	**Overall results of the five M-PCR SCC*mec* typing assays**	
106	M	7 m	Paediatric ICU	BC	NP	NP	+	-	II+IVc	II	II	II	PN	II	A
107	F	42 y	ST	Luki	+	+	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A6
108	F	24 y	Neurosurgical	CVP tip	NP	NP	+	-	IVd	IV	PN	PN	ND	IVd	A6
109	F	16 y	NP	NP	NP	NP	+	-	II	SCC*mercury* (II+SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A
110	M	13 y	Neurosurgical	BC	+	NP	+	-	SCC*mercury*	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A3
111	F	1y1 m	NP	NP	NP	NP	+	-	SCC*mercury*	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
112	F	77 y	HCM	BC	+	NP	+	-	II	SCC*mercury* (II+SCC*mercury*)	SCC*mercury*	II	PN	II+ SCC*mercury*	A
113	F	54 y	NP	NP	NP	NP	+	-	SCC*mercury*	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
114	M	4 d	Paediatric ICU	BC	+	NP	+	-	SCC*mercury*	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
115	M	31 y	ARV	Pus swab	+	+	+	-	II	SCC*mercury* (II+SCC*mercury*)	SCC*mercury*	II	PN	II+ SCC*mercury*	A
116	F	24 y	GS	Pus swab	NP	+	+	-	SCC*mercury*	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
117	M	49 y	Nephrology	Pus swab	+	NP	+	-	IVd	IV	PN	PN	ND	IVd	A
118	M	51 y	Main casualty	Pus swab	+	NP	+	-	SCC*mercury*	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
119	M	52 y	Surgery	BC	+	NP	+	-	II	SCC*mercury* (II+SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A
120	M	78 y	CS	Luki	+	NP	+	-	SCC*mercury*	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
121	F	71 y	IM	BC	+	NP	+	-	SCC*mercury*	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
122	M	23 d	Paediatric ICU	BC	+	NP	+	-	II	SCC*mercury* (II+SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A4
123	F	4 y	Neonatal ICU	Briviac tip	+	+	+	-	IVd	IV	PN	PN	ND	IVd	A3
124	F	4y3 m	PP	Sputum	+	NP	+	-	IVd	IV	PN	PN	ND	IVd	A5

BC	-Blood culture	GS	-General surgery	IM	-Internal medicine	ND	-Not detected	PP	-Paediatric pulmonology
CVP tip	-Central venous pressure tip	F	-Female	Luki	-Endotracheal aspirate	NP	-Not provided	ST	-Surgery and trauma
CS	-Cardiothoracic surgery	HCM	-High care medical	M	-Months	NT	-Not typeable	Y	-Years
D	-Days	ICU	-Intensive care unit	M	-Male	PN	-Primer not included	ARV	-Antiretroviral

Table 3.14: Overall results of the six M-PCR assays and PFGE used for the characterisation of the 194 MRSA isolates obtained from the Steve Biko Academic Hospital (Cont)

MRSA isolates	Gender	Age	Clinical wards	Specimen Type	E-test	Vitek 2 System	First M-PCR (McClure *et al.*, 2006)		SCC*mec* M-PCR typing assays						PFGE group
							16S rRNA & *mec*A	**PVL**	**Type I to V & subtype IVa to IVd** (Zhang *et al.*, 2005)	**Type I to V** (Boye *et al.*, 2007)	**Type I to III** (Oliveira and De Lencastre, 2002)	**Type II & III** (Zhang *et al.*, 2012)	**Type VIII** (McClure *et al.*, 2010)	**Overall results of the five M-PCR SCC*mec* typing assays**	
125	M	17 d	Paediatric ICU	BC	+	NP	+	-	NT	I	I	PN	PN	I	NT
126	M	6 m	Paediatric ICU	BC	+	+	+	-	II	SCC*mercury* (II+SCC*mercury*)	SCC*mercury*	II	PN	II+ SCC*mercury*	A6
127	F	28 d	Neonatal ICU	Pus swab	+	NP	+	-	SCC*mercury*	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A6
128	F	65 y	OPD	Pus swab	+	NP	+	-	II	SCC*mercury* (II+SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A
129	M	35 y	HCM	BC	+	NP	+	-	SCC*mercury*	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
130	M	49 y	ST	Luki	NP	NP	+	-	II	SCC*mercury* (II+SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A4
131	F	42 y	ST	NP	NP	NP	+	-	II	SCC*mercury* (II+SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A
132	M	60 y	IM	BC	+	NP	+	-	IVd	IV	PN	PN	ND	IVd	H
133	M	27 y	HCM	BC	+	NP	+	-	IVa	IV	PN	PN	ND	Iva	A
134	F	78 y	UG	Pus swab	+	NP	+	-	IVd	IV	PN	PN	ND	IVd	B2
135	M	28 y	HCM	Pus swab	+	NP	+	-	II	SCC*mercury* (II+SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A
136	M	31 y	Neurosurgical	CVP tip	+	+	+	-	SCC*mercury*	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A1
137	M	70 y	IM	CVP tip	+	NP	+	-	IVd	IV	PN	PN	ND	IVd	B1
138	M	35 y	Main casualty	Luki		+	+	-	IVd	IV	PN	PN	ND	IVd	I
139	F	52 d	PS	CVP tip	+	+	+	-	IVd	IV	PN	PN	ND	IVd	I
140	F	40 d	Neonatal ICU	Briviac tip	+	+	+	-	II	SCC*mercury* (II+SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A
141	F	46 y	ST	BC	NP	+	+	-	II	II	II	II	PN	II	J

ARV	-Antiretroviral	F	-Female	Luki	-Endotracheal aspitate	NP	-Not provided	PS	-Paediatric surgery
BC	-Blood culture	HCM	-High care medical	M	-Male	NT	-Not typeable	ST	-Surgery and trauma
CVP tip	-Central venous pressure tip	IM	-Internal medicine	M	-Months	OPD	-Outpatient department	UG	-Urology and gynaecology
D	-Days	ICU	-Intensive care unit	ND	-Not detected	PN	-Primer not included	Y	-Years

Table 3.14: Overall results of the six M-PCR assays and PFGE used for the characterisation of the 194 MRSA isolates obtained from the Steve Biko Academic Hospital (Cont)

MRSA isolates	Gender	Age	Clinical wards	Specimen Type	E-test	Vitek 2 System	First M-PCR (McClure *et al.*, 2006)		SCC*mec* M-PCR typing assays						PFGE group
							16S rRNA & *mec*A	**PVL**	**Type I to V & subtype IVa to IVd** (Zhang *et al.*, 2005)	**Type I to V** (Boye *et al.*, 2007)	**Type I to III** (Oliveira and De Lencastre, 2002)	**Type II & III** (Zhang *et al.*, 2012)	**Type VIII** (McClure *et al.*, 2010)	**Overall results of the five M-PCR SCC*mec* typing assays**	
142	M	27 y	Surgery male	Catheter tip	+	+	+	-	IVd	IV	PN	PN	ND	IVd	A3
143	M	45 y	ST	Pus swab	+	+	+	-	IVa	IV	PN	PN	ND	Iva	B
144	M	40 d	Neonatal ICU	BC	+	NP	+	-	II	SCC*mercury* (II+SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A
145	F	28 y	Gynaecology	Pus swab	+	+	+	-	II	SCC*mercury* (II+SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A
146	M	38 y	OP	Pus swab	+	+	+	-	IVd	IV	PN	PN	ND	IVd	A3
147	M	2y2 m	ARV	Pus swab	+	+	+	-	SCC*mercury*	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
148	F	9 d	Paediatric	BC	+	+	+	-	II	SCC*mercury* (II+SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A
149	M	39 y	OP	Tissue	NP	NP	+	-	II	SCC*mercury* (II+SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A
150	F	64 y	IM	Sputum	+	+	+	-	II	SCC*mercury* (II+SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A
151	F	60 y	HCM	Pus swab	+	+	+	-	II	SCC*mercury* (II+SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A
152	M	2y2 m	ARV	Pus swab	+	+	+	-	II	SCC*mercury* (II+SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A
153	M	52 y	ST	CVP tip	+	+	+	-	II	SCC*mercury* (II+SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A
154	F	26 d	Neonatal ICU	BC	+	+	+	-	II	SCC*mercury* (II+SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A
155	M	7 d	Paediatric ICU	BC	+	+	+	-	II	SCC*mercury* (II+SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A

ARV -Antiretroviral; BC -Blood culture; CVP tip -Central venous pressure tip; D -Days; F -Female; HCM -High care medical; ICU -Intensive care unit; IM -Internal medicine; M -Male; M -Months; ND -Not detected; NT -Not typeable; OP -Orthopaedic; PN -Primer not included; ST -Surgery and trauma; Y -Years

Table 3.14: Overall results of the six M-PCR assays and PFGE used for the characterisation of the 194 MRSA isolates obtained from the Steve Biko Academic Hospital (Cont)

MRSA isolates	Gender	Age	Clinical wards	Specimen Type	E-test	Vitek 2 System	First M-PCR (McClure *et al.*, 2006)		SCC*mec* M-PCR typing assays						PFGE group
							16S rRNA & *mec*A	PVL	Type I to V & subtype IVa to IVd (Zhang *et al.*, 2005)	Type I to V (Boye *et al.*, 2007)	Type I to III (Oliveira and De Lencastre, 2002)	Type II & III (Zhang *et al.*, 2012)	Type VIII (McClure *et al.*, 2010)	Overall results of the five M-PCR SCC*mec* typing assays	
156	M	49 y	ST	Luki		+	+	-	II	SCC*mercury* (II+SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A
157	F	46 y	ST	Urine		+	+	-	II	SCC*mercury* (II+SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A
158	M	57 y	HCM	CVP tip	+	+	+	-	II	SCC*mercury* (II+SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A
159	M	60 y	MP	Luki	+		+	-	II	SCC*mercury* (II+SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A
160	F	24 y	Main casualty	Tissue	+	+	+	-	II	SCC*mercury* (II+SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A
161	M	60 y	MP	Luki		+	+	-	NT	I	I	PN	PN	I	A3
162	F	75 y	Maxifacial	BC		+	+	-	II	SCC*mercury* (II+SCC*mercury*)	SCC*mercury*	II	PN	II+ SCC*mercury*	A
163	M	38 y	OP	Tissue	+	+	+	-	IVd	IV	PN	PN	ND	IVd	A
164	M	1y2 m	PS	CSF	+	NP	+	-	IVd	IV	PN	PN	ND	IVd	A3
165	F	64 y	MP	Sputum	+	NP	+	-	IVb	IV	PN	PN	ND	IVb	I
166	M	8 y	PU	Pus swab	+	NP	+	-	II	II	II	II	PN	II	J
167	F	58 y	Neurosurgical	Pus swab	+	+	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
168	F	42 y	Surgery	Pus swab	NP	NP	+	-	II	SCC*mercury* (II+SCC*mercury*)	SCC*mercury*	II	PN	II+ SCC*mercury*	A
169	M	32 y	HCM	BC	+	NP	+	-	SCC*mercury*	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
170	M	16 y	OP	Pus swab	+	NP	+	-	II+IVc	II	II	II	PN	II	NT
171	M	16 y	OP	Pus swab	+	NP	+	-	II	II	II	II	PN	II	I
172	M	19 d	PS	CVP tip	+	NP	+	-	II	SCC*mercury* (II+SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A6

BC	-Blood culture	F	-Female	M	-Months	NP	-Not provided	PU	-Paediatric urology
CSF	-Central nevous system	HCM	-High care medical	MP	-Medical pulmonology	OP	-Orthopaediatric	ST	-Surgery and trauma
CVP tip	-Central venous pressure tip	Luki	-Endotracheal aspirate	ND	-Not detected	PN	-Primer not included	Y	-Years
D	-Days	M	-Male	NT	-Not typeable	PS	-Paediatric surgery		

Table 3.14: Overall results of the six M-PCR assays and PFGE used for the characterisation of the 194 MRSA isolates obtained from the Steve Biko Academic Hospital (Cont)

MRSA isolates	Gender	Age	Clinical wards	Specimen Type	E-test	Vitek 2 System	First M-PCR (McClure *et al.*, 2006)		SCC*mec* M-PCR typing assays						PFGE group
							16S rRNA & *mec*A	**PVL**	**Type I to V & subtype IVa to IVd** (Zhang *et al.*, 2005)	**Type I to V** (Boye *et al.*, 2007)	**Type I to III** (Oliveira and De Lencastre, 2002)	**Type II & III** (Zhang *et al.*, 2012)	**Type VIII** (McClure *et al.*, 2010)	**Overall results of the five M-PCR SCC*mec* typing assays**	
173	F	1 y	Paediatric surgery	BC	+	NP	+	-	IVd	IV	PN	PN	ND	IVd	A
174	F	20 d	Neonatal ICU	BC	+	NP	+	-	II	SCC*mercury* (II+SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A4
175	M	16 y	OP	Tissue	+	NP	+	-	IVd	IV	PN	PN	ND	IVd	B1
176	F	72 y	HCM	Sputum	+	NP	+	-	IVd	IV	PN	PN	ND	IVd	A5
177	F	72 y	OP	Tissue	NP	NP	+	-	II	SCC*mercury* (II+SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A
178	F	33 y	MP	CVP tip	+	NP	+	-	II	SCC*mercury* (II+SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A
179	M	16 y	OP	Pus swab	NP	NP	+	-	IVd	IV	PN	PN	ND	IVd	A
180	F	69 y	OP	Tissue	+	+	+	-	II	SCC*mercury* (II+SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A5
181	F	30 y	HCM	BC	+	+	+	-	II	SCC*mercury* (II+ SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	J
182	F	25 y	Neonatal ICU	BC	+	NP	+	-	II	SCC*mercury* (II+ SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A
183	M	16 y	OP	Pus swab	+	NP	+	-	II+IVc	II	II	II	PN	II	J
184	F	47 y	ST	Pus swab	NP	NP	+	-	II	II	II	II	PN	II	J
185	F	29 y	Main casualty	Tissue	+	NP	+	-	II	II	II	II	PN	II	A
186	F	29 y	Main casualty	Tissue	+	+	+	-	II	SCC*mercury* (II+SCC*mercury*)	II+ SCC*mercury*	II	PN	II+ SCC*mercury*	A
187	F	35 y	Main casualty	Tissue	NP	NP	+	-	SCC*mercury*	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A
188	F	69 y	OP	Tissue	+	+	+	-	II	SCC*mercury* (II+SCC*mercury*)	SCC*mercury*	II	PN	II+ SCC*mercury*	A

BC -Blood culture; F -Female; M -Male; NT -Not typeable; PN -Primer not included
CVP tip -Central venous pressure tip; HCM -High care medical; M -Medical pulmonology; NP -Not provided; ST -Surgery and trauma
D -Days; ICU -Intensive care unit; ND -Not detected; OP -Orthopaediatric; Y -Years

Table 3.14: Overall results of the six M-PCR assays and PFGE used for the characterisation of the 194 MRSA isolates obtained from the Steve Biko Academic Hospital (Cont)

MRSA isolates	Gender	Age	Clinical wards	Specimen Type	E-test	Vitek 2 System	First M-PCR (McClure *et al.*, 2006)		SCC*mec* M-PCR typing assays						PFGE group
							16S rRNA & *mec*A	**PVL**	**Type I to V & subtype IVa to IVd** (Zhang *et al.*, 2005)	**Type I to V** (Boye *et al.*, 2007)	**Type I to III** (Oliveira and De Lencastre, 2002)	**Type II & III** (Zhang *et al.*, 2012)	**Type VIII** (McClure *et al.*, 2010)	**Overall results of the five M-PCR SCC*mec* typing assays**	
189	F	38 y	Neurology	BC	+	+	+	-	IVd	IV	PN	PN	ND	IVd	A
190	F	47 y	ST	BC	+	+	+	-	II+IVc	II	II	II	PN	II	J
191	F	49 y	HCM	BC	+	+	+	-	II+IVc	II	II	II	PN	II	J
192	F	47 y	ST	BC	+	+	+	-	II+IVc	II	II	II	PN	II	J
193	F	47 y	HCM	BC	+	+	+	-	II+IVc	II	II	II	PN	II	J
194	M	31 y	Main casualty	Sputum	NP	NP	+	-	NT	SCC*mercury*	SCC*mercury*	NT	PN	SCC*mercury*	A

BC -Blood culture HCM -High care medical ND -Not detected NP -Not provided ST -Surgery and trauma
F -Female M -Male NT -Not typeable PN -Primer not included Y -Years

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