The Molecular Characterisation of Methicillin-Resistant *Staphylococcus aureus* from Hospitals in Cape Town

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DECLARATION

I, Melissa Jane Jansen van Rensburg, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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Opinions expressed, and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF

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ABBREVIATIONS AND ACRONYMS

°C	Degrees Celsius
µg	Microgram
µl	Microlitre
µM	Micromolar
ACME	Arginine catabolic mobile element
AIP	Autoinducing peptide
BBA	Boiled blood agar
bp	Base pair(s)
BSA	Bovine serum albumin
BURP	Based upon repeat patterns
BURST	Based Upon Related Sequence Types
CA-MRSA	Community-associated methicillin-resistant *Staphylococcus aureus*
ca-MRSA	Community-acquired methicillin-resistant *Staphylococcus aureus*
CC	Clonal complex
CDC	Centres for Disease Control and Prevention
CIP	Ciprofloxacin
CLI	Clindamycin
CLSI	Clinical Laboratory Standards Institute
CNS	Coagulase-negative staphylococci
ddNTP	Dideoxynucleotide triphosphate
DHFR	Dihydrofolate reductase
DLV	Double locus variant
DNA	Deoxyribonucleic acid
Dnase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EF-G	Elongation factor G
EMERG	Emergency services
EMRSA	Epidemic methicillin-resistant *Staphylococcus aureus*
ERY	Erythromycin
FUS	Fusdic acid
g	gram(s)

GEN	Gentamicin
GSH	Groote Schuur Hospital
GYN	Gynaecology and obstetrics
h	Hour(s)
ha-MRSA	Hospital-acquired methicillin-resistant *Staphylococcus aureus*
HA-MRSA	Hospital-associated methicillin-resistant *Staphylococcus aureus*
hVISA	Heterogeneous vancomycin intermediate *Staphylococcus aureus*
ICU	Intensive care unit
IS	Insertion sequence
ISS	Insertion site sequence
IWG-SCC	International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements
J region	Joining region
kb	kilobase(s)
l	Litre(s)
LA-MRSA	Livestock-associated methicillin-resistant *Staphylococcus aureus*
M	Molar
MED	Medical ward
mg	Milligram(s)
mg	Milligram(s)
$MgCl_2$	Magnesium chloride
MIC	Minimum inhibitory concentration
min	Minute(s)
ml	millilitre(s)
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence typing
MLVA	Multilocus variable number tandem repeat analysis
mM	Millimolar
mm	Millimetre(s)
MMH	Mowbray Maternity Hospital

MRSA	Methicillin-resistant *Staphylococcus aureus*
MSCRAMMs	Microbial-surface components recognising adhesive matrix molecules
MSSA	Methicillin-susceptible *Staphylococcus aureus*
NaCl	Sodium chloride
NaOH	Sodium hydroxide
ND	Not determined
NHLS	National Health Laboratory Service
NP	Not provided
OD	Optical density
OPC	Outpatient clinic
ORF	Open reading frame
OXA	Oxacillin
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PEN	Penicillin
PFGE	Pulsed-field gel electrophoresis
PMN	Polymorphonuclear leukocytes
PVL	Panton-Valentine leukocidin
RCCH	Red Cross War Memorial Children's Hospital
RIF	Rifampicin
rpm	Revolutions per minute
RRDR	Rifampicin resistance-determining region
RSA	Republic of South Africa
s	Second(s)
SCC	Staphylococcal cassette chromosome
SCC*Hg*	Staphylococcal cassette chromosome mercury
SCC*mec*	Staphylococcal cassette chromosome mec
SCV	Small colony variant
SDS	Sodium dodecyl sulphate
SLV	Single locus variant
SNP	Single nucleotide polymorphism
spa-CC	*spa* clonal complex
ST	Sequence type
SUR	Surgical ward

SXT	Co-trimoxazole
TAE	Tris-acetate EDTA
TBE	Tris-borate EDTA
TE	Tris-EDTA
Tn	Transposon
U	Enzyme unit
UCT	University of Cape Town
UCTPH	University of Cape Town Private Academic Hospital
UK	United Kingdom
UPGMA	Unweighted pair-group method using arithmetic averages
USA	United States of America
UV	Ultraviolet
V	Volts
VAN	Vancomycin
VH	Victoria Hospital
VISA	Vancomycin intermediate *Staphylococcus aureus*
VRSA	Vancomycin resistant *Staphylococcus aureus*

PREFACE

Comprehensive molecular epidemiological data are prerequisite for establishing control over methicillin-resistant *S. aureus* (MRSA) in the hospital setting; however, there is currently a paucity of molecular epidemiological data available on MRSA from South Africa. A molecular characterisation of one hundred MRSA isolates collected between January 2007 and December 2008 from patients in five hospitals in Cape Town was carried out in this study.

PCR assays did not detect the Panton-Valentine leukocidin toxin in any of the isolates. The majority (92.00 %) of the MRSA segregated into six pulsed-field gel electrophoresis clusters, each of which contained isolates from at least 2 hospitals, suggesting the transmission of MRSA within and between hospitals in Cape Town. A combination of SCC*mec* typing, multilocus sequence typing, *spa* typing and *dru* typing was used to further characterise the MRSA. The six PFGE clusters and five of the sporadic isolates correspond to four clones, while the remaining three MRSA were consistent with sporadic clones. Three of the predominant clones corresponded to frequently described pandemic clones: ST239-MRSA-III, ST36-MRSA-II and ST5-MRSA-I. ST239-MRSA-III and ST36-MRSA-II were minor clones, accounting for 4.00 % and 12.00 % of the isolates, respectively. ST5-MRSA-I was the second-most prevalent clone identified in this study and accounted for 37.00 % of the isolates. The sporadic clones included ST22-MRSA-IVh, ST72-MRSA-V and ST650-MRSA-IVb. While ST22-MRSA-IVh also corresponded to a pandemic clone, ST72-MRSA-V and ST650-MRSA-IVb both represent unusual MRSA genotypes.

The dominant clone identified in this study was the infrequently described multidrug-resistant ST612-MRSA-IVd, which was detected in all five hospitals. This clone appears to be endemic to South Africa, but has otherwise only been reported infrequently in Australia and the United Kingdom. The identification of the same uncommon rifampicin resistance genotype in ST612-MRSA-IV from Cape Town and isolates previously described in South Africa and Australia, combined with the *spa* typing and *dru* typing data, suggest that ST612-MRSA-IV has undergone clonal expansion in local hospitals.

CHAPTER 1

Literature Review

1.1 The staphylococci and their clinical relevance

The bacterial genus *Staphylococcus* includes more than 40 species of non-spore-forming, non-motile, Gram-positive cocci (Winn *et al.*, 2006; Rogers *et al.*, 2009). The genus name is derived from the Greek *staphyle* meaning "grapes", and *kokkos* meaning "berries", because, while staphylococci occur singly, in pairs, tetrads and chains, they are most commonly observed in grape-like clusters (Winn *et al.*, 2006; Brooks and Carroll, 2007b; Mathema *et al.*, 2009). Most staphylococci are facultative anaerobes and are also typically catalase-positive, which distinguishes members of the genus from other Gram-positive cocci, such as the streptococci and enterococci (Winn *et al.*, 2006; Brooks and Carroll, 2007b).

The staphylococci are frequent colonisers of the skin and mucous membranes of humans and other animals, and have the potential to cause disease when the host's immune system is compromised (Oliveira *et al.*, 2002; Aires-de-Sousa and de Lencastre, 2004; Winn *et al.*, 2006; Gordon and Lowy, 2008). *Staphylococcus aureus* is recognised as the most important human pathogen within the *Staphylococcus* genus (Winn *et al.*, 2006). When grown on rich media, *S. aureus* tends to form large colonies that are white-grey to golden-yellow in colour, hence the species name *aureus*, meaning "golden" (Brooks and Carroll, 2007b; Mathema *et al.*, 2009; van Belkum *et al.*, 2009). *S. aureus* is distinguished from other staphylococci by the production of coagulase, a virulence factor that clots blood plasma and aids in immune evasion (Winn *et al.*, 2006; Brooks and Carroll, 2007b).

In contrast to *S. aureus*, the coagulase-negative staphylococci (CNS) typically form relatively small grey-white colonies when grown on rich media (Brooks and Carroll, 2007b). In the past, these less virulent species were not deemed important human pathogens, but that perception has gradually shifted with the CNS emerging as an important cause of nosocomial infections (Huebner and Goldmann, 1999; Livermore, 2000; Garza-González *et al.*, 2010; Rogers *et al.*, 2009). The CNS commonly cause

infections in immunocompromised individuals, and are also frequently implicated in infections associated with indwelling medical devices (Huebner and Goldmann, 1999; Livermore, 2000; Rogers *et al.*, 2009; Garza-González *et al.*, 2010). Seven CNS species are known human pathogens, the most important of which are *Staphylococcus epidermidis*, *Staphylococcus haemolyticus, Staphylococcus lugdunensis* and *Staphylococcus saprophyticus* (Rogers *et al.*, 2009). Additionally, certain CNS species are known veterinary pathogens that, although not frequently isolated from humans, have zoonotic potential as they have been found to infect those working in close contact with animals (Winn *et al.*, 2006; Garza-González *et al.*, 2010).

1.2 *S. aureus*: pathogen extraordinaire

Although certain CNS species are now recognised as common human pathogens, *S. aureus* remains the most important member of the staphylococci (Winn *et al.*, 2006). *S. aureus* can be found on inanimate objects in the environment, and as a component of the normal flora of humans and other animals (Lowy, 1998; Livermore, 2000; Winn *et al.*, 2006; Gordon and Lowy, 2008). In humans, *S. aureus* commonly colonises the anterior nares, axillae, perineum, vagina and gastrointestinal tract (Lowy, 1998; Winn *et al.*, 2006; Gordon and Lowy, 2008). Studies of *S. aureus* nasal colonisation rates have estimated that approximately 20 % of individuals are persistently colonised, and a further 30 – 60 % intermittently colonised, with these individuals serving as vehicles of transmission in both the hospital and community settings (Livermore, 2000; Aires-de-Sousa and de Lencastre, 2004; Albrich and Harbarth, 2008; Gordon and Lowy, 2008; Struelens *et al.*, 2009).

S. aureus is an opportunistic pathogen that can be carried asymptomatically for extended periods of time on mucous membranes, or briefly on the skin (Archer, 1998). Once the barriers of the host's immune system are breached and *S. aureus* is provided access to adjacent tissues or to the bloodstream, this organism is capable of causing a wide variety of infections (Archer, 1998; Lowy, 1998; Harris and Richards, 2006; Gordon and Lowy, 2008). These infections range from relatively benign skin and soft tissue infections to more severe, life-threatening infections such as bacteraemia, pneumonia, endocarditis and osteomyelitis and, like the CNS, *S. aureus* is a common cause of chronic indwelling device-related infections (Lowy, 1998; Livermore, 2000; Aires-de-Sousa and de Lencastre, 2004; Harris and Richards, 2006; Gordon and Lowy, 2008). Due to the production of toxins, certain *S. aureus* strains also cause a variety of toxinoses (Lowy, 1998; Gordon and Lowy, 2008), as will be described later in this chapter [1.3].

In addition to causing disease in humans, *S. aureus* is also a significant veterinary pathogen (Winn *et al.*, 2006). Particular *S. aureus* lineages are known to cause disease in companion animals, and also in economically important livestock, such as cows, chicken, goats, pigs, rabbits and sheep (Winn *et al.*, 2006; Pantosti and Venditti, 2009; Rogers *et al.*, 2009; McCarthy and Lindsay, 2010). Given their zoonotic potential, several of these lineages are thought to represent potential public

health concerns (Ben Zakour *et al.*, 2008). A selection of these lineages will be discussed further in the context of *S. aureus* evolution later in this chapter [1.7.3].

1.3 The contribution of virulence factors to *S. aureus* pathogenesis

S. aureus has the ability to cause a broad spectrum of infections involving any organ in its human host (Archer, 1998). The extensive array of virulence factors available to *S. aureus* is recognised as a pivotal factor in its pathogenesis (Archer, 1998; Lowy, 1998; Foster, 2005; Gordon and Lowy, 2008; Skrupky *et al.*, 2009; Mathema *et al.*, 2009). While the precise combination of virulence factors varies from strain to strain, the majority of virulence determinants can be assigned to one of five groups based on their roles in *S. aureus* pathogenesis, as defined by Gordon and Lowy (2008) (Table 1.1). These five categories group virulence factors that are involved in attachment, persistence, immune evasion, tissue invasion and toxin-mediated disease. In essence, virulence factors enable *S. aureus* to colonise a host, establish a local infection, disseminate and cause disease (Archer, 1998).

Table 1.1 Five major categories of *S. aureus* virulence factors defined according to roles in pathogenesis[a]

Role in pathogenesis	Associated clinical syndromes	Selected examples of virulence factors (gene)[b]
Attachment (during colonisation and early stages of infection)	Device-related infections; osteomyelitis; septic arthritis; endocarditis	MSCRAMMs including clumping factors (*clfA*; *clfB*), fibronectin-binding proteins (*fnbA*; *fnbB*), collagen-binding protein (*cna*), and bone sialprotein-binding protein (*bbp*)
Persistence	Device-related infections; osteomyelitis; septic arthritis; endocarditis; recurrent infections	Aminolevulinic acid dehydratase (*hemB*) mutants (SCV phenotype); polysaccharide intercellular adhesion (*ica*) and accessory gene regulator (*agr*) (biofilm production); intracellular persistence
Destruction and/or evasion of components of host's immune system	Necrotising pneumonia; invasive skin and soft tissue infections; recurrent infections	Panton-Valentine leukocidin (*lukS-PV*; *lukF-PV*); γ-toxin (*hlg*); leukotoxin D, E (*lukD*; *lukE*); staphylokinase (*sak*); capsular polysaccharides, particularly 5 and 8 (*cap5* and *cap8* gene clusters); protein A (*spa*); phenol soluble modulins (*psm*-α gene cluster); coagulase (*coa*); CHIPS (*chp*); extracellular adherence protein (*eap*); MHC class II analogous protein (*map*); staphylococcal inihibitor of complement (*scn*); aureolysin (*aur*)
Tissue invasion (during later stages of infection)	Destruction of host tissue; metastasis	Proteases (*V8*); nucleases; lipases; elastases/metalloproteases (*sepA*); phospholipase C (*plc*); hyaluronate lyase (*hysA*)
Toxinoses and/or sepsis	Toxic shock syndrome; bullous impetigo; staphylococcal scalded skin syndrome; food poisoning; sepsis	Toxic shock syndrome toxin-1 (*tstH*); exfoliative toxins A, B and D (*eta*; *etb; etd*); entertoxins A – Q, excluding F (*sea* – *seq*, but not *sef*); α-toxin (*hla*); peptidoglycan; lipoteichoic acid

[a] Adapted from Foster (2005), Winn *et al.* (2006), Gordon and Lowy (2008), and Malachowa and DeLeo (2010).

[b] MSCRAMMs, microbial-surface components recognising adhesive matrix molecules; SCV, small colony variant; CHIPS, chemotaxis inhibitory protein.

A number of virulence factors play an important role during colonisation and the early stages of infection as they promote bacterial adhesion. These cell-surface proteins are known as "microbial-surface components recognising adhesive matrix molecules" or MSCRAMMs (Table 1.1) (Archer, Foster and Höök, 1998; Lowy, 1998; Gordon and Lowy, 2008). The MSCRAMMs bind extracellular matrix components including fibronectin, fibrinogen and collagen (Foster and Höök, 1998; Gordon and Lowy, 2008). These plasma components are known to coat the surfaces of indwelling medical devices, thereby making it possible for *S. aureus* to adhere to both host and indwelling-device surfaces (Dancer, 2008). Due to their adhesive properties, the MSCRAMMs appear to be particularly important in establishing device-related infections, bone and joint infections and endovascular infections (Foster and Höök, 1998; Gordon and Lowy, 2008).

After establishing an infection, *S. aureus* exploits several virulence strategies to persist within its host. An important requirement for persistence is immune evasion and, during the earlier stages of infection, *S. aureus* produces a number of virulence factors that help thwart the host's immune system (Table 1.1) (Foster, 2005). *S. aureus* employs several other strategies to persist within its host, including biofilm production and the invasion of host cells (Table 1.1). Biofilms consist of sessile populations of bacteria within an extracellular matrix, which shields the bacteria from antimicrobial agents and host defences (Yarwood *et al.*, 2004; Harris and Richards, 2006; Dancer, 2008). The ability to persist intracellularly in both phagocytic and non-phagocytic cells is thought to play an important role in chronic and recurrent infections as *S. aureus* is protected from host immune responses and certain therapeutic agents (Foster, 2005; Harris and Richards, 2006; Gordon and Lowy, 2008). The formation of small-colony variants (SCVs) is thought to play a similar role in persistent and recurrent infections (Gordon and Lowy, 2008; Sendi and Proctor, 2009). SCVs show increased uptake by host cells and survive intracellularly because they cause minimal damage to the cell. SCVs also exhibit decreased activation of host immune responses, and are refractory to the host's defences and certain antimicrobial agents (Sendi and Proctor, 2009).

Although the expression of bacterial adhesins is vital during the early stages of infection, it becomes important to alter the expression of these virulence genes during the later stages of infection (Lowy, 1998; Gordon and Lowy, 2008). In order

for *S. aureus* to disseminate to peripheral sites, it is necessary for the organism to down-regulate the expression of genes involved in adhesion and up-regulate the expression of those involved in tissue invasion and penetration (Table 1.1) (Lowy, 1998; Novick and Geisinger, 2008). The up-regulation of proteases, lipases, elastases and other cytotoxins enables *S. aureus* to penetrate host tissues, disseminate to peripheral sites and establish additional foci of infection (Foster, 2005; Gordon and Lowy, 2008; Skrupky, 2009). *S. aureus* is also able to interact with and activate the host's immune system and coagulation pathways to cause septic shock (Gordon and Lowy, 2008).

Several regulatory systems have been implicated in the temporal control of virulence factor expression in *S. aureus*, including the accessory gene regulator (*agr*), the staphylococcal accessory regulator (*sar*), the repressor of toxins or Rot (*rot*), the multiple gene regulator (*mgr*) and SaeRS (*saeRS*) (Lowy, 1998; Gordon and Lowy, 2008). Of these regulatory systems, the *agr* global regulator, an auto-inducing two-component system, is undoubtedly the best understood. As the number of bacteria present at the site of infection increases, so does the concentration of the autoinducing peptide (AIP). Once the AIP reaches a threshold concentration, the *agr* system is activated resulting in the down-regulation of adhesins accompanied by the up-regulation of secreted virulence factors that enable tissue invasion and penetration (Lowy, 1998; Yarwood *et al.*, 2004; Novick and Geisinger, 2008).

Although not present in all strains, *S. aureus* isolates may also carry combinations of additional virulence determinants that cause various toxinoses (Table 1.1) (Lowy, 1998; Foster, 2005; Gordon and Lowy, 2008; Skrupky *et al.*, 2009). These virulence factors are often located on mobile genetic elements and are disseminated by horizontal gene transfer (Fitzgerald *et al.*, 2001; Malachowa and DeLeo, 2010; Novick *et al.*, 2010). For example, the exfoliative toxins that cause staphylococcal scalded-skin syndrome or bullous impetigo have been described on plasmids, phages and genomic islands (Gordon and Lowy, 2008; Malachowa and DeLeo, 2010). Similarly, enterotoxins known to cause food poisoning, as well as the toxic shock syndrome toxin and the Panton-Valentine leukocidin (PVL) toxin, which will be described in detail in Chapter 4 [4.1], have been described on phages and pathogenicity islands in *S. aureus* (Gordon and Lowy, 2008; Malachowa and DeLeo, 2010; Novick *et al.*, 2010).

1.4 An overview of antimicrobial chemotherapy and resistance in *S. aureus*

Although empirical treatments against bacterial infections have been in use since the 1600s, antimicrobial chemotherapy only emerged as a science in the early twentieth century (Bryskier, 2005d; Brooks and Carroll, 2007a; Greenwood *et al.*, 2007). At the dawn of the antibiotic era, the medical community was optimistic that all infectious diseases would be treatable in the near future; however, this optimism was short-lived as bacterial strains resistant to the newly introduced antimicrobial agents soon emerged (Lowy, 2003; Levy and Marshall, 2004; Greenwood *et al.*, 2007). Humans and bacteria have since been engaged in an ongoing battle as bacteria have consistently acquired resistance mechanisms to clinically relevant antimicrobial agents, which has necessitated the development of novel therapeutic agents, thus perpetuating the cycle (Greenwood *et al.*, 2007).

Extensive research on chemotherapeutic agents has resulted in the discovery of numerous classes of antibiotics. The existing classes of antimicrobial agents target five major bacterial processes including cell wall, protein and nucleic acid synthesis, metabolic pathways, and cell membrane function (Levy and Marshall, 2004; Brooks and Carroll, 2007a; Greenwood *et al.*, 2007). Bacterial mechanisms of antimicrobial resistance to these antimicrobial agents vary depending on the bacterium and antibiotic in question, but can be divided into three categories: enzymatic degradation or modification of the antimicrobial agent, alteration of the drug target, and altered access to the drug target by decreased uptake, or increased efflux of the antimicrobial agent (Levy and Marshall, 2004; Smith, 2004; Greenwood *et al.*, 2007).

S. aureus has proved particularly adept at acquiring resistance mechanisms against clinically relevant antibiotics with multidrug-resistant strains posing a serious therapeutic challenge and economic burden (Oliveira *et al.*, 2002; Ito *et al.*, 2003; Lowy, 2003; Levy and Marshall, 2004; Shorr, 2007; Segreti, 2009). In *S. aureus*, as in other bacteria, resistance mechanisms arise due to chromosomal mutations or horizontal transfer of mobile genetic elements encoding resistance determinants (Chambers, 1997; Ito *et al.*, 2003; Lowy, 2003; Greenwood *et al.*, 2007; Malachowa and DeLeo, 2010). This organism's ability to consistently acquire resistance mechanisms to all major classes of antimicrobial agents has, in conjunction with its

repertoire of virulence strategies, been a major contributor to its longstanding success as a pathogen (Oliveira *et al.*, 2002). A brief overview of major classes of chemotherapeutic agents used in mono- or combination-therapies for the treatment of *S. aureus* infections in Cape Town hospitals, as well as important resistance strategies, will be provided here.

1.4.1.1 β-lactams

The β-lactam class of antimicrobial agents comprises the largest group of bacterial cell wall synthesis inhibitors, including the penams, penems, cephems and monocyclic β-lactams (Bryskier, 2005b). The most clinically relevant families of β-lactam antimicrobial agents include the penicillins (penams), carbapenems (penems) and cephalosporins (cephems) (Figure 1.1) (Levy and Marshall, 2004; Bryskier, 2005b).

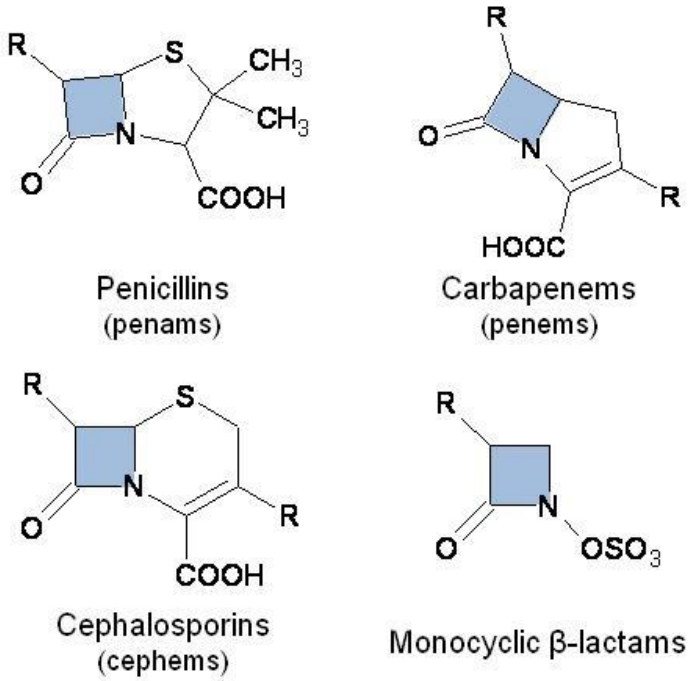


Figure 1.1 General structures of four major families of β-lactam antibiotics. The characteristic β-lactam ring essential for the antimicrobial activity of these groups is shown in blue. The families are differentiated by the structure of the ring attached to the β-lactam ring, and by the side chains attached to that ring. Adapted from Mims *et al.* (2004) and Russell *et al.* (2004).

The penams, penems, cephems and monocyclic β-lactams share a common basic structure centred around the characteristic β-lactam ring that is crucial for their antimicrobial activity (Figure 1.1) (Bryskier, 2005b; Greenwood *et al.*, 2007). The

four families are differentiated on the basis of the ring bound to the central β-lactam ring (Figure 1.1) (Bryskier, 2005b). These antimicrobial agents exert a bactericidal effect on *S. aureus* as they inhibit the penicillin-binding proteins (PBPs) responsible for transpeptidation of the peptidoglycan layer, thereby disrupting cell wall integrity (Brooks and Carroll, 2007a).

The introduction of penicillin into clinical practice in 1940 correlated with decreased morbidity and mortality in patients with *S. aureus* infections; however, resistance to penicillin emerged rapidly in this organism with the first reports published in 1942 (Oliveira *et al.*, 2002; Lowy, 2003). It has been suggested that up to 80 – 95 % of *S. aureus* strains are currently penicillin-resistant (Bryskier, 2005c; Sakoulas and Moellering, 2008; Malachowa and DeLeo, 2010). Penicillin-resistant *S. aureus* isolates produce β-lactamase, an enzyme that hydrolyses the β-lactam ring that is essential to the antimicrobial activity of the β-lactams (Lowy, 2003; Bryskier, 2005c; Greenwood *et al.*, 2007). In *S. aureus*, the *blaZ* gene that encodes β-lactamase is typically plasmid-borne, but has also been described on transposons, or within the bacterial chromosome (Lowy, 2003; Malachowa and DeLeo, 2010). A number of β-lactamase-stable β-lactam agents have been introduced into clinical practice in an attempt to combat penicillin-resistant *S. aureus* strains. Among these agents are methicillin and the the isoxazolylpenicillins, including oxacillin and flucloxacillin (Bryskier, 2005e; Greenwood *et al.*, 2007). Resistance to these antibiotics emerged due to the acquisition of an alternative PBP, as will be discussed further later in this chapter [1.5]. Most recently, it has been shown that ceftaroline and ceftobiprole, investigational fifth generation cephalosporins, appear to have potential against isoxazolylpenicillin-resistant *S. aureus* strains as they are able to inhibit the alternative PBP (Ratnaraja and Hawkey, 2008; Skrupky *et al.*, 2009).

1.4.1.2 Aminoglycosides

The aminoglycosides comprise a large family of protein synthesis inhibitors that exert a bactericidal effect mediated by binding to the 30S ribosomal subunit. Aminoglycoside binding is thought to impact on protein synthesis is several ways, one of which is by preventing the formation of the initiation complex (Lambert, 2004; Mims *et al.*, 2004). Additionally, aminoglycosides may inhibit translocation, and also tend to cause the production of non-functional proteins due to misreading of

particular mRNA codons (Lambert, 2004; Greenwood *et al.*, 2007). In *S. aureus*, aminoglycoside resistance commonly arises subsequent to the acquisition of a mobile genetic element, typically a plasmid or transposon, which encodes one or more aminoglycoside-modifying enzyme (Chambers, 1997; Malachowa and DeLeo, 2010). Aminoglycoside acetyltransferases, phosphotransferases and nucleotidyltransferases comprise the three known types of aminoglycoside-modifying enzymes, all of which have been described in *S. aureus* to date (Chambers, 1997; Lowy, 2003; Greenwood *et al.*, 2007; Malachowa and DeLeo, 2010).

1.4.1.3 Macrolides, lincosamides and streprogramins

The macrolides, lincosamides and streptogramins comprise three classes of protein synthesis inhibitors that act at a similar point in the synthetic pathway, and all bind to the 50S ribosomal subunit (Lambert, 2004; Mims *et al.*, 2004; Greenwood *et al.*, 2007). The macrolides are thought to inhibit translocation and cause the peptide chain to dissociate from the ribosome (Greenwood *et al.*, 2007), while the lincosamides and streptogramins inhibit elongation of the peptide chain (Lambert, 2004). Given that these three classes of antimicrobial agents have highly similar binding sites, it is hardly surprising that cross-resistance commonly occurs (Mims, *et al.*, 2004). In *S. aureus*, the most common mechanism of cross-resistance is methylation of the 23S subunit of the bacterial ribosome due to the activity of ribosomal methylases encoded by plasmid- or transposon-encoded *erm* genes (Lowy, 2003; Bryskier, 2005c; Malachowa and DeLeo, 2010). Resistance may also arise due to acquisition of the plasmid-borne *msrA* and *vga* genes that encode components involved in the efflux of macrolides and streptogramins, and lincosamides, respectively (Malachowa and DeLeo, 2010). Additionally, *S. aureus* is known to acquire plasmid-encoded acetyltransferases that modify the streptogramins (Lowy, 2003).

1.4.1.4 Fusidic acid

Fusidic acid is a protein synthesis inhibitor often used in combination therapies for the treatment of deep-seated *S. aureus* infections (Greenwood *et al.*, 2007). Of all the protein synthesis inhibitors, fusidic acid acts latest in the pathway (Mims *et al.*, 2004) by interacting with elongation factor G (EF-G) on the ribosome to inhibit translocation (Greenwood *et al.*, 2007; Lannergård *et al.*, 2009). Resistance to

fusidic acid emerges rapidly in *S. aureus* with two known mechanisms of resistance. Chromosomal mutations in *fusA* and *rplF*, which encode EF-G and the ribosomal protein L6, respectively, are known to decrease drug-target interactions (Greenwood *et al.*, 2007; Lannergård *et al.*, 2009). The second mechanism of resistance involves the protection of the drug target due to acquisition of the plasmid-encoded *fusB* determinant (Lannergård *et al.*, 2009; Malachowa and DeLeo, 2010).

1.4.1.5 Quinolones

The quinolones inhibit nucleic acid synthesis by targeting topoisomerase IV and DNA gyrase with the former serving as the primary target in *S. aureus* (Chambers, 1997; Greenwood *et al.*, 2007). These enzymes play an integral role during replication and transcription as they ensure that bacterial DNA is in the correct conformation and that its integrity is maintained (Lowy, 2003; Lambert, 2004; Mims *et al.*, 2004). In *S. aureus*, resistance to the quinolones usually arises due to the acquisition of chromosomal mutations in the *grlA*, *gyrA* and *gyrB* genes that result in the alteration of the drug target (Chambers, 1997; Lowy, 2003).

1.4.1.6 Rifamycins

The rifamycins constitute an important class of nucleic acid synthesis inhibitors, of which the semi-synthetic rifampicin has the greatest clinical importance (Greenwood *et al.*, 2007). The rifamycins inhibit bacterial transcription by binding to the β-subunit of DNA-dependent RNA polymerase (Brooks and Carroll, 2007b). Resistance to the rifamycins arises due to alteration of the drug target and is mediated by the acquisition of chromosomal mutations in the *rpoB* gene, which encodes the β-subunit of the bacterial RNA polymerase (Greenwood *et al.*, 2007). Resistance to rifampicin in *S. aureus* will be discussed further in Chapter 6.

1.4.1.7 Sulphonamides and diaminopyrimidines (trimethoprim)

The sulphonamides and diaminopyrimidines represent two classes of antimicrobial agents that target tetrahydrofolate synthesis, a vital bacterial metabolic pathway (Figure 1.2) (Lambert, 2004; Mims *et al.*, 2004).

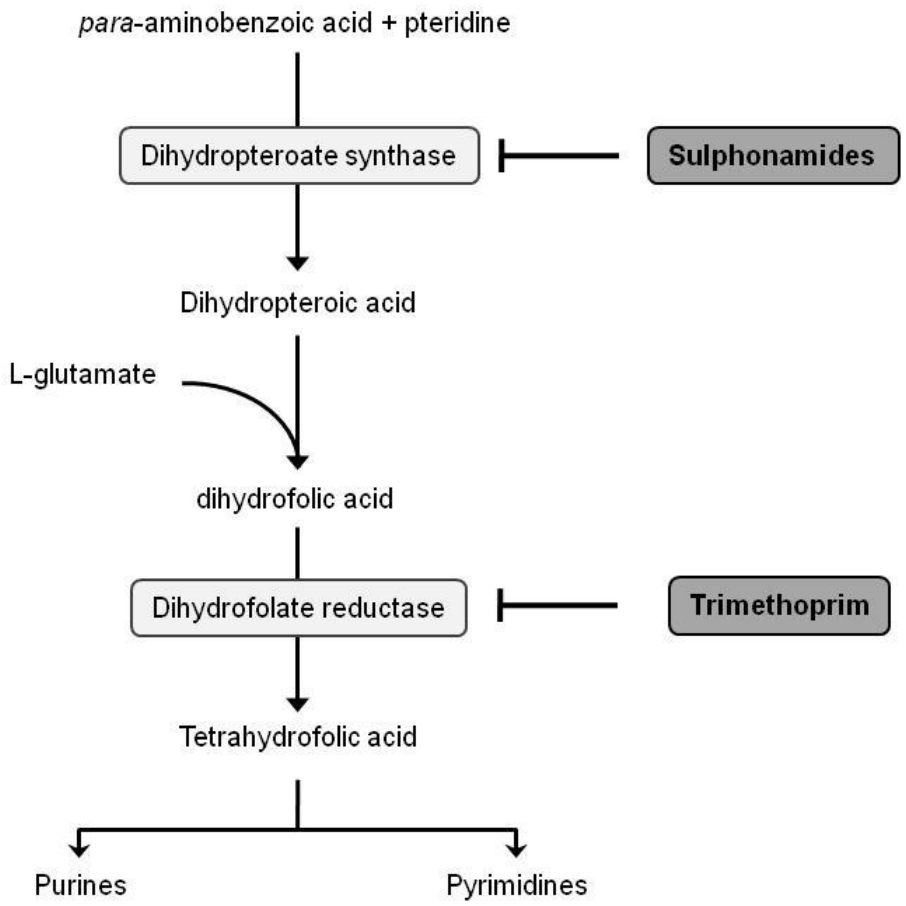


Figure 1.2 Flow-diagram illustrating the tetrahydrofolate synthesis pathway essential for the production of purines and pyrimidines in bacteria. The key enzymes dihydropteroate synthase and dihydrofolate reductase are inhibited by the sulphonamides and trimethoprim, respectively, as indicated. Adapted from Mims *et al.* (2004)

The tetrahydrofolate synthesis pathway is essential for the production of the purines and pyrimidines required for nucleic acid synthesis; therefore disruption of the pathway indirectly inhibits nucleic acid synthesis (Mims *et al.*, 2004). The sulphonamides inhibit dihydropteroate synthetase, preventing the production of dihydropteroic acid, an essential intermediate in the pathway (Figure 1.2) (Mims *et al.*, 2004; Lambert; 2004). The diaminopyrimidines, the most important of which is trimethoprim, act later in the pathway inhibiting dihydrofolate reductase (DHFR) to prevent the production of tetrahydrofolic acid (Figure 1.2) (Mims *et al.*, 2004; Greenwood *et al.*, 2007). The sulphonamides and trimethoprim act synergistically and, as a result, are prescribed in combination therapies with co-trimoxazole representing the most common combination (Greenwood *et al.*, 2007).

In *S. aureus*, resistance may arise to either the sulphonamide or trimethoprim components of co-trimoxazole. The acquisition of chromosomal mutations in the *sulA* gene encoding dihydropteroate synthase confers resistance to the sulphonamides through the over-production of dihydropteroic acid. In the case of trimethoprim resistance, chromosomal mutations within the *dfrB* gene encoding DHFR result in decreased drug-target interactions. Alternatively, resistance to co-trimoxazole may be mediated by the plasmid-borne *dfrA* and *dfrK* genes that encode alternative copies of DHFR with a low affinity for trimethoprim (Lowy, 2003; Malachowa and DeLeo, 2010).

1.4.1.8 Glycopeptides

The glycopeptides comprise the other major class of bacterial cell wall inhibitors, and are important in the treatment of drug-resistant *S. aureus* infections (Greenwood *et al.*, 2007). The most commonly used glycopeptides include vancomycin and teicoplanin, which is in fact a lipoglycopeptide (Bryskier, 2005b). These molecules are large with a complex structure centred around a multi-peptide backbone (Greenwood *et al.*, 2007). The glycopeptides exert their bactericidal effect by binding to the D-alanyl-D-alanine peptide residues present in peptidoglycan precursors, thereby inhibiting transpeptidation (Skrupky *et al.*, 2009).

The increasingly frequent use of vancomycin for the treatment of infections caused by *Clostridium difficile*, enterococci and multidrug-resistant staphylococci has inevitably led to the emergence of vancomycin-resistant staphylococci (Lowy 2003). Vancomycin resistance was first reported in a clinical *S. haemolyticus* isolate in 1987 (Scwalbe *et al.*, 1987). A decade later, the first vancomycin intermediate *S. aureus* (VISA) isolate was reported in Japan (Hiramatsu *et al.*, 1997). Heterogeneous VISA (hVISA) strains give rise to both vancomycin-intermediate and -susceptible subpopulations, and are thought to represent precursors of true VISA strains (Liu and Chambers, 2003). VISA and hVISA are characterised by decreased susceptibility to vancomycin with MICs ranging between 8 and 16mg/L and, importantly, these strains have been associated with treatment failure (Liu and Chambers, 2003; Appelbaum, 2007). Since the 1990s, there have been reports of VISA and hVISA strains worldwide (Liu and Chambers, 2003). Of even greater concern has been the more recent emergence of vancomycin resistant *S. aureus* (VRSA). The first VRSA isolate

was reported in the United States of America (USA) in 2002 with additional isolates subsequently reported in the USA (Appelbaum, 2007; Sievert et al, 2008), Brazil (Oliveira *et al.*, 2001), India (Tiwari and Sen, 2006), Jordan (Bataineh, 2006) and Iran (Aligholi *et al.*, 2008).

It has been shown that the mechanism of reduced vancomycin susceptibility differs in VISA and VRSA isolates (Lowy, 2003). In the case of VISA and hVISA isolates, reduced vancomycin susceptibility arises due to altered peptidoglycan synthesis. These isolates exhibit increased levels of peptidoglycan synthesis and decreased cross-linkage of the peptidoglycan chains. As a result, more D-alanyl-D-alanine residues are available to bind and sequester vancomycin within the outer limits of the bacterial cell wall. The sequestered vancomycin then aids in preventing additional vancomycin molecules reaching their targets and the cell wall, although thicker and irregularly shaped, remains intact (Lowy, 2003; Sakoulas and Moellering, 2008). In VRSA isolates, vancomycin resistance is due to the acquisition of a plasmid-borne *van* operon obtained from vancomycin resistant enterococci (Malachowa and DeLeo, 2010). The presence of the *van* operon results in the production of peptidoglycan precursors with D-alanyl-D-lactate terminal residues, which have a reduced affinity for vancomycin compared to D-alanyl-D-alanine residues; therefore cell wall synthesis is unaffected by the presence of this antimicrobial agent (Lowy, 2003; Malachowa and DeLeo, 2010). The emergence of vancomycin-resistant staphylococci has driven the search for alternative antimicrobial agents and, as a result, several promising alternatives to vancomycin and teicoplanin are currently under investigation, including the glycopeptides telavancin and oritavancin, and the lipoglycopeptide dalbavancin (Ratnaraja and Hawkey, 2008; Skrupky *et al.*, 2009).

1.4.2 Waiting in the wings: newly available classes of antimicrobial agents

In recent years the threat of multidrug-resistant *S. aureus* strains, particularly VISA and VRSA, has propelled investigations into new classes of antimicrobial agents, including the oxazolidinones, glycylcyclines and lipopeptides. As a result, a number of new antimicrobial agents with activity against resistant *S. aureus*, including VISA and VRSA strains have recently been introduced into clinical practice, including linezolid, daptomycin and tigecycline (Ratnaraja and Hawkey, 2008; Skrupky *et al.*, 2009; Rossolini *et al.*, 2010). Linezolid is an oxazolidinone that binds to the 23S

ribosomal subunit to prevent the formation of the initiation complex, thereby inhibiting protein synthesis (Greenwood *et al.*, 2007; Ratnaraja and Hawkey, 2008). Daptomycin is a cyclic lipopeptide that exerts its activity by disrupting the phospholipid structure of the cell membrane (Bryskier, 2005a; Greenwood *et al.*, 2007). Tigecycline is a glycylcycline that inhibits protein synthesis by interacting with the 30S ribosomal subunit (Brooks and Carroll, 2007a; Ratnaraja and Hawkey, 2008). Resistance to these three antimicrobial agents appears to be rare, although there are concerns regarding the reduced susceptibility of certain VISA strains to daptomycin, which is most likely due to the drug being sequestered by the thickened cell wall (Ratnaraja and Hawkey, 2008; Sakoulas and Moellering, 2008; Rossolini *et al.*, 2010).

1.4.3 Does antimicrobial chemotherapy represent a double-edged sword in the case of *S. aureus*?

Antimicrobial chemotherapy has undoubtedly reduced morbidity and mortality due to bacterial infections; however, it is possible that, at least in the case of *S. aureus*, antimicrobial chemotherapy may represent a double-edged sword. It appears that, in addition to providing the selective pressure that fosters the development of antimicrobial resistance, inappropriate or insufficient chemotherapy may in fact enhance *S. aureus* pathogenicity. Linezolid and clindamycin are known to encourage *S. aureus* intracellular persistence, which, as described earlier, is an important feature of the pathogenesis of this organism. It has also been suggested that the emergence of SCVs may be associated with failed antimicrobial therapies. Interestingly, it has been found that the horizontal transfer of mobile genetic elements is stimulated during the *S. aureus* SOS response, which is induced by certain β-lactams, fluoroquinolones and trimethoprim. Additionally, *in vitro* studies have shown that exposure to sub-inhibitory concentrations of β-lactam or fluoroquinolone antibiotics results in increased expression of *hla* and *pvl* encoding the α-toxin and PVL, respectively. These findings suggest that inappropriate or insufficient chemotherapy may encourage intracellular persistence, enhance the transmission of mobile genetic elements carrying virulence factors and resistance elements, and lead to increased production of particular *S. aureus* virulence factors (Dancer, 2008). While additional studies are required to further investigate these issues, it may well be prudent for clinicians to be more judicious in their prescription of antibiotics in future.

1.5 Methicillin-resistant *Staphylococcus aureus* (MRSA)

1.5.1 The emergence and clinical significance of MRSA

In 1940, the introduction of penicillin correlated with improved outcomes in patients with *S. aureus* infections; however, these effects were transient as penicillin-resistant *S. aureus* isolates were first reported in 1942 (Lowy, 2003). The prevalence of penicillin-resistant *S. aureus* increased during the 1950s, which provided impetus for the search for β-lactamase-stable antimicrobial agents (Oliveira *et al.*, 2002; Greenwood *et al.*, 2007). One such agent was methicillin, a semi-synthetic penicillin derivative with methoxy groups substituted at positions 2' and 6' of the benzene ring, thus rendering the molecule resistant to the action of β-lactamases (Bryskier, 2005e).

Methicillin was introduced into clinical practice in Europe between 1959 and 1961 in an attempt to combat penicillin-resistant *S. aureus* strains (Oliveira *et al.*, 2002; Lowy, 2003; Bryskier 2005c). The success of methicillin against *S. aureus* infections was short-lived with the first resistant isolates described in England in 1961 (Jevons, 1961). Methicillin-resistant *S. aureus* (MRSA) went on to be described in other European countries and the USA by the end of the 1960s (Bryskier, 2005c; Grundmann *et al.*, 2006; Deurenberg and Stobberingh, 2008). By the close of the 1970s, MRSA had been reported worldwide and MRSA prevalence rates have increased steadily over the past forty years (Ito *et al.*, 2003; Bryskier, 2005c; Deurenberg and Stobberingh, 2008; Witte *et al.*, 2008). Although MRSA gained notoriety as a nosocomial pathogen, this organism is no longer confined to the hospital setting. Since the 1990s, MRSA has been reported with increasing frequency in the community (Deurenberg and Stobberingh, 2008; Witte, 2009). Community-associated MRSA (CA-MRSA) will be discussed in more detail later in this chapter in the context of *S. aureus* evolution [1.7.3], and also in Chapter 4.

MRSA is a human pathogen of major clinical importance and currently constitutes a global public health concern (Aires-de-Sousa and de Lencastre, 2004; van Belkum *et al.*, 2009). In comparison with infections caused by methicillin-susceptible *S. aureus* (MSSA), MRSA infections are associated with increased morbidity and mortality (Lowy, 2003; Shorr, 2007; Witte *et al.*, 2008). This is largely due to the fact that MRSA strains represent a therapeutic challenge as they are frequently resistant to several classes of antimicrobial agents (Lowy, 2003; Gordon and Lowy, 2008).

Vancomycin remains the mainstay of chemotherapy for MRSA infections; however, the recent emergence of VISA and VRSA is of concern (Ratnaraja and Hawkey, 2008; Lindsay, 2010). The VISA and VRSA isolates described to date have been methicillin-resistant, which is worrying given the importance of vancomycin for the treatment of MRSA infections; however, the situation is not yet dire as antimicrobial agents recently introduced into clinical practice remain active against VISA and VRSA [1.4.2], while new fifth generation cephalosporins, glycopeptides and lipoglycopeptides with activity against MRSA are currently under investigation [1.4.1.1 and 1.4.1.8] (Ratnaraja and Hawkey, 2008).

In addition to being associated with therapeutic challenges and poorer patient outcomes, MRSA infections also represent an economic burden (Aires-de-Sousa and de Lencastre, 2004; Shorr, 2007; van Belkum *et al.*, 2009). These infections are associated with elevated treatment costs that arise due to the need for prolonged hospitalisation and additional procedures, diagnostic tests and antimicrobial agents (Aires-de-Sousa and de Lencastre, 2004; Grundmann *et al.*, 2006; Shorr *et al.*, 2007). The costs incurred during the containment of nosocomial MRSA outbreaks vary depending on the prescribed interventions, but tend to be significant (Grundmann *et al.*, 2006). Additionally, there are also indirect societal costs associated with MRSA infections that arise due to short- and long-term losses of productivity, and also due to mortality (Grundmann *et al.*, 2006).

1.5.2 The global prevalence of MRSA

While MRSA is prevalent worldwide, surveillance studies indicate that prevalence rates vary greatly between and even within countries (Oliveira *et al.*, 2002). Grundmann *et al.* (2006) reviewed antimicrobial surveillance studies carried out between 1998 and 2006 to provide an estimate of global MRSA prevalence rates. The authors found that only certain Scandinavian countries, such as Norway, Sweden and Iceland, reported exceptionally low MRSA prevalence rates of less than 1 %. Denmark, the Netherlands, Finland, Estonia and Canada had slightly higher MRSA prevalence rates of between 1 and 10 %, while Mexico, Morocco, Tunisia and much of northern Europe had moderate prevalence rates ranging from 10 to 25 %. In comparison, the UK, the USA, Turkey, Algeria, Egypt, Australia, Brazil, Argentina and most of southern Europe, had higher prevalence rates ranging from 25 to 50 %.

Finally, the highest MRSA prevalence rates were reported for countries such as Columbia, Iraq, Japan, Singapore, South Korea and Romania and exceeded 50 %.

As indicated by Grundmann *et al.* (2006), the absence of data from developing countries, particularly those in Africa and Asia, makes it challenging to obtain an accurate comparison of global MRSA prevalence rates. As South Africa has previously participated in international antimicrobial surveillance studies, it is one of the few African countries for which MRSA prevalence data is available. Zinn *et al.* (2004) detected a 39 % MRSA prevalence rate among isolates obtained during 1996. The SENTRY study carried out between 1998 and 1999 reported that 41.5 % of *S. aureus* isolates from South Africa were methicillin-resistant (Bell *et al.*, 2002). Similarly, the PEARLS study carried out between 2001 and 2002 reported that the MRSA prevalence rate in South Africa was 33.3 % (Bouchillon *et al.*, 2004). These findings should, however, be interpreted cautiously because the data were typically representative of only a small number of institutions from certain regions of the country, and, as mentioned previously, MRSA prevalence rates may vary markedly within a given country (Oliveira *et al.*, 2002). Antimicrobial surveillance has become more common in South Africa in recent years with local studies being initiated in both the public and private sectors (Perovic *et al.*, 2006; Shittu and Lin, 2006; Brink *et al.*, 2007; Groome *et al.*, 2009; Marais *et al.*, 2009). In perhaps the most comprehensive national study carried out to date in this country, the National Antimicrobial Surveillance Forum reported that the nationwide prevalence of MRSA from *S. aureus* blood culture isolates was 36 % in the private healthcare sector between January and June 2006 (Brink *et al.*, 2007).

1.5.3 The genetic basis and mechanism of methicillin resistance

In *S. aureus*, methicillin resistance arises subsequent to the acquisition of a large mobile genetic element (approximately 21 – 67kb), which is known as the staphylococcal cassette chromosome *mec* (SCC*mec*) (Katayama *et al.*, 2000; Berglund *et al.*, 2008; Deurenberg and Stobberingh, 2008; Zhang *et al.*, 2009; Malachowa and DeLeo, 2010). The SCC*mec* element undergoes site-specific integration into the *S. aureus* chromosome (Ito *et al.*, 1999; Katayama *et al.*, 2000; Ito *et al.*, 2001). The site of integration is at the 3 prime end of *orfX*, an open reading frame (ORF) of unknown function, which is located near to the origin of replication,

between the *spa* and *purA* loci (Ito *et al.*, 1999; Ito *et al.*, 2003). Eleven SCC*mec* types (I – XI), and many subtypes thereof, have been reported to date (Ito *et al.*, 2009; http://www.sccmec.org/Pages/SCC_TypesEN.html); however, only SCC*mec* types I – VIII have been described in detail to date (Ito *et al.*, 2001; Ma *et al.*, 2002; Ito *et al.*, 2004; Oliveira *et al.*, 2006; Berglund *et al.*, 2008; Zhang *et al.*, 2009). Studies have shown that SCC*mec* types I – VIII, with the exception of SCC*mec* type VII, all share a common basic genetic structure (Ito *et al.*, 2009), as illustrated in Figure 1.3.

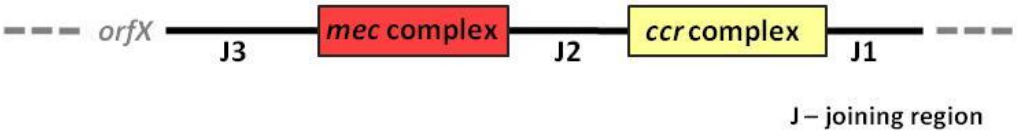


Figure 1.3 Schematic diagram illustrating the basic genetic structure common to SCC*mec* types I – VI and VIII. The *S. aureus* chromosome is shown in grey and the staphylococcal cassette chromosome *mec* (SCC*mec*) element in bold. Adapted from de Lencastre *et al.* (2007).

The basic structure of SCC*mec* elements I – VI and VIII can be summarised, starting from the 3 prime end of *orfX*, as "J3 – *mec* complex – J2 – *ccr* complex – J1", where the *mec* complex encodes the methicillin resistance determinant, the *ccr* complex confers mobility on the element and the joining or J (formerly junkyard) regions comprise non-essential components that may carry additional resistance determinants (Ito *et al.*, 2003; de Lencastre *et al.*, 2007; Deurenberg and Stobberingh, 2008; Ito *et al.*, 2009). SCC*mec* type VII deviates from the common basic structure as, although it contains the same components, the positions of the *mec* and *ccr* complexes are reversed [3.1] (Berglund *et al.*, 2008).

In accordance with the guidelines of the International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC), SCC*mec* types are differentiated based on the combination of the class of *mec* complex and *ccr* allotype present, while differences in the J regions are used to define SCC*mec* subtypes (Ito *et al.*, 2009). A detailed description of the information available on the genetic structure, unique features and epidemiology of SCC*mec* types I – XI, focusing on SCC*mec* types I – VIII, will be provided in Chapter 3 [3.1]. The current section of this literature review will focus on the roles and basic

components of the *mec* and *ccr* complexes that are common to all extant SCC*mec* types.

1.5.3.1 The mec *complex*

Six classes of *mec* complex have been identified to date, and are designated A, B, C1, C2, D and E (Hanssen and Ericson Sollid, 2006; Deurenberg and Stobberingh, 2008). These *mec* complexes all contain the methicillin resistance determinant, *mecA*, as well as various regulatory components and insertion sequences (Hanssen and Ericson Sollid, 2006; Ito *et al.*, 2009). The exogenous *mecA* gene is 2.1kb in size and encodes a 78kDa PBP, known as PBP2' or PBP2A (Brown and Reynolds, 1980; Reynolds and Brown, 1985; Beck *et al.*, 1986; Ubukata *et al.*, 1985; Utsui *et al.*, 1985; Ito *et al.*, 1999). PBP2' has a very low affinity for methicillin and other β-lactam antibiotics (Brown and Reynolds, 1980; Hartman and Tomasz, 1981; Reynolds and Brown, 1985; Utsui *et al.*, 1985; Ubukata *et al.*, 1989; Lowy, 2003). Crystallisation studies indicate that the active site of PBP2' is able to retain its transpeptidase activity whilst preventing β-lactam binding (Lim and Strynadka, 2002). As a result, PBP2', in conjunction with the penicillin-insensitive transglycosylase domain of the endogenous PBP2, acts as a custodian of transpeptidation, thereby maintaining cell wall integrity in the presence of β-lactam antibiotics (Pinho *et al.*, 2001a; Pinho *et al.*, 2001b; Berger-Bächi and Rohrer, 2002). It would, however, appear that PBP2' does not function as effectively as the endogenous *S. aureus* PBPs because the peptidoglycan layer of MRSA isolates is not as extensively cross-linked in the presence of β-lactams (Berger-Bächi and Rohrer, 2002).

The class A *mec* complex is thought to be the prototypic *mec* complex and includes *mecA* as well as intact copies of the *mecI* and *mecR1* regulatory components (Ito *et al.*, 2009). The *mecI* and *mecR1* genes are responsible for the regulation of *mecA* expression, and encode the MecI repressor and MecR1 sensor-transducer, respectively (Berger-Bächi and Rohrer, 2002; Lowy, 2003). In the absence of β-lactam antibiotics, MecI binds to the operator region of *mecA*, thereby inhibiting transcription. The presence of β-lactams results in the autocatalytic cleavage of MecR1, which activates its cytoplasmic metalloprotease domain. In turn, the metalloprotease domain of MecR1 is responsible for MecI cleavage, thereby derepressing *mecA* to induce the expression of methicillin resistance (Berger-Bächi

and Rohrer, 2002). It has been shown that mutations or deletions in the *mecI* gene result in constitutive rather than inducible methicillin resistance (Berger-Bächi and Rohrer, 2002; Lowy, 2003).

Of the six known classes of *mec* complex, all except A contain remnants of *mecI* and *mecR1* due to deletions, or the integration of insertion sequences within that region of the *mec* complex, as illustrated in Figure 1.4 (Hanssen and Ericson Sollid, 2006; Deurenberg and Stobberingh, 2008).

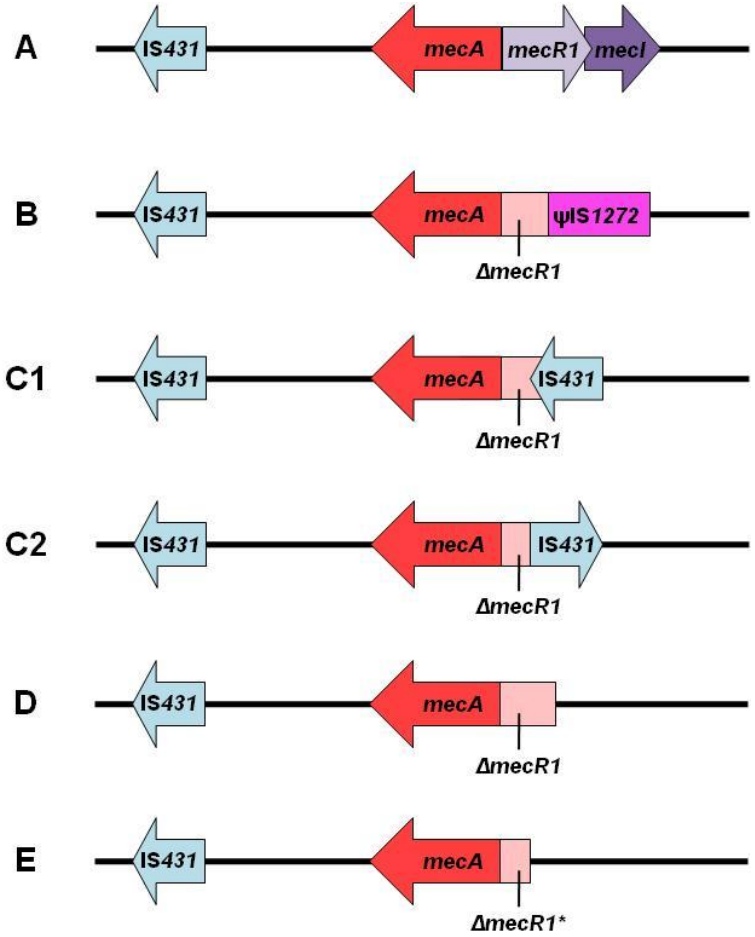


Figure 1.4 Schematic representation of the genetic structures of *mec* complexes A – E. Arrowheads indicate the orientation of certain *mec* complex components. *ΔmecR1**, 976 bp deletion compared to *ΔmecR1* in the class D mec complex. Adapted from Hanssen and Ericson Sollid (2006).

All known *mec* classes, except class D, have been described in SCC*mec* elements isolated from *S. aureus* strains [3.1] (Ito *et al.*, 2009; http://www.sccmec.org/Pages/SCC_TypesEN.html). In the prototypic class A *mec*

complex, *mecR1* and *mecI* are located upstream of *mecA*, while the insertion sequence IS*431* is located downstream of the methicillin-resistance determinant (Figure 1.4). In comparison, the class B *mec* complex also includes *mecA* and the downstream copy of IS*431*, but *mecR1* is truncated following the integration of insertion sequence IS*1272* (Figure 1.4). The class C1 and C2 *mec* complexes are similar, but distinct: both consist of *mecA* with a downstream copy of IS*431*, but *mecR1* is truncated due to the upstream insertion of an additional copy of IS*431* (Figure 1.4). The orientation of the second copy of IS*431* differentiates classes C1 and C2. In *mec* class C1, both copies of IS*431* are in the same orientation, while in C2 the upstream copy of IS*431* is in the reverse orientation to the downstream copy (Figure 1.4) (Ito *et al.*, 2009). The class D and E *mec* complexes are also similar as they both contain *mecA* with a downstream copy of IS*431*, as well as a partial copy of *mecR1* upstream of the methicillin-resistance determinant (Figure 1.4). The partial copies of the *mecR1* genes distinguish these *mec* complexes as that present in class E carries a 976 bp deletion compared to that present in class D (Hanssen and Ericson Sollid, 2006; Deurenberg and Stobberingh, 2008). Variants within the major *mec* classes have been described, and are characterised by the presence of additional insertion sequences. These variants are indicated by subscript numerals following the *mec* class, for example, B_2 (Ito *et al.*, 2009).

1.5.3.2 The ccr *complex*

The *ccr* complex consists of the *ccr* gene(s), as well as any associated ORFs (Deurenberg and Stobberingh, 2008; Ito *et al.*, 2009). In *S. aureus*, three phylogenetically distinct *ccr* genes have been described: *ccrA*, *ccrB* and *ccrC* (Ito *et al.*, 2009). The *ccrA* and *ccrB* genes have been further classified into four and six allotypes, respectively; however, only one *ccrC* allotype has been described to date (Ito *et al.*, 2009; http://www.sccmec.org/Pages/SCC_TypesEN.html). The known *ccr* complexes can be separated into two distinct groups: those that contain adjacent copies of *ccrA* and *ccrB*, and those that contain *ccrC*. The *ccr* complex allotypes most commonly found in *S. aureus* include type 1 (*ccrA1B1*), type 2 (*ccrA2B2*), type 3 (*ccrA3B3*), type 4 (*ccrA4B4*) and type 5 (*ccrC*) (Ito *et al.*, 2009). Additionally, the IWG-SCC has communicated the identification of *ccr* allotypes 6 (A5B3), 7 (A1B6) and 8 (A1B3) via their website (http://www.sccmec.org/Pages/SCC_TypesEN.html), although only allotypes 7 and 8 have been described in MRSA-associated *ccr* complexes [3.1].

The *ccr* genes encode recombinases of the invertase/resolvase family, which confer mobility on the SCC*mec* element (Ito *et al.*, 1999; Katayama *et al.*, 2000; Ito *et al.*, 2001; Ito *et al.*, 2004; Oliveira *et al.*, 2006). The recombinases mediate the site-specific integration and excision of SCC*mec* at the insertion site sequence (ISS), also known as the SCC*mec* attachment site (*attB*), which is located at the 3 prime end of *orfX* (Ito *et al.*, 1999; Ito *et al.*, 2001; Ito *et al.*, 2009). The precise location of the integration site within the ISS has been determined to within four nucleotides. These four nucleotides comprise part of a 15 bp direct repeat sequence that is characteristic of the ISS with its counterpart situated in the terminal portion of the J3 region of SCC*mec* (Ito *et al.*, 1999; Ito *et al.*, 2001). The 15 bp direct repeats, in addition to the 27 bp imperfect inverted repeat sequences that are also present at the terminals of the SCC*mec* element, appear to be targeted by the recombinases mediating integration and excision (Ito *et al.*, 1999; Ito *et al.*, 2001; Ito *et al.*, 2009). Similarly, the composition of regions flanking the ISS also appears to affect an isolate's ability to acquire SCC*mec* (Noto *et al.*, 2008).

1.5.3.3 The origin of mecA *and possible reservoirs of SCC*mec

Early studies showed that there was no allelic equivalent of *mecA* in MSSA isolates (Stewart and Rosenblum, 1980). Combined with the detection of skewed GC content at the third codon position, and atypical codon usage within several SCC*mec* ORFs, this suggested that the methicillin resistance determinant was obtained from another organism (Hiramatsu *et al.*, 2001). Screening different staphylococcal species for the presence of *mecA* resulted in the detection of homologues with 80 and 91 % nucleotide identities in *Staphylococcus sciuri* (Couto *et al.*, 1996) and *Staphylococcus vitulinus* (Schnellmann *et al.*, 2006), respectively; however, these homologues were not components of an SCC*mec* element, or a *mec* complex.

Based on the available data, it was predicted that the origin of *mecA* would be traced to a close relative of *S. sciuri* and *S. vitulinus*. *Staphylococcus fleurettii*, an animal commensal belonging to the *S. sciuri* species group, was recently identified as the origin of *mecA* (Tsubakishita *et al.,* 2010). The nucleotide identities shared by *mecA* homologues detected in *S. fleurettii* strains and the MRSA strain N315 ranged from 99 to 100 %. Examination of the flanking chromosomal regions in *S. fleurettii* also

revealed that the *mecA* locus in this organism is highly similar to the prototypic class A *mec* complex. It appears that *S. fleurettii*, *S. sciuri* and *S. vitulinus* acquired *mecA* from a common ancestor, and that the gene underwent molecular differentiation after these species diverged, resulting in the three alleles observed today. At present, the origin of the SCC*mec* element remains unknown, but it seems likely that it arose when the *S. fleurettii*-derived *mec* complex fused with a *mecA*-negative SCC element. It is possible that this process occurs frequently, and that novel SCC*mec* elements are produced on a regular basis. Alternatively, SCC*mec* may have emerged on a limited number of occasions with subsequent genetic diversification generating the wide variety of SCC*mec* elements described to date (Tsubakishita *et al.,* 2010).

Although the origins of SCC*mec* are currently under debate, it is widely accepted that the CNS represent a reservoir of SCC*mec* elements (Hiramatsu *et al.*, 2001; Hanssen and Ericson Sollid, 2006; de Lencastre *et al.*, 2007; Garza-González *et al.*, 2010). Several observations support this hypothesis, including the fact that SCC*mec* elements are more prevalent, and more diverse, in CNS than *S. aureus* (Garza-González *et al.*, 2010). Other evidence cited in support of the hypothesis includes the fact that there has been species-independent conservation of certain *ccr* genes (Garza-González *et al.*, 2010), and also that the insertion sequence IS*1272* found in the class B *mec* complex [1.5.3.1] is more commonly described in *S. haemolyticus* and *S. epidermidis* than in *S. aureus*, suggesting that these elements may have been transferred from the CNS species to *S. aureus* (Hiramatsu *et al.*, 2001; Hanssen and Ericson Sollid, 2006; Garza-González *et al.*, 2010).

1.6 *S. aureus* epidemiology

It has long been acknowledged that, in order to control a pathogen such as *S. aureus*, it is prerequisite to obtain an understanding of its epidemiology, population genetics and evolution (Aires-de-Sousa and de Lencastre, 2004; Deurenberg and Stobberingh, 2008; Robinson *et al.*, 2010). Given that *S. aureus* is an important pathogen in both hospitals and communities, it is crucial to have accurate and reliable epidemiological tools available for use during outbreak investigations (Shopsin and Kreiswirth, 2001; Oliveira *et al.*, 2002; Aires-de-Sousa and de Lencastre, 2004). Additionally, epidemiological data is essential for the evaluation and modification of existing antibiotic prescription guidelines and infection control practices in the pursuit of controlling *S. aureus* dissemination (van Belkum *et al.*, 2009).

Early *S. aureus* epidemiological studies were reliant upon phenotypic typing techniques; however, advances in molecular biology have led to the development of several new techniques that have revolutionised the field of *S. aureus* epidemiology (Oliveira *et al.*, 2002; Aires-de-Sousa and de Lencastre, 2004). *S. aureus* typing is no longer based on phenotypic methods, but rather on genotypic methods that are able to detect underlying genetic variation (Aires-de-Sousa and de Lencastre, 2004; van Belkum *et al.*, 2009). Over the past few decades, a global emphasis on the importance of *S. aureus* molecular epidemiology has led to the development of numerous band- and DNA sequence-based genotypic techniques (Shopsin and Kreiswirth, 2001; Aires-de-Sousa and de Lencastre, 2004; Smyth and Robinson, 2010). Band-based genotypic techniques require visual comparisons of restriction patterns or PCR amplification profiles to determine the relatedness of isolates. These methods indirectly detect underlying genetic variation that occurs in the form of restriction enzyme recognition sequences or primer-binding sites (Shopsin and Kreiswirth, 2001; Smyth and Robinson, 2010). Due to their shortcomings, the popularity of band-based techniques has waned over the years, and these methods have largely been replaced by DNA sequence-based techniques (Aires-de-Sousa and de Lencastre, 2004; Deurenberg and Stobberingh, 2008). Reasons for the transition from band- to sequence-based typing techniques will be discussed further in Chapter 5 [5.1].

The inclusion of particular typing techniques in an epidemiological study is primarily dependent on the questions to be addressed (Aires-de-Sousa and de Lencastre, 2004; Cookson *et al.*, 2007; van Belkum *et al.*, 2009). The available methods have varying powers of discrimination as they index genetic variation that accumulates at different rates; therefore some techniques are best suited to local or short-term epidemiology, while others are better suited to global or long-term epidemiology, while others still provide information valuable to either type of study (Deurenberg and Stobberingh, 2008; van Belkum *et al.*, 2009). Additional factors that may be considered when selecting molecular typing methods include cost, time to result and whether the technique is to be used in a clinical or research laboratory (Aires-de-Sousa and de Lencastre, 2004). Molecular typing techniques popular worldwide for the characterisation of *S. aureus* isolates include pulsed-field gel electrophoresis, SCC*mec* typing and subtyping, multilocus sequence typing, and *spa* typing (Aires-de-Sousa and de Lencastre, 2004; Deurenberg and Stobberingh, 2008; van Belkum *et al.*, 2009). A brief overview of the major techniques currently used in *S. aureus* molecular epidemiology will be provided here, while more detailed descriptions of the methods used in this study will be included in subsequent chapters.

1.6.1 Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) is a highly discriminatory band-based typing technique, which indexes variation that accumulates rapidly throughout the *S. aureus* genome (Spratt, 1999; Deurenberg and Stobberingh, 2008; van Belkum *et al.*, 2009). Briefly, PFGE involves the comparison of macrorestriction patterns that are generated after pulsed-field gel electrophoresis is used to separate the large DNA fragments produced by restriction enzyme digestion of intact chromosomal DNA (Schwartz and Cantor, 1984; Trindade *et al.*, 2003). A more detailed description of the technique is provided in Chapter 2 [2.1]. PFGE is one the oldest genotypic techniques, but is still commonly used in studies concerned with local or short-term epidemiology (Deurenberg and Stobberingh, 2008). The method has remained popular because of its utility in outbreak investigations, and in studies tracing hospital-to-hospital transmission (Struelens *et al.*, 1992; Cookson *et al.*, 2007; Deurenberg and Stobberingh, 2008).

1.6.2 SCC*mec* typing and subtyping

Information regarding the SCC*mec* content of MRSA isolates can be valuable in both local and global epidemiological studies. A variety of PCR-based SCC*mec* typing and subtyping assays have been developed, with the choice of method dependent on the epidemiological questions to be addressed (Aires-de-Sousa and de Lencastre, 2004; de Lencastre *et al.*, 2007; Deurenberg and Stobberingh, 2008). The most popular SCC*mec* typing and subtyping assays are multiplex PCRs, which include primers for the detection of SCC*mec* type- or subtype-specific loci, resulting in unique, easily identifiable amplification patterns after the PCR products are separated by agarose gel electrophoresis (de Lencastre *et al.*, 2007). Further information on techniques that are currently popular for SCC*mec* typing and subtyping is provided in Chapter 3 [3.1].

1.6.3 Multilocus sequence typing

Multilocus enzyme electrophoresis (MLEE) was one of the first molecular typing techniques used in studies investigating the global epidemiology of *S. aureus* (Spratt, 1999; Smyth and Robinson, 2010). MLEE indirectly indexes variation that accumulates in housekeeping genes by examining the electrophoretic mobilities of their products. Multilocus sequence typing (MLST) represents an extension of MLEE that was made possible by advances in DNA sequencing, and catalogues underlying genetic variation that accumulates within seven housekeeping genes (Spratt, 1999). In MLST, internal fragments of the seven housekeeping genes are amplified by PCR, sequenced directly, and compared to existing sequences present in the online *S. aureus* MLST database (http://saureus.mlst.net/). The isolate's sequence type (ST) is then determined, based on the combination of housekeeping alleles present, and is identified by a unique number (Maiden *et al.*, 1998; Spratt, 1999; Enright *et al.*, 2000). A more comprehensive description of MLST can be found in Chapter 5 [5.1].

The eBURST algorithm, a revised version of the BURST (based upon related sequence types) algorithm, was developed to detect relationships between STs, and is freely available on the MLST website (http://saureus.mlst.net/eburst/) (Feil *et al.*, 2004; Aanensen and Spratt, 2005). The algorithm groups STs into clonal complexes (CCs); a CC includes all strains that share at least six of the seven MLST loci with one or more ST in the group, and is assigned a unique identifying number (Feil and

Enright, 2004; Feil *et al.*, 2004). In most cases, it is possible to assign a putative founding genotype to a CC, which is defined as the ST with the most single locus variants (SLVs). The larger CCs commonly contain a number of SLVs and double locus variants (DLVs) that have become dominant within particular regions, forming subgroups with their own SLVs and DLVs (Feil *et al.*, 2004). As MLST indexes genetic variation that accumulates slowly over time, the technique is useful for the detection of underlying relationships between isolates; therefore MLST and the eBURST algorithm have proved invaluable in global or long-term epidemiological studies, as described later in this chapter [1.7] (Spratt, 1999; Deurenberg and Stobberingh, 2008; van Belkum *et al.*, 2009; Lindsay, 2010).

1.6.4 *spa* typing

In recent years, *spa* typing, a single-locus sequence-based method, has become increasingly popular for use in *S. aureus* epidemiological investigations. It has been shown that *spa* typing has a discriminatory power between that of PFGE and MLST and can be used for studying both local and global *S. aureus* epidemiology [5.1] (Deurenberg and Stobberingh, 2008). The technique also has the advantage of being less expensive than MLST and less laborious than either MLST or PFGE. In brief, *spa* typing entails PCR amplification and direct sequencing of the hypervariable X-region of *spa*, which encodes protein A (Frénay *et al.*, 1996; Shopsin *et al.*, 1999; Shopsin and Kreiswirth, 2001; Harmsen *et al.*, 2003). The X-region is comprised of repeats [5.1] and variation arises due to duplications and deletions of these repeats, as well as point mutations (Harmsen *et al.*, 2003). Ridom StaphType, a software package for the analysis of *spa* sequencing data, has become popular worldwide for the rapid assignment of *spa* types [5.1] (Deurenberg and Stobberingh, 2008). The software package is linked to well-curated online database to ensure that the universal nomenclature is maintained, and also to facilitate inter-laboratory comparisons and data sharing (Harmsen *et al.*, 2003; Deurenberg and Stobberingh, 2008). Additional details on *spa* typing and Ridom StaphType are available in Chapter 5 [5.1].

As for MLST, it is possible to carry out cluster analyses to define *spa* clonal complexes (*spa*-CCs) using the BURP (based upon repeat pattern) algorithm in Ridom StaphType (Mellmann *et al.*, 2008). Studies have detected congruence

between *spa*-CCs identified using the BURP algorithm and those identified using MLST and the eBURST algorithm (Strommenger *et al.*, 2006a; Cookson *et al.*, 2007; Hallin *et al.*, 2007; Mellmann *et al.*, 2008); however, this approach may, at times, lack discriminatory power (Deurenberg and Stobberingh, 2008; Strommenger *et al.*, 2008). It has been suggested that *spa* typing be used in conjunction with other molecular typing tools in order to obtain the most accurate description of *S. aureus* epidemiology (Hallin *et al.*, 2007; Strommenger *et al.*, 2008).

1.6.5 Other techniques currently available for *S. aureus* epidemiology

In addition to the techniques described above for *S. aureus* typing, many more have been developed recently and are increasing in popularity. One example is *dru* typing, a single-locus sequence-based technique that indexes variation within the SCC*mec*-associated series of imperfect direct repeat units present in most MRSA isolates [5.1] (Goering *et al.*, 2008a). The technique is becoming increasingly popular because of its ability to subtype isolates indistinguishable by PFGE, providing a degree of resolution that is important during outbreak investigations (Goering *et al.*, 2008a; Shore *et al.*, 2010). There has also been a move to update multilocus variable-number tandem repeat analysis (MLVA), a band-based typing method (van Belkum *et al.*, 2009). Groups have updated MLVA by using software packages to estimate PCR product size after agarose gel electrophoresis (Pourcel *et al.*, 2009), or by using an automated DNA sequencer to determine the sizes of fluorescently labelled amplicons (Schouls *et al.*, 2009). It has been suggested that methods based on MLVA will, like *dru* typing, aid in the differentiation of isolates indistinguishable by PFGE (Holmes *et al.*, 2010).

Further advances in DNA sequencing technologies have led to the development of new typing methods, which have the potential to once again revolutionise the field of *S. aureus* epidemiology (van Belkum *et al.*, 2009; Lindsay, 2010). A recent study demonstrated the value of whole genome sequencing in *S. aureus* epidemiological studies by examining the fine-scale transmission of a particular MRSA clone within a Thai hospital (Harris *et al.*, 2010). The same study indicated the value of whole genome sequencing for global MRSA epidemiology. The increasing availability of whole genome data has also aided the development of multi-strain DNA microarrays for *S. aureus* genotyping (Lindsay, 2010). At present, whole genome analyses and

DNA microarray genotyping remain expensive; therefore these technologies are mainly restricted to developed countries.

1.7 The population genetics and evolution of *S. aureus*

The field of bacterial population genetics is, in part, concerned with describing the phylogenies or natural groups or lineages that have descended from a unique common ancestor within a particular species. This information serves as the framework for evolutionary hypotheses explaining the patterns of descent and the accumulation of genetic variation within the given population (Smyth and Robinson, 2010). A detailed understanding of a pathogen's population structure and evolution is important as it may be used to mitigate the disease burden caused by the organism. For instance, the identification of dominant bacterial lineages and their associated pathogenic characteristics and molecular markers may be valuable when investigating new therapeutic targets or diagnostic markers. Similarly, a pathogen's population structure may provide information regarding its spread that can be used to prevent further dissemination. Additionally, this type of information can be used to predict how a pathogen may evolve in the future, thereby providing a tactical advantage in the ongoing battle between humans and bacteria (Lyndsay, 2010; Smyth and Robinson, 2010; Robinson *et al.*, 2010).

1.7.1 The population structure of *S. aureus*

Given the propensity of *S. aureus* to cause infections in the hospital setting, an understanding of its population genetics and evolution has been pursued for decades in the hope of controlling this pathogen (Smyth and Robinson, 2010). Progress in the field was initially hampered by the lack of appropriate techniques; however, remarkable progress has been made in the past decade due to the development of molecular typing methods, as well as the advent of whole genome sequencing and comparative genomic microarrays (Lindsay, 2010; Smyth and Robinson, 2010).

As mentioned previously, MLST is well-suited to the detection of underlying genetic relationships between apparently unrelated isolates obtained over extended time periods or great geographical distances (Spratt, 1999; Deurenberg and Stobberingh, 2008). Enright *et al.* (2002) used MLST and the eBURST algorithm to provide the first detailed DNA sequence-based description of the *S. aureus* population structure. The strain collection characterised by Enright *et al.* (2002) included 912 *S. aureus* isolates obtained from 20 countries between 1961 and 1999. The study has since been supplemented by other researchers who have added their data to the MLST

website (http://saureus.mlst.net/), thus providing a more detailed description of the *S. aureus* population structure. As of December 2010, an eBURST analysis of the *S. aureus* MLST database detected forty-two major CCs, as well as eighteen minor CCs lacking putative ancestral STs, and two hundred and forty-six singleton STs not associated with a CC (http://saureus.mlst.net/eburst/). Of the known *S. aureus* CCs, CC1, CC5, CC8, CC9, CC12, CC15, CC22, CC25, CC30, CC45 and CC51 have been identified as major lineages relevant in humans (Lindsay, 2010; McCarthy and Lindsay, 2010).

Subsequent studies have examined the relatedness of *S. aureus* lineages and have shown that the extant CCs can be separated into two groups, both containing clinically important and widely disseminated clones (Robinson and Enright, 2003; Cooper and Feil, 2006; Smyth and Robinson, 2010). It has been suggested that different evolutionary processes may have moulded these two groups, but further studies are required for confirmation (Smyth and Robinson, 2010). Also of interest in these studies were two CCs that did not fall into either subgroup: CC152 and CC75 (Smyth and Robinson, 2010). CC152 represents a divergent lineage predominant in Mali (Ruimy *et al.*, 2008), while CC75 is thought to represent an ancestral *S. aureus* lineage confined to Australia (Ng *et al.*, 2009; Smyth and Robinson, 2010).

1.7.2 The population structure and evolution of methicillin-resistant *S. aureus*: focusing on healthcare-associated lineages

In 1993 it was proposed that all extant MRSA arose from a single MSSA ancestor that had acquired SCC*mec* (Kreiswirth *et al.*, 1993). Almost ten years later, the MLST-based study carried out by Enright *et al.* (2002) overturned the single-clone hypothesis. This study, and that of Robinson and Enright (2003), provided the first detailed description of the population structure and evolution of hospital-associated MRSA (HA-MRSA) lineages. Robinson and Enright (2003) supplemented MLST with *sas* typing (a multilocus sequence typing method that indexes variation within seven *S. aureus* surface associated proteins) and *spa* typing to show that SCC*mec* had been acquired, and in some cases re-acquired, on at least twenty occasions by a limited number of *S. aureus* lineages, namely CC5, CC8, CC22, CC30 and CC45. Further support for the multi-clone hypothesis was provided by Fitzgerald *et al.* (2001) who demonstrated by DNA microarray that SCC*mec* had been acquired on at

least five occasions by different lineages. Early studies also showed that SCC*mec* was typically acquired by MSSA lineages already successful in the hospital setting (Gomes *et al.*, 2006). These lineages appeared to possess some genetic advantage prior to the acquisition of the methicillin resistance determinant; however, certain lineages, such as ST239 of CC8, have become increasingly successful subsequent to the acquisition of SCC*mec* (Lyndsay, 2010).

Enright *et al.* (2002) found that, although epidemic MRSA (EMRSA) clones dominant in distant regions over a period of nearly forty years were distinguishable by PFGE, several clones in fact shared the same ST and SCC*mec* type, suggesting underlying genetic relationships. These findings, supported by the work of Robinson and Enright (2003), suggested that several major EMRSA clones arose from a common ancestor, and have since been disseminated worldwide. Table 1.2 provides an overview of important pandemic HA-MRSA clones, indicating those that are thought to have descended from a common ancestor.

Table 1.2 An overview of important hospital-associated methicillin-resistant *Staphylococcus aureus* (HA-MRSA) clones[a]

Clone[b]	Clonal complex	Common names	Geographical distribution[c]
ST5-MRSA-I	5	UK-EMRSA-3	Arg, Den, Ger, Jap, Nor, Par, Pol, Por, Slo, Tai, UK
ST5-MRSA-II	5	New York/ Japan/ USA100	Ast, Bel, Can, Chn, Den, Fin, Fra, Ger, Hun, Ire, Isr, Jap, Kor, Mex, Nor, Por, SA, Sin, Swe, Swi, Tai, Uru, UK, USA
ST5-MRSA-IV	5	Paediatric/ USA800	Alg, Arg, Ast, Aus, Bra, Col, Den, Fin, Fra, Ger, Gre, Kor, Nor, Pol, Por, Spa, Swe, Tur, Uru, UK, USA
ST228-MRSA-I	5	Southern Germany	Aus, Bel, Den, Fin, Ger, Hun, Ita, Pol, Slo, Spa, Swi
ST8-MRSA-II	8	Irish-1	Ast, Can, Ire, UK, USA
ST8-MRSA-IV	8	UK-EMRSA-2/ UK-EMRSA-6/ USA500	Ast, Aus, Bel, Can, Den, Fin, Fra, Ger, Hun, Ire, Isr, Jap, Net, Nor, Swi, Tai, UK, USA
ST239-MRSA-III	8	Brazilian/Hungarian/ Portuguese/ Viennese/ UK-EMRSA-1/ UK-EMRSA-4/ UK-EMRSA-11	Alg, Arg, Ast, Aus, Bra, Can, Chi, Chn, Cze, Den, Fin, Ger, Gre, Hun, Ind, Ids, Kor, Mon, Net, Nor, Par, Pol, Por, RoG, Rus, SA, Sin, Slo, Spa, Sri, Swe, Tai, Tha, UK, Uru, USA, Vie
ST247-MRSA-I	8	Iberian	Aus, Bel, Cro, Cze, Den, Fin, Fra, Ger, Hun, Isr, Ita, Net, Nor, Pol, Por, Slo, Spa, Swe, Swi, UK, USA
ST250-MRSA-I	8	Archaic	Ast, Can, Den, Ger, Swi, Uga, UK, USA
ST22-MRSA-IV	22	UK-EMRSA-15/ Barnim	Ast, Aus, Bel, Can, Chn, Cze, Den, Fin, Ger, Hun, Ire, Kuw, Mal, NZ, Nor, Por, Sin, Spa, Swe, UK
ST45-MRSA-IV	45	Berlin/ USA600	Arm, Ast, Aus, Bel, Chn, Den, Fin Ger, Hun, Isr, Net, Nor, Spa, Swe, Swi, USA
ST36-MRSA-II	30	UK-EMRSA-16/ USA200	Ast, Aus, Bel, Can, Den, Fin, Ger, Gre, Ire, Mex, Nor, Por, Spa, Swe, Swi, UK, USA

[a] Adapted from Deurenberg and Stobberingh (2008); Gordon and Lowy (2008); Witte *et al.* (2008).

[b] Clone name as determined using the universal nomenclature proposed by Enright *et al.* (2002) following the form (Sequence Type-methicillin resistance phenotype-SCC*mec* type).

[c] Alg, Algeria; Arg, Argentina; Arm, Armenia; Ast, Australia; Aus, Austria; Bel, Belgium; Bra, Brazil; Can, Canada; Chi, Chile; Chn, China; Col, Columbia; Cro, Croatia; Cze, Czech Repubic; Den, Denmark; Fin, Finland; Fra, France; Ger, Germany; Gre, Greece; Hun, Hungary; Ind, India; Ids, Indonesia; Ire, Ireland; Isr, Israel; Jap, Japan; Kor, Korea; Kuw, Kuwait; Mal, Malta; Mon, Mongolia; Net, Netherlands; NZ, New Zealand; Nor, Norway; Par, Paraguay; Pol, Poland; Por, Portugal; RoG, Republic of Georgia; Rus, Russia; SA, Saudi Arabia; Sin, Singapore; Slo, Slovenia; Spa, Spain; Sri, Sri Lanka; Swe, Sweden; Swi, Switzerland; Tai, Taiwan; Tha, Thailand; Tur, Turkey; Uga, Uganda; UK, United Kingdom; Uru, Uruguay; USA, United States of America; Vie, Vietnam.

Historically, EMRSA clones were named according to their geographical origins. Given that many EMRSA clones appear to share common ancestors, this somewhat arbitrary system has been replaced with a universal nomenclature (Enright *et al.*, 2002). The revised nomenclature is based on an isolate's ST and SCC*mec* type, and also indicates whether the isolate is MSSA, MRSA or glycopeptide intermediate *S. aureus* (GISA). For example, the Archaic clone, which corresponds to ST250 and carries SCC*mec* type I, is described as ST250-MRSA-I using the universal nomenclature. Table 1.2 includes the revised universal nomenclature, as well as the common names, of important HA-MRSA clones. The universal nomenclature is now widely used, making underlying relationships between clones more apparent. This is well illustrated by the ST239-MRSA-III clone, which corresponds to the former Brazilian, Hungarian and Portuguese clones. These clones were responsible for MRSA epidemics in their respective countries and have certain distinguishing molecular characteristics, but in fact share a common ancestor (Enright *et al.*, 2002; Robinson and Enright, 2003; Harris *et al.*, 2010).

The results of the studies discussed above suggested that the worldwide prevalence of MRSA is due to the dissemination of a limited number of highly successful pandemic clones. MLST does, however, only monitor genetic diversity within a very small portion of the *S. aureus* genome (approximately 3.2kb) (Enright *et al.*, 2000). A more recent study examining variation within a larger proportion of the genome (46kb) has suggested that MRSA evolution may be more complex than MLST-based studies suggested (Nübel *et al.*, 2008). Nübel *et al.* (2008) showed that, in the case of ST5, the frequency of SCC*mec* acquisition has been underestimated, and ST5-MRSA lineages have emerged on numerous occasions in distinct geographical locations. Further studies are required to determine whether the same is true of other MRSA lineages but, if this is the case, it may be necessary to revise the existing nomenclature to include the region of origin in to distinguish different subpopulations within a given clonal type.

Although the results reported by Nübel *et al.* (2008) are interesting, the contribution of dissemination to the global prevalence of MRSA cannot be dismissed. Recent studies investigating the population structure of ST239-MRSA-III have indicated that dissemination, rather than repeated emergence, is the the cause of its global

prevalence (Smyth *et al.*, 2009; Gray *et al.*, 2010; Harris *et al.*, 2010). As mutation discovery and comparative genomics become more accessible, the relative contributions of dissemination and local emergence to the global MRSA population structure will gradually be resolved.

1.7.3 The evolution of community-associated methicillin-resistant *S. aureus* (MRSA) and companion animal- and livestock-associated MRSA

Although MRSA first emerged in the hospital setting, there have been increasingly frequent reports of CA-MRSA since the 1990s (Deurenberg and Stobberingh, 2008; DeLeo *et al.*, 2010). CA-MRSA tends to infect previously healthy individuals without prior healthcare exposure or other risk factors for MRSA infection and outbreaks commonly occur among individuals in the military, prisoners, athletes involved in contact sports, certain indigenous populations (Native Americans, Pacific Islanders and Aborigines), men who have sex with men, children in day-care centres and intravenous drug users (Millar *et al.*, 2007; Deurenberg and Stobberingh, 2008; DeLeo *et al.*, 2010). CA-MRSA isolates are typically distinguished by distinctive molecular characteristics: they are generally susceptible to non-β-lactam antibiotics, carry SCC*mec* types IV, V and VII, are often associated with the PVL toxin, and generally belong to genetic lineages distinct from HA-MRSA clones [4.1] (Millar *et al.*, 2007; Deurenberg and Stobberingh, 2008). These strains have gradually been introduced into hospitals and, similarly, HA-MRSA clones have been introduced into communities [4.1]. As a result, HA-MRSA and CA-MRSA can now be acquired in the hospital or community settings (Millar *et al.*, 2007; Deurenberg and Stobberingh, 2008; Elston and Barlow, 2009; Witte, 2009).

As mentioned previously in this chapter, *S. aureus* is also a major veterinary pathogen (Witte *et al.*, 2008; Ben Zakour *et al.*, 2008; Witte, 2009). Companion animals are commonly colonised or infected with MRSA, and these strains typically belong to the major human HA-MRSA lineages previously mentioned in this chapter [1.7.2], suggesting transmission within the household setting (Strommenger *et al.*, 2006b; Pantosti and Venditti, 2009; van Belkum *et al.*, 2009). Given their zoonotic potential and economic importance, there is also an increasing emphasis on the study of the evolution of livestock-associated MRSA (LA-MRSA) (Ben Zakour *et al.*, 2008). Certain LA-MRSA strains correspond to major human genotypes, such as

ST5, which has been isolated from poultry (McCarthy and Lindsay, 2010), while other genotypes appear to be restricted to certain hosts, such as the bovine lineages CC97, CC130, CC151, CC188 and CC771, or the ST398 genotype, which represents a major coloniser of pigs, but is seldom detected in humans (Witte *et al.*, 2008; Witte, 2009; Lindsay, 2010; McCarthy and Lindsay, 2010; Smyth and Robinson, 2010). In this context, host adaptation, and the effects of recent changes in agricultural practices and animal husbandry on the evolution and population structure of LA-MRSA, is of ongoing interest.

CA- and LA-MRSA strains both represent an emerging threat to public health with potential for major economic repercussions; therefore, it is important to attempt to obtain a greater understanding of the evolution of and population structure of these MRSA subgroups, as different strategic approaches may be required for their control (van Belkum *et al.*, 2009).

1.8 Study relevance, aims and objectives

As emphasised above, comprehensive local epidemiological data are prerequisite for the development of effective antibiotic prescription policies and infection control guidelines that will ultimately result in the control of MRSA within the hospital setting. In the global context, studies on *S. aureus* molecular epidemiology have provided valuable preliminary data that have served as a foundation for more extensive studies on the evolution and population structure of certain clones. It is envisioned that an improved understanding of the population structure and evolution of *S. aureus*, particularly MRSA, will alleviate the global disease burden caused by this organism.

At the onset of this study, a limited number of studies investigating the epidemiology of MRSA from Africa had been carried out, and the field was in its infancy in South Africa. To the best of the author's knowledge, the only molecular epidemiological data on MRSA from South Africa available at the time was presented at the 17th European Congress of Clinical Microbiology and Infectious Diseases in Munich, Germany, in 2007. That study included only *spa* typing and SCC*mec* typing, but no comprehensive data was available on MRSA present in South African hospitals or

communities (Oosthuysen *et al.*, 2007). The field has, however, grown during the course of the current study as evidenced by the publication of a number of reports describing aspects of the molecular epidemiology of MRSA from South Africa (Essa *et al.*, 2009; Shittu *et al.*, 2009; Makgotlho *et al.*, 2009; Moodley *et al.*, 2010), which will be discussed in detail later in this thesis. Nevertheless, there remained a paucity of data on MRSA circulating in hospitals in Cape Town, in the Western Cape province of South Africa.

1.8.1 Study aim

The primary aim of this study was to describe the epidemiology of MRSA circulating in Cape Town hospitals by performing a molecular characterisation of one hundred isolates obtained from local hospitals between January 2007 and December 2008.

1.8.2 Study objectives

- Describe the local epidemiology of MRSA from hospitals in Cape Town using PFGE, SCC*mec* typing and subtyping, and analyse the data in conjunction with the laboratory data provided by the National Health Laboratory Service.
- Describe the epidemiology of MRSA from hospitals in Cape Town within the context of global MRSA epidemiology using a combination of multilocus sequence typing, *spa* typing, and *dru* typing.

CHAPTER 2

The Epidemiology of Methicillin-Resistant *S. aureus* from Hospitals in Cape Town

2.1 Introduction

Between January 2004 and June 2010, MRSA was responsible for 42.16 % of *S. aureus* bacteremias identified by the National Health Laboratory Service (NHLS) microbiology laboratory based at Groote Schuur Hospital, which serves five public hospitals in Cape Town (A. Whitelaw, unpublished data). In comparison to previous reports from South Africa (Bell *et al.*, 2002; Bouchillon *et al.*, 2004; Zinn *et al.*, 2004; Perovic *et al.*, 2006; Shittu and Lin, 2006; Brink *et al.*, 2007) and other countries worldwide (Grundmann *et al.*, 2006), this figure suggests a relatively high prevalence of MRSA in hospitals in Cape Town; therefore, the aim of this study was to obtain a comprehensive understanding of the epidemiology of MRSA from local hospitals. One of the methods employed to achieve this was pulsed-field gel electrophoresis (PFGE). PFGE is a highly discriminatory molecular typing technique that is currently considered the gold standard for *S. aureus* epidemiological investigations, particularly for those concerned with local epidemiology (Spratt, 1999; Deurenberg and Stobberingh, 2008; Struelens *et al.*, 2009; van Belkum *et al.*, 2009).

PFGE was developed by Schwartz and Cantor (1984), and has since been adapted for many organisms, including *S. aureus* (Ichiyama *et al.*, 1991). The method entails the lysis of bacteria within an agarose plug in order to obtain intact chromosomal DNA. The DNA is digested with a restriction enzyme that cleaves the bacterial chromosome infrequently, generating between twelve and twenty high molecular weight fragments, which are separated on an agarose gel by pulsed-field gel electrophoresis (Schwartz and Cantor, 1984; Ichiyama *et al.*, 1991; Trindade *et al.*, 2003). The resulting macrorestriction profiles are visualised and the levels of similarity between isolates can be assessed manually using the Tenover criteria (Tenover *et al.*, 1995), or with a software package, such as GelCompar II by Applied Maths (Reed *et al.*, 2007).

Despite developments in DNA sequence-based typing techniques, PFGE remains the method of choice for MRSA outbreak investigations worldwide (Deurenberg and Stobberingh, 2008). Since 2005, PFGE has been used to investigate nosocomial outbreaks in neonatal and paediatric units in France (El Helali *et al.*, 2005), Italy (Bertini *et al.*, 2006) and Taiwan (Lin *et al.*, 2007). In all three cases, PFGE confirmed a nosocomial outbreak and the epidemiological data were used to inform intervention strategies. This approach has also been used in community-based epidemiological investigations, as was reported after outbreaks of an epidemic MRSA clone, known as USA300, among prisoners and professional athletes in the USA during 2002 and 2003, respectively (Kazakova *et al.*, 2005; Turabelidze *et al.*, 2006).

In addition to being widely used for outbreak investigations, PFGE has also been used to monitor the composition of MRSA clones within a particular hospital, country or region (Struelens *et al.*, 2009). Using PFGE, Trzciński *et al.* (1997) showed that two MRSA clones were dominant across four hospitals in Warsaw, Poland, between 1991 and 1994. In a similar study, Teixeira *et al.* (1995) used PFGE to show that one particular MRSA clone had become dominant across six distant Brazilian hospitals during the 1990s. PFGE also proved useful for marking changes in the composition of MRSA clones in Portugal during the 1990s when the technique was used to document the emergence of the Brazilian clone in three Portuguese hospitals (Aires-de-Sousa *et al.*, 1998). On a larger scale, PFGE was used to show that the Iberian, EMRSA-15 and EMRSA-16 clones were present throughout Europe (Murchan *et al.*, 2003).

2.2 Experimental Protocol

2.2.1 Bacterial isolates and collection data

One hundred clinical MRSA isolates, collected between January 2007 and December 2008, were included in this study. These isolates represented 13.6 % of all MRSA isolated during the collection period by the NHLS microbiology laboratory based at Groote Schuur Hospital. The isolates were collected randomly, without duplication, from patients at 5 hospitals linked to the University of Cape Town (UCT) academic complex, namely Groote Schuur Hospital (GSH, *n* = 51), Red Cross War Memorial Children's Hospital (RCCH, *n* = 21), Mowbray Maternity Hospital (MMH, *n* = 19), UCT Private Academic Hospital (UCTPH, *n* = 5) and Victoria Hospital (VH, *n* = 4). The available laboratory data for all study isolates can be found in Appendix A.

The isolates were obtained from patients in various wards, including intensive care units (*n* = 23), surgical (*n* = 23), medical (*n* = 16), obstetrics and gynaecology (*n* = 16), outpatient clinics (*n* = 15), and emergency services (*n* = 4). The clinical request form did not specify a ward for 3 of the isolates. The proportions of isolates from the included hospitals reflected the proportions of MRSA isolated from patients at each hospital. The isolates were cultured from a variety of specimens including pus and pus swabs (*n* = 64), respiratory tract specimens (*n* = 13), urine (*n* = 9), central venous catheter tips (*n* = 7) and blood (*n* = 7). These proportions of specimen types were representative of those from which all MRSA was cultured during the collection period.

2.2.2 Isolate identification and antimicrobial susceptibility testing

Isolate identification and antimicrobial susceptibility testing was carried out by the staff of the NHLS microbiology laboratory at GSH. Isolates were identified as *S. aureus* either by the VITEK 2 (BioMerieux, Marcy l'Etoile, France) or by a positive DNase test. Antimicrobial susceptibility testing was carried out by disc diffusion (Oxoid Ltd, Basingstoke, UK), E-test strips (AB Biodisk, Solna, Sweden), or on the VITEK 2 for the following antimicrobial agents: penicillin, cloxacillin, erythromycin, clindamycin, rifampicin, co-trimoxazole, ciprofloxacin, gentamicin, fusidic acid and vancomycin. The choice of method was determined by the standard operating procedure of the diagnostic laboratory. Zone sizes, E-test MICs and VITEK 2 MICs

were interpreted according to Clinical Laboratory Standards Institute guidelines (Clinical Laboratory Standards Institute, 2007; Clinical Laboratory Standards Institute, 2008).

2.2.3 Pulsed-field gel electrophoresis

The method described by Reed *et al.* (2007) was optimised for use in this study.

2.2.3.1 Isolate sub-culture

All isolates were sub-cultured from 25 % glycerol stocks. An inoculum was streaked for single colonies on a BBA plate (NHLS, Cape Town, South Africa) and incubated aerobically at 37°C overnight.

2.2.3.2 Preparation of agarose plugs

Cell suspensions were prepared by re-suspending a sweep of colonies in 2 ml of TEN Buffer (Appendix B). One millilitre of cell suspension was standardised to an OD_{600} reading that ranged between 0.825 and 0.875 using a Biomate 5 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Standardisation of the bacterial suspensions ensured that a uniform amount of DNA was obtained for each isolate, thereby increasing the accuracy of the final comparison of macrorestriction profiles.

To weaken the bacterial cell wall, 5 µl of lysostaphin (1 mg/ml) (Sigma Aldrich, Inc., St Louis, MO, USA) was added to a 250 µl aliquot of standardised cell suspension, followed by 250 µl of molten 2 % SeaKem Gold agarose (Cambrex Bio Science Rockland, Inc., Rockland, ME), which had been equilibrated at 60°C for 10 min. The mixture was then transferred into sealed plug moulds and the plugs were allowed to solidify at 4°C for 10 min. Any excess agarose was removed using a surgical blade, and the plugs were transferred to 2 ml eppendorfs in preparation for cell lysis.

2.2.3.3 Bacterial lysis

In order to obtain intact chromosomal DNA, *in situ* lysis of *S. aureus* was carried out as follows: two millilitres of EC Buffer (Appendix B), pre-warmed to 37°C, was added to cover the agarose plugs, which were incubated at 37°C for 4 h to allow digestion of the cell wall and membrane to proceed. The EC Buffer was replaced with an equal volume of ESP Buffer (Appendix B), and the disruption of the cell wall and membrane, as well as the degradation of cellular proteins, was facilitated by incubation at 55°C for 16 h.

After the 16 h incubation period, the plugs were transferred to sterile universal bottles. A volume (10 ml) of TE Buffer (Appendix B) was added and the plugs were washed on a compact rocker CR300t (FINEPCR, Gunpo-si, South Korea) for 30 min at 35 rpm. The TE buffer was decanted and the wash was repeated 4 times with fresh TE buffer. After the fourth wash, the protocol was either stopped by storing the plugs in TE Buffer at 4°C, or was continued by proceeding with the restriction enzyme digest.

2.2.3.4. SmaI *restriction enzyme digest*

The intact chromosomal DNA obtained after bacterial lysis was digested with the restriction enzyme *Sma*I, which cleaves the *S. aureus* chromosome infrequently, producing between 12 and 20 fragments, which is ideal for comparison (Ichyama *et al.*, 1991; Reed *et al.*, 2007).

A section (width, 5mm; height, 2mm; depth, 1mm) was trimmed from each agarose plug in preparation for the restriction enzyme digest. The chromosomal DNA was digested with 30U *Sma*I (Roche Diagnostics GmbH, Mannheim, Germany) in a final volume of 125 µl, with 1 µl BSA (Roche Diagnostics GmbH, Mannheim, Germany), using the buffer supplied by the manufacturer at a 1X final concentration. The digest was allowed to proceed at room temperature for 3 h.

2.2.3.5 Preparation of the agarose gel and electrophoresis buffer

A volume (150 ml) of 1 % pulsed-field certified ultra pure DNA grade agarose (BioRad Laboratories, Inc, Hercules, CA, USA) was prepared with 0.5X TBE buffer (Appendix B) and equilibrated at 60°C for 10 min. The agarose was poured into the gel mould and allowed to set at room temperature for 2.5 h. Two and a half litres of 0.5X TBE buffer was poured into the chamber of the electrophoresis apparatus. The cooling unit was set to 14°C and the pump was turned on to allow the buffer to circulate and cool for 2 h prior to electrophoresis.

2.2.3.6 Sample loading

The first and last 3 lanes of each gel were not used due to poor migration in these regions. The Lambda Ladder PFG Marker (New England BioLabs, Inc, Ipswich, MA, USA) (Appendix C) was loaded into the first and last wells used in each gel. The *S. aureus* strain NCTC8325, used as a PFGE reference strain, was loaded in every fifth lane. The plugs were inserted against the front of the wells using a sealed Pasteur pipette. The wells were sealed using excess molten 2 % SeaKem Gold plug agarose and, once the agarose had set, the gel platform was placed into the electrophoresis chamber.

2.2.3.7 Pulsed-field gel electrophoresis

PFGE was used to separate the DNA fragments obtained after *Sma*I digestion. In PFGE, the direction of the electrical field is changed or "switched" periodically to facilitate size-dependent migration of the large DNA fragments. In this study, electrophoresis was carried out using a CHEF-DRII GeneNavigator (Amersham Biosciences, Fairfield, CT, USA), with an initial switch time of 5 s, and a final switch time of 60 s. The duration of the run was 22 h at 200V with the circulating buffer at a constant temperature of 14°C.

2.2.3.8 Visualisation of macrorestriction profiles

After electrophoresis, the gel was transferred to a plastic container to which 500 ml of 0.5X TBE buffer was added. Ethidium bromide (Fluka, Sigma Aldrich, Inc., St Louis, MO, USA) was added to a final concentration of 1 µg/ml and the gel was placed on a

compact rocker CR300t (FINEPCR, Gunpo-si, South Korea) set at 15rpm for 30 min. The gel was destained in 400 ml of distilled water for 20 min. The macrorestriction profiles were visualised using UV transillumination and photographed with a UVIpro Silver (UVItec Ltd, Cambridge, UK) or a G:BOX (Syngene, Frederick, MD, USA), and the digital image was converted to TIFF file format for use in the computer-assisted comparison of macrorestriction profiles.

2.2.3.9 Analysis of macrorestriction profiles

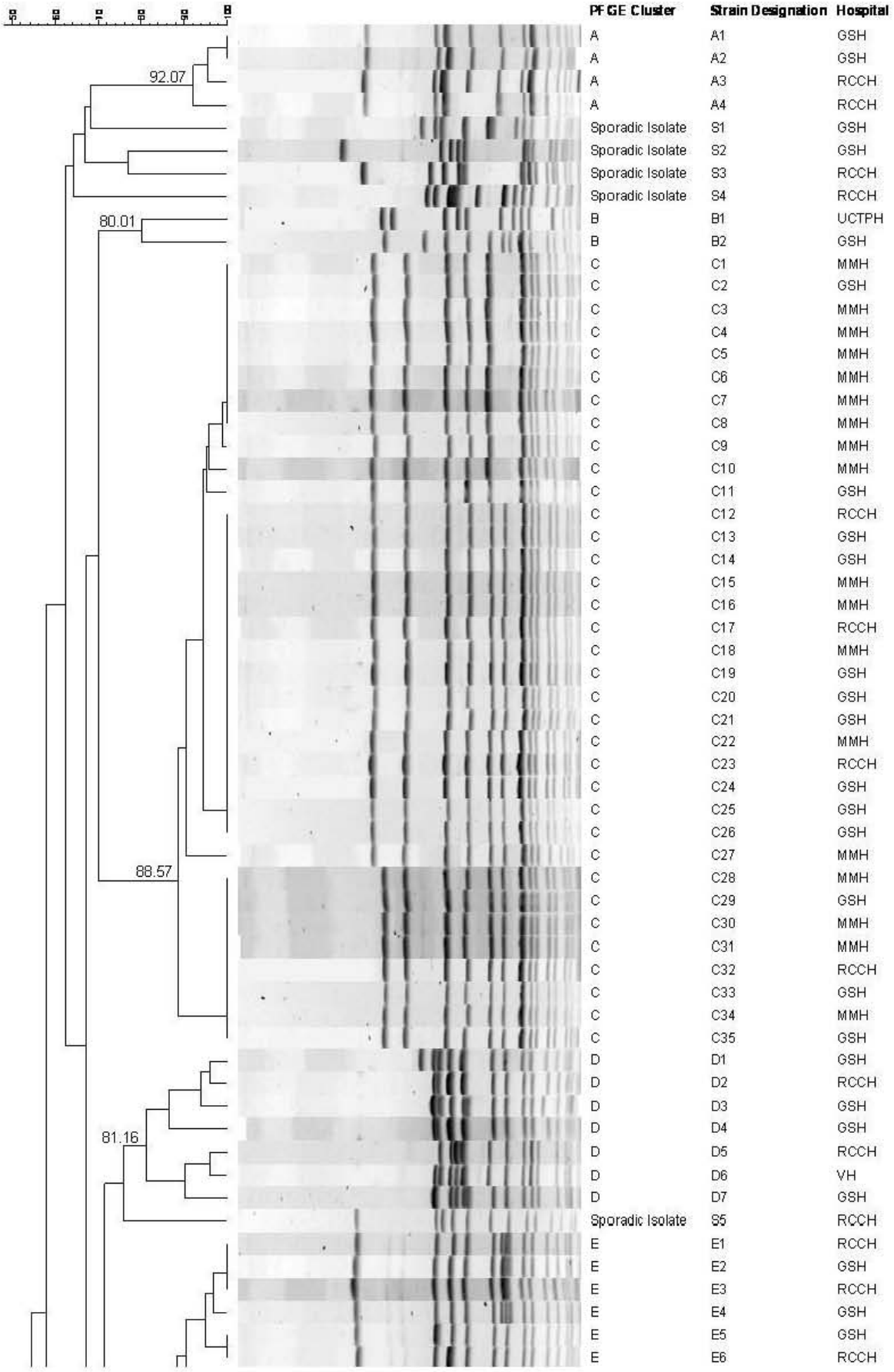
The digital images were analysed using GelCompar II (version 4.6) by Applied Maths (BVBA, Sint-Martens-Latem, Belgium). The guidelines supplied by Applied Maths and the method described by Reed *et al.* (2007) were followed. Once the image had been cropped and converted to greyscale, each lane was defined manually, and background noise was reduced by carrying out a background subtraction. The NCTC8325 control strains loaded in every fifth lane were then used to normalise the gel to correct for any inconsistencies in migration. The molecular weights of the *Sma*I restriction fragments for NCTC8325 are known and the first 11 fragments were used for normalisation in this study (Appendix D). Distortion bars were used to confirm the accuracy of the normalisation as described in the GelCompar II manual. After normalisation, bands were assigned using the automatic search function and were then checked manually. Only DNA fragments within the normalised region of the gel, that is between the first (674kb) and eleventh (76kb) NCTC8325 *Sma*I fragments, were included in the analysis (Murchan *et al.*, 2003). Processing was completed by linking the macrorestriction profiles to the database.

Once all gel images had been processed, a composite comparison of macrorestriction profiles obtained for the 100 MRSA isolates was carried out. The NCTC8325 strains were used as global references to enable inter-gel comparisons. A dendrogram indicating the levels of similarity between the isolates was created with the unweighted pair-group method using arithmetic averages (UPGMA) using the Dice similarity coefficient. The optimisation and band tolerance settings were at 0.5 % and 1 %, respectively (Reed *et al.*, 2007). Due to the highly clonal nature of *S. aureus*, a higher similarity threshold is used to define clusters compared to other bacteria. A similarity threshold of 80 % was used to define PFGE clusters in this study (Murchan *et al.*, 2003).

2.3 Results

2.3.1 Comparison of PFGE macrorestriction profiles

Using the 80 % similarity threshold, the 100 MRSA isolates were separated into 6 PFGE clusters (A – F), and 8 sporadic isolates, which were designated S1 – S8 (Figure 2.1). Clusters C (*n* = 35) and E (*n* = 33) were the largest clusters identified, and collectively accounted for 68 % of the isolates. The outstanding isolates assigned to PFGE clusters were distributed across clusters A (*n* = 4), B (*n* = 2), D (*n* = 7) and F (*n* = 11).



PFGE Cluster	Strain Designation	Hospital
A	A1	GSH
A	A2	GSH
A	A3	RCCH
A	A4	RCCH
Sporadic Isolate	S1	GSH
Sporadic Isolate	S2	GSH
Sporadic Isolate	S3	RCCH
Sporadic Isolate	S4	RCCH
B	B1	UCTPH
B	B2	GSH
C	C1	MMH
C	C2	GSH
C	C3	MMH
C	C4	MMH
C	C5	MMH
C	C6	MMH
C	C7	MMH
C	C8	MMH
C	C9	MMH
C	C10	MMH
C	C11	GSH
C	C12	RCCH
C	C13	GSH
C	C14	GSH
C	C15	MMH
C	C16	MMH
C	C17	RCCH
C	C18	MMH
C	C19	GSH
C	C20	GSH
C	C21	GSH
C	C22	MMH
C	C23	RCCH
C	C24	GSH
C	C25	GSH
C	C26	GSH
C	C27	MMH
C	C28	MMH
C	C29	GSH
C	C30	MMH
C	C31	MMH
C	C32	RCCH
C	C33	GSH
C	C34	MMH
C	C35	GSH
D	D1	GSH
D	D2	RCCH
D	D3	GSH
D	D4	GSH
D	D5	RCCH
D	D6	VH
D	D7	GSH
Sporadic Isolate	S5	RCCH
E	E1	RCCH
E	E2	GSH
E	E3	RCCH
E	E4	GSH
E	E5	GSH
E	E6	RCCH

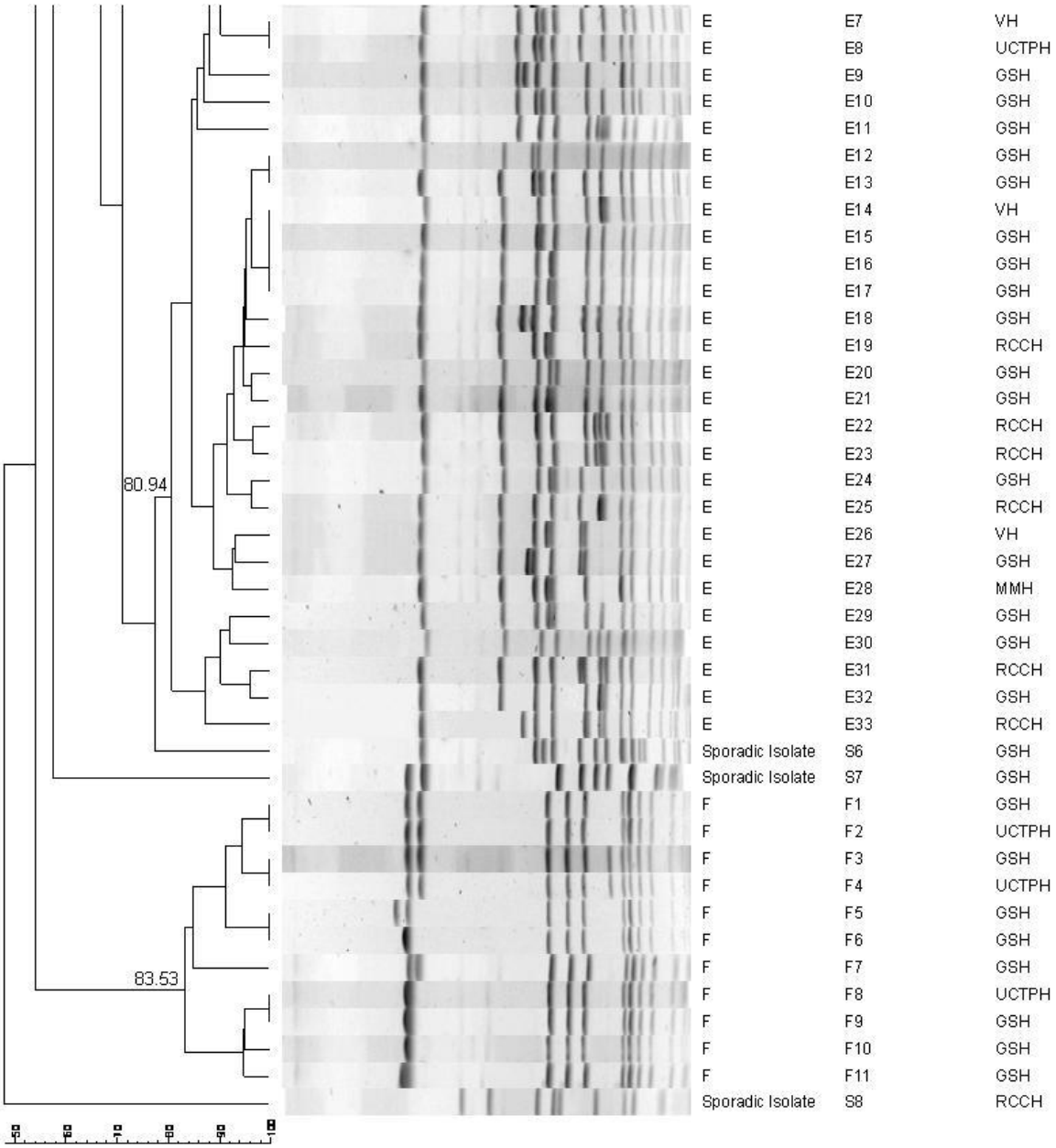


Figure 2.1. Pulsed-field gel electrophoresis macrorestriction profiles obtained for 100 methicillin-resistant *S. aureus* from 5 hospitals in Cape Town, and the dendrogram showing the levels of similarity between the isolates. The PFGE data were analysed using GelCompar II version 4.6 (Applied Maths), and the dendrogram was created using the unweighted pair-group method using arithmetic averages (UPGMA) with the Dice similarity coefficient. Scale bars above and below the dendrogram indicate the levels of similarity (%) between isolates. Using a similarity threshold of 80 %, six clusters (A – F), and 8 sporadic isolates, were identified. Cluster names are indicated adjacent to the macrorestriction profiles, and similarity levels (%) are shown on the dendrogram adjacent to the relevant nodes. The second column indicates strain designations used throughout this thesis. The hospitals of origin are shown in the final column (GSH, Groote Schuur Hospital; RCCH, Red Cross War Memorial Children's Hospital; MMH, Mowbray Maternity Hospital; UCTPH, University of Cape Town Private Academic Hospital; VH, Victoria Hospital).

The PFGE results were analysed in conjunction with the available laboratory data, including hospital and ward of origin, as well as date of isolation. The laboratory and experimental data for individual isolates are available in Appendix A. When the isolates were stratified according to hospital of origin, clusters A, B and F included isolates from 2 hospitals, clusters C and D contained isolates from 3 hospitals, and cluster E included isolates from all 5 hospitals, as summarised in Table 2.1.

Table 2.1 Stratification of pulsed-field gel electrophoresis clusters by hospital of origin [a]

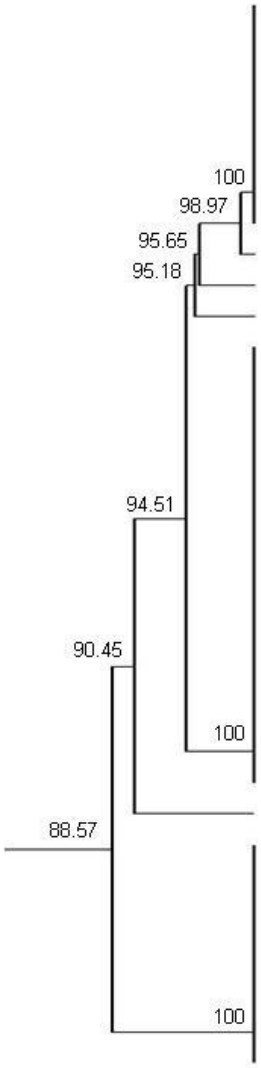
PFGE cluster (no. isolates)	Distribution of strains across hospitals (no. isolates (%))				
	GSH	RCCH	MMH	UCTPH	VH
A (4)	2 (50.00)	2 (50.00)	-	-	-
B (2)	1 (50.00)	-	-	1 (50.00)	-
C (35)	13 (37.14)	4 (11.43)	18 (51.43)	-	-
D (7)	4 (57.14)	2 (28.57)	-	-	1 (14.29)
E (33)	19 (57.58)	9 (27.27)	1 (3.03)	1 (3.03)	3 (9.09)
F (11)	8 (72.73)	-	-	3 (27.27)	-

[a] GSH, Groote Schuur Hospital; RCCH, Red Cross War Memorial Children's Hospital; MMH, Mowbray Maternity Hospital; UCTPH, University of Cape Town Private Academic Hospital ; VH, Victoria Hospital

Since sample sizes from individual wards were small, it was difficult to detect clustering by ward of origin. There was generally no clustering according to date of isolation; however, there were a few minor exceptions, and these isolates are indicated in Appendix A. A noteworthy exception in both of the previously described analyses was cluster C.

Scrutiny of cluster C (35 isolates) detected several groups of highly similar MRSA with similarity levels ranging from 88.57 to 100 % (Figure 2.2). These MRSA isolates were obtained within short periods of time from patients in gynaecology and obstetrics wards and neonatal units at MMH and GSH (Figures 2.2). Significantly, 18 out of 19 MRSA from MMH were included in cluster C, with the remaining isolate assigned to cluster E (Figure 2.1; Appendix A). While isolates from MMH comprised the majority of MRSA from cluster C (*n* = 18, 51.43 %), an additional 7 (20.00 %) of the remaining isolates from this cluster were from patients in the maternity services

and neonatal units at GSH. Therefore 71.43 % of isolates from cluster C was obtained from patients in the maternity services at MMH and GSH. Additionally, 3 groups of indistinguishable isolates were identified in cluster C, each including several subgroups of MRSA that appeared to cluster according to dates of isolation (Figure 2.2).



MRSA Isolate	Hospital	Ward	Date of Isolation
C1	MMH	Gynaecology and Obstetrics	17/06/2007
C2	GSH	Gynaecology and Obstetrics	23/06/2007
C3	MMH	Gynaecology and Obstetrics	26/06/2007
C4	MMH	ICU (Neonatal)	07/10/2007
C5	MMH	Gynaecology and Obstetrics	14/12/2007
C6	MMH	Gynaecology and Obstetrics	13/04/2008
C7	MMH	Gynaecology and Obstetrics	26/04/2008
C8	MMH	ICU (Neonatal)	16/07/2008
C9	MMH	Gynaecology and Obstetrics	05/10/2007
C10	MMH	Gynaecology and Obstetrics	22/04/2008
C11	GSH	Gynaecology and Obstetrics	11/06/2008
C12	RCCH	Not Specified	15/02/2007
C13	GSH	Gynaecology and Obstetrics	27/02/2007
C14	GSH	ICU (Neonatal)	28/02/2007
C15	MMH	Gynaecology and Obstetrics	12/06/2007
C16	MMH	Gynaecology and Obstetrics	12/06/2007
C17	RCCH	Outpatient Clinic	18/06/2007
C18	MMH	Gynaecology and Obstetrics	07/09/2007
C19	GSH	ICU (Neonatal)	12/12/2007
C20	GSH	ICU (Neonatal)	12/12/2007
C21	GSH	Surgical	22/01/2008
C22	MMH	ICU (Neonatal)	16/04/2008
C23	RCCH	Surgical	04/06/2008
C24	GSH	Surgical	26/06/2008
C25	GSH	Surgical	24/07/2008
C26	GSH	Surgical	28/07/2008
C27	MMH	ICU (Neonatal)	26/02/2007
C28	MMH	Gynaecology and Obstetrics	30/05/2008
C29	GSH	Emergency Services	11/06/2008
C30	MMH	Outpatient Clinic	17/06/2008
C31	MMH	Gynaecology and Obstetrics	20/06/2008
C32	RCCH	Surgical	27/09/2008
C33	GSH	Outpatient Clinic	28/09/2008
C34	MMH	ICU (Neonatal)	28/09/2008
C35	GSH	Gynaecology and Obstetrics	14/12/2008

Figure 2.2 Clustering of methicillin-resistant *S. aureus* isolates from the maternity and neonatal services within pulsed-field gel electrophoresis cluster C. An enlarged portion of the PFGE dendrogram showing only cluster C is shown on the left, with similarity levels (%) indicated at the nodes. Strain designations used in the text are indicated adjacent to the dendrogram, and isolates obtained from patients in the maternity and neonatal services at MMH and GSH are highlighted in grey. The hospital and ward of origin, as well as the date of isolation, is shown for each isolate. In the column indicating dates of isolation, groups of MRSA isolates obtained within 3 weeks of each other are demarcated with a black border, and highlighted according to year of isolation (blue, 2007; orange, 2008).

2.3.2 Comparison of antimicrobial susceptibility profiles across PFGE clusters

Antimicrobial susceptibility profiles were examined within and across PFGE clusters. By definition, all of the study isolates were resistant to the β-lactam antibiotics tested (penicillin and cloxacillin); all isolates were also susceptible to vancomycin. The antimicrobial susceptibility profiles of isolates within the same PFGE cluster were highly similar, if not identical, as summarised in Table 2.2, and indicated in full in Appendix A.

Table 2.2 Antimicrobial resistance profiles across pulsed-field gel electrophoresis clusters A – F

PFGE cluster (no. isolates)	Antimicrobial agents [a] (no. isolates (%) resistant)									
	PEN	OXA	ERY	CLI	RIF	SXT	CIP	GEN	FUS	VAN
A (4)	4 (100)	4 (100)	4 (100)	4 (100)	0	4 (100)	4 (100)	4 (100)	0	0
B (2)	2 (100)	2 (100)	2 (100)	2 (100)	0	0	1 (50.00)	1 (50.00)	0	0
C (35)	35 (100)	35 (100)	32 (91.43)	32 (91.43)	1 (2.86)	1 (2.86)	0	10 (28.57)	0	0
D (7)	7 (100)	7 (100)	5 (71.43)	5 (71.43)	7 (100)	7 (100)	1 (14.29)	5 (71.43)	0	0
E (33)	33 (100)	33 (100)	13 (39.39)	11 (33.33)	33 (100)	31 (93.94)	28 (84.85)	31 (93.94)	1[b] (3.03)	0
F (11)	11 (100)	11 (100)	11 (100)	11 (100)	0	0	10 (90.91)	3 (27.27)	0	0

[a] PEN, penicillin; OXA, cloxacillin; ERY, erythromycin; CLI, clindamycin; RIF, rifampicin; SXT, co-trimoxazole; CIP, ciprofloxacin; GEN, gentamicin; FUS, fusidic acid; VAN, vancomycin.

[b] Intermediate level of resistance to fusidic acid.

The predominant PFGE clusters, C and E, had notably disparate antimicrobial susceptibility profiles. Isolates from cluster C were typically susceptible to rifampicin, co-trimoxazole, ciprofloxacin, gentamicin and fusidic acid, but a large proportion (91.43 %) was resistant to erythromycin and clindamycin (Table 2.2). In contrast, isolates from cluster E were typically resistant to several classes of antimicrobial agents with 32 out of 33 (96.97 %) isolates classified as multidrug-resistant (resistant to 3 or more classes of antimicrobial agents in addition to the β-lactam antibiotics). These isolates were universally resistant to rifampicin and almost universally resistant to co-trimoxazole (*n* = 31, 93.94 %) with a large proportion also resistant to the other antimicrobial agents, with the exception of erythromycin and clindamycin (Table 2.2). It was noted that all MRSA isolates from cluster D had similar multidrug-resistant profiles. These isolates were also resistant to rifampicin and co-trimoxazole, but a smaller proportion was resistant to ciprofloxacin compared to isolates from cluster E (Table 2.2).

Four sporadic isolates (S1, S4, S5 and S6) were also multidrug-resistant with antimicrobial susceptibility profiles similar to those observed for isolates from clusters D and E (Table 2.3). This contrasted sharply with the antimicrobial susceptibility profile of isolate S8 that was pan-susceptible to the non-β-lactam antimicrobial agents (Table 2.3), and was also defined as the PFGE outlier as it shared the lowest similarity level with all other study isolates (54.12 %) (Figure 2.1).

Table 2.3 Antimicrobial resistance profiles of sporadic isolates identified by pulsed-field gel electrophoresis

Sporadic isolate [a]	Antimicrobial resistance profile [b]
S1	PEN, OXA, RIF, SXT, CIP, GEN
S2	PEN, OXA, ERY, CLI
S3	PEN, OXA, ERY, CLI, CIP, GEN
S4	PEN, OXA, ERY, CLI, RIF, SXT, CIP, GEN
S5	PEN, OXA, RIF, SXT, GEN
S6	PEN, OXA, RIF, SXT, GEN
S7	PEN, OXA, ERY, CLI, CIP, FUS [c]
S8	PEN, OXA

[a] Strain designations as used in the text and indicated in Figure 2.1.
[b] PEN, penicillin; OXA, cloxacillin; ERY, erythromycin; CLI, clindamycin; RIF, rifampicin; SXT, co-trimoxazole; CIP, ciprofloxacin; GEN, gentamicin; FUS, fusidic acid.
[c] Intermediate level of resistance to fusidic acid.

2.4 Discussion

Six clusters of closely related MRSA and eight sporadic isolates were identified among the hundred isolates obtained from hospitals in Cape Town. The two dominant clusters, C and E, contained thirty-five and thirty-three isolates, respectively. Similar levels of homogeneity have recently been reported in other studies that have used PFGE to investigate the molecular epidemiology of MRSA from South Africa. Shittu *et al.* (2009) showed that sixty-one MRSA obtained from thirteen healthcare institutions between 2001 and 2003 in the KwaZulu-Natal province were assigned to seven PFGE clusters, with the predominant cluster containing 62.3 % of the isolates. More recently, Moodley *et al.* (2010) reported that 82 % of three hundred and twenty MRSA isolates, obtained from state and private diagnostic microbiology laboratories throughout South Africa between 2005 and 2006, were distributed across five major PFGE clusters. On the other hand, far greater levels of genetic diversity among nosocomial MRSA have also been reported. For instance, twenty-three distinct clusters were identified among fifty-four MRSA isolates from a teaching hospital in Portugal (Couto *et al.*, 1995).

Stratification of PFGE clusters according to hospital showed that all clusters contained isolates from at least two hospitals, suggesting transmission of MRSA between hospitals in Cape Town during the collection period. Previous studies have shown that staff and patient transfer plays a major role in the dissemination of MRSA between healthcare institutions (Murchan *et al.*, 2004; Henderson, 2006; Lin *et al.*, 2007). A noteworthy example was the transmission of the EMRSA-16 clone across the United Kingdom (UK) during the 1990s. It was reported that the transfer of infected patients and colonised staff led to the dissemination of EMRSA-16 from Northamptonshire, England, initially to neighbouring counties, and then to further regions of the UK (Murchan *et al.*, 2004). It has also been shown that overcrowding and understaffing in hospitals leads to decreased infection control compliance, as well as increased movement of staff and patients between wards, resulting in increased transmission of MRSA (Bertini *et al.*, 2006; Clements *et al.*, 2008). In Cape Town, it is common practice for healthcare workers to rotate through multiple hospitals within the UCT academic complex, which represents one possible route of transmission of MRSA within and between local hospitals. Healthcare workers also often provide after-hours cover at different hospitals. Additionally, patients are

frequently transferred between the included hospitals, representing a second possible mode of dissemination of MRSA between hospitals in Cape Town.

Although it was not possible to detect any trends in clustering by ward of origin, the fact that closely related isolates within each cluster were obtained from a large number of wards and floors in each hospital was of concern. This pattern of dissemination may have been due to a combination of environment-to-person or person-to-person transmission of MRSA, and may be indicative of poor adherence to infection control guidelines. Previous studies showed that MRSA isolated from healthcare workers and their patients belonged to the same PFGE cluster, confirming that MRSA can be transmitted from patient to healthcare worker and *vice versa* (El Helali *et al.*, 2005; Bertini *et al.*, 2006; Lin *et al.*, 2007). Many hospitals in the USA, Canada, Europe and New Zealand screen asymptomatic healthcare workers and high-risk patients for MRSA (Herwaldt, 1999; Gavaldà *et al.*, 2006; Humphreys, 2007; Albrich and Harbarth, 2008). Colonised healthcare workers are typically treated with antibiotics and, in some cases, are restricted to MRSA-dedicated wards, or are removed from duty and are only permitted to return once the bacteria have been eradicated. Likewise, colonised patients are generally treated with antibiotics, or cohorted in MRSA-dedicated wards, or depending on local policies, both interventions may be utilised (Albrich and Harbarth, 2008; Struelens *et al.*, 2009). These policies are not implemented in the included hospitals, primarily due to resource constraints. While it is likely that poor adherence to infection control guidelines contributed to the dissemination of MRSA observed in this study, it is also possible that colonisation of healthcare workers and patients may have played a role in the transmission of these isolates.

Based on the findings of this study, infection control practices in all five hospitals are of concern, but dissemination of MRSA within the maternity and neonatal services is particularly worrying. As shown in PFGE cluster C (Figure 2.2), several groups of highly similar, if not indistinguishable, MRSA were identified, including isolates obtained within short time periods from patients in the maternity and neonatal services at GSH and MMH. The remaining isolates from cluster C were obtained from patients at RCCH. Staff and patient movements between the three hospitals are common, representing likely modes of transmission of MRSA within the maternity and neonatal services. The clustering of isolates within cluster C strongly suggested

poor adherence to infection control guidelines, which merits further investigation and surveillance. A study is currently underway to investigate the association of cluster C isolates with maternity and neonatal services.

A comparison of antimicrobial susceptibility profiles across the six PFGE clusters and eight sporadic isolates indicated that, with the exception of the PFGE outlier, S8, the isolates tended to be resistant to β- and non-β-lactam antibiotics. These antimicrobial susceptibility profiles are typical of HA-MRSA clones which, historically, have been resistant to non-β-lactam antibiotics (Deurenberg and Stobberingh, 2008; Ratnaraja and Hawkey, 2008). There were marked similarities in antimicrobial susceptibility profiles among isolates from the same PFGE cluster. This is not always the case, as illustrated by Laplana *et al.* (2007) who identified differences in antimicrobial susceptibility profiles among isolates from the same PFGE cluster.

Multidrug-resistant MRSA is currently problematic worldwide (Levy and Marshall, 2004) and South Africa is no exception, as shown by Marais *et al.* (2009) who found that 81.5 % of MRSA obtained from patients at public and private healthcare institutions throughout South Africa were multidrug-resistant. Nevertheless, it was cause for concern that multidrug-resistant isolates were identified in all six PFGE clusters. It was particularly worrying that 96.97 and 100 % of isolates from clusters D and E were multidrug-resistant, and were observed in all five local hospitals (Appendix A). Isolates from both of these clusters were universally resistant to rifampicin, and all but two were resistant to co-trimoxazole; however, resistance to these particular antimicrobial agents was largely absent among the remaining isolates (Appendix A).

High proportions of MRSA resistant to rifampicin and co-trimoxazole have also been reported in studies carried out in the KwaZulu-Natal and Gauteng provinces (Shittu *et al.*, 2009; Groome *et al.*, 2009). In this context, rifampicin is commonly prescribed in South Africa for the treatment of *Mycobacterium tuberculosis* infections (South African Department of Health, 2004), while co-trimoxazole is prescribed for the prophylaxis or treatment of *Pneumocystis* infections in HIV-positive persons (South African Department of Health, 2010). A study carried out in Japan suggested that the use of rifampicin for the treatment of tuberculosis selected for MRSA resistant to this

antimicrobial agent (Sekiguchi *et al.*, 2006). Further, it has been suggested that the use of co-trimoxazole in HIV-positive persons selects for co-trimoxazole-resistant, and multidrug-resistant, *S. aureus* strains (Martin *et al.*, 1999). It is quite possible that frequent use of these antibiotics in hospitals in Cape Town and other regions in South Africa has selected for rifampicin- and co-trimoxazole-resistant MRSA, which are best fitted to the local environment.

In summary, PFGE provided data regarding the epidemiology of MRSA from hospitals in Cape Town. These data suggested the transmission of multidrug-resistant MRSA within and between local hospitals. This approach also provided an overview of the relatedness of the isolates, which served as a starting point for the complete molecular characterisation of the MRSA, as described in subsequent chapters of this thesis.

CHAPTER 3

SCC*mec* Content of Local Methicillin-Resistant *S. aureus*: The Devil is in the Detail

3.1 Introduction

Characterisation of the staphylococcal cassette chromosome *mec* (SCC*mec*) content of MRSA isolates is essential for a complete description of their molecular epidemiology (Enright *et al.*, 2002; Cookson *et al.*, 2007). The role of SCC*mec* in methicillin resistance, common structural components of the known SCC*mec* types, and the site-specific integration and excision of the element were described in Chapter 1 [1.5.3]. The focus of this chapter will be the classification of SCC*mec* types, and the strategies used to differentiate these elements. Additionally, details regarding the global distribution and structural components of individual SCC*mec* types will be provided here.

In December 2009, the IWG-SCC published a comprehensive set of guidelines regarding the classification of SCC*mec* elements (Ito *et al.*, 2009). In accordance with the IWG-SCC guidelines, SCC*mec* types are defined by the combination of *mec* class and *ccr* allotype present, while subtypes are defined by differences in their respective J regions, as will be discussed later in this chapter. The IWG-SCC also recommended that the existing SCC*mec* nomenclature be revised to make the system more informative. It was suggested that the Roman numerals should be retained, but *ccr* allotype (currently 1 – 5, 7 and 8 in SCC*mec* elements in *S. aureus*) and *mec* class (currently A, B, C1, C2 and E in SCC*mec* elements in *S. aureus*) should also be indicated, in that order, in parentheses (Ito *et al.*, 2009; http://www.sccmec.org/Pages/SCC_TypesEN.html). The revised names for SCC*mec* types I – XI are, therefore, I (1B), II (2A), III (3A), IV (2B), V (5C2), VI (4B), VII (5C1), VIII (4A), IX (1C2), X (7C1) and XI (8E), respectively. The only information currently available on the most recently reported SCC*mec* types IX – XI is the combination of *mec* and *ccr* complexes that they contain (http://www.sccmec.org/Pages/SCC_TypesEN.html); therefore only SCC*mec* types I – VIII will be discussed in detail in this chapter. Additionally, the revised

nomenclature is not yet commonly used and, for ease of reading, only Roman numerals will be used in the remainder of this thesis.

Although, with the exception of SCC*mec* type VII, the genetic organisation of SCC*mec* types I – VIII is identical [1.5.3], there are numerous differences in the underlying sequences of the basic structural components present in these elements, as shown in Figure 3.1 (Ito *et al.*, 2009). Additionally, differences in the epidemiology of SCC*mec* types I – VIII have also been described. SCC*mec* type I (34.3kb) was isolated from the first MRSA strain reported in England in 1961, and is defined by the presence of a class B *mec* complex and type 1 *ccr* allotype (Figure 3.1) (Ito *et al.*, 2001). In general, SCC*mec* type I does not carry additional antibiotic resistance genes within its J regions; therefore this mobile genetic element typically does not confer resistance to antimicrobial agents besides the β-lactam antibiotics (Ito *et al.*, 2001; Ito *et al.*, 2003; Ito *et al.*, 2009)

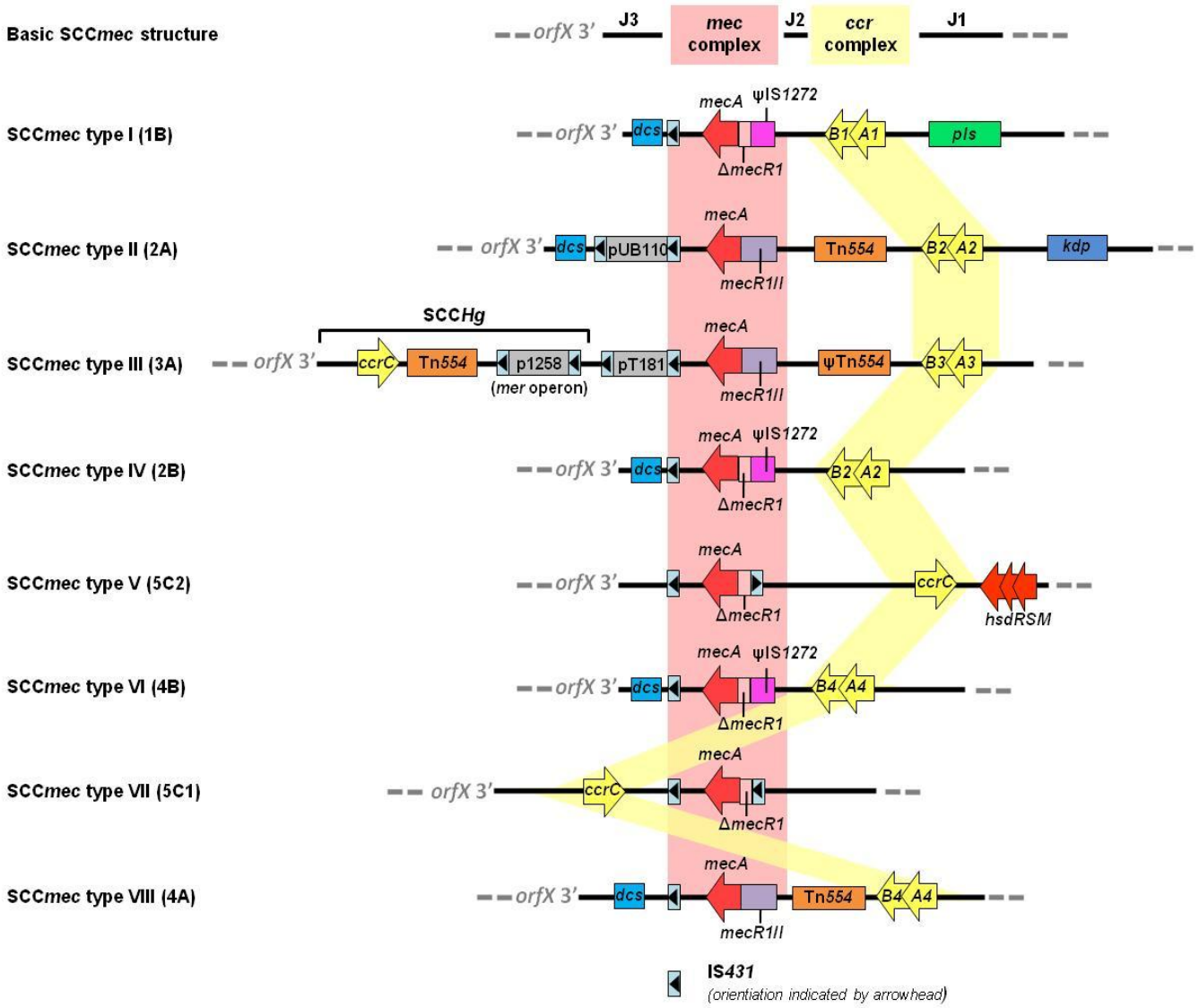


Figure 3.1 Schematic representation of SCC*mec* types I – VIII. The basic structure of SCC*mec* is shown at the top. Regions corresponding to the *mec* and *ccr* complexes are highlighted in pink and yellow, respectively. J1, J2, and J3 indicate the joining regions. The *S. aureus* chromosome is shown in grey. The revised nomenclature for SCC*mec* types I – VIII is shown on the left (*ccr* allotype and class of *mec* complex indicated, in order, in parentheses). Arrowheads indicate orientation. IS, insertion sequence; p, linearised plasmid; Tn, transposon; SCC*Hg*, staphylococcal cassette chromosome mercury. Adapted from Chen *et al.* (2009) and Ito *et al.* (2009).

On the other hand, SCC*mec* types II and III, which both carry the class A *mec* complex but contain *ccr* allotypes 2 and 3, respectively, commonly carry additional resistance determinants within their J regions (Figure 3.1) (Ito *et al.*, 2001; Ito *et al.*, 2003; Ito *et al.*, 2009). In SCC*mec* type II (53kb), these regions contain plasmid pUB110, which encodes resistance to several aminoglycosides, including tobramycin, kanamycin and bleomycin, as well as a copy of transposon Tn*554*, which confers resistance to the macrolides, lincosamides and streptogramins (Ito *et al.*, 2003). The genetic organisation of SCC*mec* type III (66.9kb) is more complex in that it is a composite element consisting of two smaller SCC elements: SCC*Hg* (formerly SCC*mercury*), and the SCC*mec* III element, which contains a class A *mec* complex and *ccr* allotype 3 (Figure 3.1) (Ito *et al.*, 2009). The SCC*Hg* component carries Tn*554*, as well as p1258, which contains the *mer* operon, conferring resistance to mercury (Ito *et al.*, 2003). The SCC*mec* III component of the composite element includes ψTn*554*, which confers cadmium resistance, and pT181, which encodes resistance to tetracycline (Ito *et al.*, 2001; Ito *et al.*, 2003). SCC*mec* types II and III were first described in strains isolated in Japan and New Zealand, respectively (Ito *et al.*, 2001), and, as with SCC*mec* type I, have since been described frequently worldwide, particularly in association with HA-MRSA isolates (Deurenberg and Stobberingh, 2008; Ratnaraja and Hawkey, 2008).

SCC*mec* type IV is defined by the presence of the class B *mec* complex and *ccr* allotype 2 (Figure 3.1) (Ma *et al.*, 2002). This element typically does not include additional antibiotic resistance determinants (Ma *et al.*, 2002; Deurenberg and Stobbering, 2008). Ranging from 20.9kb to 24.3kb, SCC*mec* type IV is one of the smallest SCC*mec* elements, which may explain why it is the most frequently transferred SCC*mec* type and is prevalent worldwide (Ma *et al.*, 2002; Okuma *et al.*, 2002; Robinson and Enright, 2003; Deurenberg and Stobberingh, 2008). SCC*mec* type IV was first described in CA-MRSA (Daum *et al.*, 2002) and has been commonly associated with these lineages, although there have also been reports of HA-MRSA strains carrying this element (de Lencastre *et al.*, 2007; Ratnaraja and Hawkey, 2008). A number of variants of SCC*mec* types I – III have been described based on differences in the J regions of elements carrying the same *mec*-*ccr* combination (Deurenberg and Stobberingh *et al.*, 2008; Ito *et al.*, 2009); however, SCC*mec* type IV has been shown to be most variable with ten subtypes (IVa – d, IVE, IVF and IVg – IVj) described to date (Ma *et al.*, 2002; Ito *et al.*, 2003; Kwon *et al.*, 2005; Shore

et al., 2005; de Lencastre *et al.*, 2007; Berglund *et al.*, 2009). It is thought the frequent horizontal transfer of SCC*mec* type IV, and the genetic background of the MRSA lineages carrying this element, have contributed to the highly polymorphic nature of this SCC*mec* type (Ma *et al.*, 2002; Okuma *et al.*, 2002; Robinson and Enright, 2003; Jansen *et al.*, 2006).

The current nomenclature systems used for describing SCC*mec* subtypes are relatively unwieldy: lowercase letters denote differences in the J1 region (eg. IVa), uppercase letters indicate the presence or absence of mobile genetic elements (eg. IA), and Arabic numerals denote differences in each J region (eg. II.1.1.1, II.1.1.2 and II.2.1.1). Unsurprisingly, there has been a move to simplify and improve subtype nomenclature through the creation of a website dedicated to identifying molecular markers within the J regions (http://www.SCCmec.org), which will be used to differentiate subtypes and assign subtype numbers in a more informative manner (Ito *et al.*, 2009).

SCC*mec* types V and VI are two of the smaller SCC*mec* elements at 28kb (Ito *et al.*, 2004) and 20.9kb (Oliveira *et al.*, 2006), respectively, and also typically only confer resistance to β-lactam antibiotics (Figure 3.1) (Deurenberg and Stobberingh, 2008). SCC*mec* type V (class C2 *mec* complex, *ccr* allotype 5) has commonly been reported in CA-MRSA strains (Ito *et al.*, 2004; de Lencastre *et al.*, 2007; Deurenberg and Stobberingh, 2008), while the distribution of SCC*mec* type VI (class B *mec* complex, *ccr* allotype 4) appears more limited with the element reported in continental Portugal and its island territories, and also in France (Oliveria *et al.*, 2006; Dauwalder *et al.*, 2008; Conceiçao *et al.*, 2010).

SCC*mec* types VII and VIII represent the most recently reported SCCmec elements that have been described in full in the literature (Berglund *et al.*, 2008; Zhang *et al.*, 2009; Ito *et al.*, 2009). SCC*mec* type VII (35.9kb) is defined by the presence of a class C1 *mec* complex and *ccr* allotype 5, and deviates from the common structural organisation observed for all other SCC*mec* types as the *ccr* complex precedes the *mec* complex (Figure 3.1). This element only confers resistance to β-lactam antibiotics and was described in a putative CA-MRSA isolate from Sweden (Berglund *et al.*, 2008). SCC*mec* type VIII (32kb), an epidemic MRSA isolate from Canada,

represents the SCC*mec* element most recently described in full (Zhang *et al.*, 2009). SCC*mec* type VIII contains the class A *mec* complex and *ccr* allotype 4, and includes Tn*554* in the J2 region (Figure 3.1), which, as in SCC*mec* types II and III, confers resistance to the macrolides, lincosamides and streptogramins, (Ito *et al.*, 2009; Zhang *et al.*, 2009).

The classification of SCC*mec* types is most commonly carried out using PCR-based assays (de Lencastre *et al.*, 2007; Deurenberg and Stobberingh, 2008). Techniques used for the identification of SCC*mec* type and subtype commonly include multiplex PCR assays such as those described by Milheiriço *et al.* (2007a and 2007b), which are currently among the most frequently used SCC*mec* typing and SCC*mec* type IV subtyping strategies. The SCC*mec* typing multiplex PCR assay is used for the detection of SCC*mec* types I – VI (Milheiriço *et al*,. 2007a), while the SCC*mec* type IV subtyping assay is used for the detection of subtypes IVa – IVh (Milheiriço *et al.*, 2007b). These strategies were used in the current study to characterise the SCC*mec* content of the MRSA described in Chapter 2. SCC*mec* types VII – XI, and subtypes IVi and IVj, were identified after these typing strategies were developed by Milheiriço *et al.* (2007a and 2007b), which illustrates how existing assays constantly need to be updated to include novel SCC*mec* types and subtypes. The most recently developed SCC*mec* typing strategy is a Real-Time multiplex PCR assay for the detection of SCC*mec* types I – VI and VIII (Chen *et al.*, 2009); however, a multiplex PCR assay that detects all eleven SCC*mec* types is yet to be developed.

3.2 Experimental Protocol

3.2.1 The isolation of *S. aureus* genomic DNA

S. aureus was sub-cultured from 25 % glycerol stocks as described in Chapter 2 [2.2.3.3], and genomic DNA was isolated using the QIAamp DNA Mini Kit (QIAGEN, Valencia, CA, USA). The protocol provided by the manufacturer required minor modifications. An extra initial incubation step was included, prior to the addition of Buffer ATL, to weaken the bacterial cell wall; a sweep of pure colonies was added to 200 µl of pre-treatment solution containing 25 µg/ml of lysostaphin (Sigma Aldrich, Inc., St Louis, MO, USA), 2 mM EDTA and 20 mM Tris-Cl, and incubated at 37°C in a ThermoMixer Compact (Eppendorf, Hamburg, Germany), with shaking at 800rpm, for 30 min, which was found to be optimal in our laboratory. After the initial incubation step, the bacteria were harvested by centrifugation at 7500rpm at room temperature for 15 min in an Eppendorf 5417 C bench-top centrifuge (Eppendorf, Hamburg, Germany), the supernatant was discarded and the manufacturer's protocol resumed with the addition of 180 µl of Buffer ATL. In the final elution step, 50 µl of Buffer AE was applied to the MiniSpin column, which was incubated at room temperature for 5 min prior to centrifugation to increase the final yield of genomic DNA. The concentration of the genomic DNA was determined using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

3.2.2 Agarose gel electrophoresis

The integrity of the *S. aureus* genomic DNA was confirmed by conventional electrophoresis using 1 % agarose gels. The SeaKem LE agarose (Lonza Biologics Plc, Slough, UK) was dissolved in 1X TAE buffer (Appendix B) with ethidium bromide (Fluka, Sigma Aldrich, Inc., St Louis, MO, USA) at a final concentration of 0.5 µg/ml. Five microlitres each of genomic DNA and 5X loading buffer (Bioline, London, UK) were loaded into the wells. An electrical field (5V/cm) was applied to the gel for 1.5 h, causing negatively charged DNA molecules to migrate towards the positive terminal of the electrophoresis chamber with the rate of migration dependent on the molecular weights of the fragments. A molecular weight marker, Hyperladder I (Bioline, London, UK) (Appendix C), was included to facilitate the estimation of DNA fragment sizes. DNA was visualised by UV transillumination and photographed using a UVIpro Silver (UVItec Ltd, Cambridge, UK) or a CHEMI-Genius Bioimaging System (Syngene, Frederick, MD, USA).

3.2.3 Methods used for the characterisation of SCC*mec* content of MRSA isolates

The characterisation of the SCC*mec* content of the 100 MRSA isolates required several methodological approaches that were carried out sequentially, as depicted in Figure 3.2.

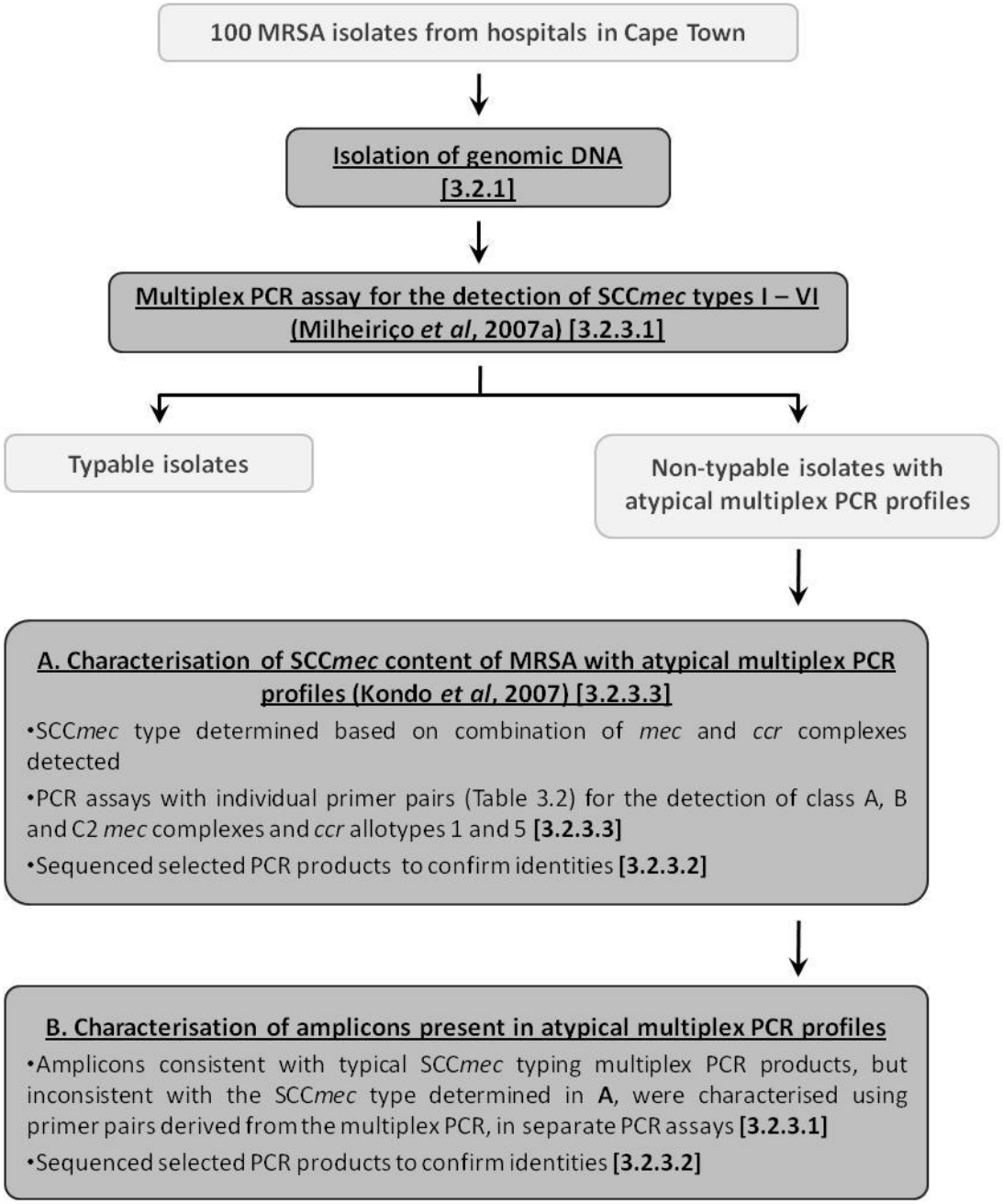


Figure 3.2 Flow-diagram depicting methods used to characterise the SCC*mec* content of 100 methicillin-resistant *S. aureus* isolates.

*3.2.3.1. Multiplex PCR assay for the detection of SCC*mec *Types I – VI*

The multiplex PCR assay developed by Oliveira and de Lencastre (2002) and updated by Milheiriço *et al.* (2007a), for the detection of SCC*mec* types I – VI, was initially used for SCC*mec* typing. The assay was optimised by Dr Eliya Madikane and Mkunde Chachage, and carried out on 41 of the 100 MRSA isolates prior to the start of this study. The SCC*mec* types of the remaining 59 isolates were determined by the author. SCC*mec* types VII – XI had not been described at the onset of this study; therefore primers for the detection of these SCC*mec* elements were not included in the assay.

The multiplex PCR assay was carried out in a volume of 50 µl, which contained a final concentration of 1X Super-Therm PCR buffer and 1.5 mM $MgCl_2$ (JMR Holdings, London, UK), with 160 µM deoxynucleotide triphosphate (dNTP) mix (Thermo Scientific, Wilmington, DE, USA). Five nanograms of genomic DNA [3.2.1] and 1.25 U of Super-Therm *Taq* polymerase (JMR Holdings, London, UK) were added to each reaction. The primers CIF2 F2, CIF2 R2, RIF5 F10 and RIF R13 were included at a final concentration of 0.6 µM, while primers kdp F1, kdp R1, dcs F2, dcs R1, mecI P2, mecI P3, ccrC F2, ccrC R2, ccrB2 F2, ccrB2 R2, SCC*mec* III J1F, SCC*mec* III J1R, SCC*mec* V J1F and SCC*mec* V J1R were included at a final concentration of 0.8 µM. For the amplification of *mecA*, the internal control, primers mecA P4 and mecA P7 were included at a final concentration of 0.2 µM. All primers were synthesised at the Synthetic DNA Laboratory at the University of Cape Town (Cape Town, South Africa), and the primer sequences and corresponding control strains are shown in Appendix E. Table 3.1 summarises the primer target sequences, the SCC*mec* types detected by each primer pair, and also indicates the expected PCR product sizes. Where necessary, individual primer pairs were used in separate PCR assays to confirm the origin of additional PCR products identified in the multiplex PCR profiles of particular isolates (Figure 3.2).

Table 3.1. Primers used in the SCC*mec* typing multiplex PCR assay for the detection of SCC*mec* types I – VI

Primer pair[a]	Complementary sequence [a] (location within SCC*mec*[b])	SCC*mec* types containing complementary sequence						Expected product size (bp)
		I	II	III	IV	V	VI	
CIF2 F2 CIF2 R2	Sequence downstream of *pls* (J1)	■						495
ccrC F2 ccrC R2	Internal to *ccrC* (*ccr* complex)					■		449
RIF5 F10 RIF5 R13	Region between Tn*554* and *orfX* (SCC*Hg*)			■				414
SCC*mec* V J1F SCC*mec* V J1R	Not specified (J1)					■		377
dcs F2 dcs R1	Internal to *dcs* gene (J3)	■	■		■		■	342
ccrB2 F2 ccrB2 R2	Internal to *ccrB* allotype 2 (*ccr* complex)		■		■			311
kdp F1 kdp R1	Internal to *kdp* operon (J1)		■					284
SCC*mec* III J1F SCC*mec* III J1R	Not specified (J1)			■				243
mecI P2 mecI P3	Internal region of *mecI* (*mec* complex)		■	■				209
mecA P4 mecA P7	Internal region of *mecA*[c] (*mec* complex)	■	■	■	■	■	■	162

[a] Milheiriço *et al.* (2007a) (primer sequences available in Appendix E).

[b] SCC*mec*, staphylococcal cassette chromosome *mec*; J1, joining region 1; SCC*Hg*, staphylococcal cassette chromosome mercury; J3, joining region 3.

[c] Internal positive control.

PCR amplification was carried out using an Applied Biosystems 2720 Thermocycler (Applied Biosystems, Carlsbad, CA, USA), including an initial denaturation step at 94°C for 3 min, followed by 30 cycles of 30 s at 94°C, 30 s at 53°C, and 30 s at 72°C, with a final extension step of 3 min at 72°C. The following prototypic *S. aureus* strains containing SCC*mec* types I – VI were included in each experiment: COL (I), BK2464 (II), ANS46 (III), MW2 (IV), WIS (V) and HDE288 (VI) (Oliveira and de Lencastre, 2002; Milheiriço *et al.*, 2007a). These control strains were obtained from Professor Hermínia de Lencastre (Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Oeiras, Portugal) with the exception of WIS, which was obtained from Professor Keiichi Hiramatsu (Department of Bacteriology, Juntendo University, Tokyo, Japan).

The PCR products were separated by conventional electrophoresis on 2 % gels that were prepared by dissolving SeaKem LE agarose (Lonza Biologics Plc, Slough, UK) in 0.5X TBE buffer (Appendix B), with 0.5 µg/ml ethidium bromide (Fluka, Sigma Aldrich, Inc., St Louis, MO, USA). The electrophoresis and visualisation of PCR products was carried out as previously described [3.2.2], except that 10 µl of the final volume (50 µl) of PCR product and 2 µl of 5X loading buffer (Bioline, London, UK) were loaded into the wells. Hyperladder IV (Bioline, London, UK) (Appendix C) was included as the molecular weight marker and electrophoresis proceeded at 5V/cm for 2.5 h.

3.2.3.2. DNA sequencing of PCR products

When necessary, PCR products obtained in multiplex PCR assays, or in reactions containing individual primer pairs, were sequenced to confirm their identities. Twenty microlitres of PCR product, and 4 µl of 5 X loading buffer (Bioline, London, UK), were loaded into the wells of a 2 % agarose gel prepared as for the visualisation of these products. The products were separated by electrophoresis at 3V/cm for 3 h. The DNA was visualised using a UV lightbox (FotoDyne Inc, Hartland, WI, USA) and PCR products of interest were identified with the aid of the included molecular weight marker. Using a surgical blade, the fragments of interest were carefully excised from the agarose gel so as to avoid carry-over of non-specific products. The DNA was purified for direct sequencing using the MinElute Gel Extraction Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. The DNA was

eluted in 10 µl of molecular grade water (Fluka, Sigma Aldrich, Inc., St Louis, MO, USA) and quantified using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

The purified PCR products and corresponding primers were sent to the Central Analytical Facility at the University of Stellenbosch where both strands were sequenced using the Sanger or dideoxy method. The Sanger method includes a cycle sequencing reaction in which dNTPs and dideoxynucleotide triphosphates (ddNTPs) are available for incorporation into the growing DNA strand. Each of the 4 ddNTPs has a specific fluorescent tag, and lacks a 3 prime hydroxyl group; therefore, elongation is terminated upon addition of a ddNTP, thereby generating sequencing products that vary in size depending on where the ddNTP was incorporated (Hartwell *et al.*, 2004). At the Central Analytical Facility, automated sequencing was carried out on an ABI3730xl DNA analyser, which uses capillary electrophoresis for the fine-scale size-dependent separation of the resulting sequencing products. As a product exits the capillary, a UV laser excites the fluorescent tag of the terminal ddNTP, and in this way the full sequence of the amplicon of interest is gradually determined (Hartwell *et al.*, 2004).

The chromatograms provided by the Central Analytical Facility were viewed and sequences edited in BioEdit Sequence Alignment Editor (version 7.0.5.2) (Hall, 1999). A nucleotide-BLAST query of the nucleotide collection database was carried out for the sequences of interest, using the default settings (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

*3.2.3.3 Characterisation of the SCC*mec *content of MRSA isolates with atypical multiplex PCR profiles*

Thirty-seven of the 100 study isolates had amplification patterns that did not correspond to those of the control strains and were, therefore, non-typable by the SCC*mec* typing multiplex PCR assay of Milheiriço *et al.* (2007a). The SCC*mec* typing strategy described by Kondo *et al.* (2007) was modified for use in this study to resolve the identities of the non-typable isolates. The strategy of Kondo *et al.* (2007) includes individual multiplex PCR assays for the detection of class A, B, C1 and C2 *mec* complexes and *ccr* allotypes 1 – 5, thereby characterising the combination of

mec and *ccr* complexes present in an isolate in order to determine its SCC*mec* type. The primer pairs described for the detection of class A, B and C2 *mec* complexes, and *ccr* allotypes 1 and 5, were used individually in separate assays as necessary to confirm the SCC*mec* content of the non-typable isolates (Figure 3.2).

For the detection of *mec* classes A, B and C2, all reactions were carried out in a volume of 50 µl with 1X SuperTherm PCR buffer, 2 mM $MgCl_2$ (JMR Holdings, London, UK), 200 µM of each dNTP (Thermo Scientific, Wilmington, DE, USA), 20 ng of template DNA [3.2.1] and 2.5 U of *Taq* polymerase (JMR Holdings, London, UK). Each primer from the pairs mA7/ mI6, mA7/ IS7, and mA7/ IS2 (iS-2) (Synthetic DNA Laboratory, University of Cape Town, Cape Town, South Africa) was added at a final concentration of 0.1 µM. The common primer, mA7, is specific for *mecA*, while primers mI6, IS7 and IS2 (iS-2) are specific for regions upstream of the methicillin-resistance determinant that are unique to *mec* classes A, B and C2, respectively, as summarised in Table 3.2 (Appendix E). *S. aureus* control strains ANS46 (class A *mec* complex), COL (class B *mec* complex) and WIS (class C2 *mec* complex) were included as appropriate. The PCRs were carried out in an Applied Biosystems 2720 Thermocycler (Applied Biosystems, Carlsbad, CA, USA) with the cycling conditions for each assay including an initial denaturation step of 2 min at 94°C followed by 30 cycles of denaturation for 2 min at 94°C, annealing for 1 min at 57°C and extension for 2 min at 72°C, with a final extension step of 2 min at 72°C.

Table 3.2 Primers for the classification of SCC*mec* types based on the combination of *mec* class and *ccr* allotype present

Primer pair [a]	Region targeted [a]	*mec* class/ *ccr* complex detected	SCC*mec* types containing *mec* class/*ccr* complex								Expected product size (bp)
			I	II	III	IV	V	VI	VII	VIII	
mA7 mI6	*mecA – mecI*	*mec* class A		■	■					■	1963
mA7 IS7	*mecA – IS1272*	*mec* class B	■			■		■			2872
mA7 IS2(iS-2)	*mecA* – IS*431* (copy inserted upstream of *mecA*)	*mec* class C2					■				804
α1 βc	*ccrA1 – ccrB*	*ccr* type 1	■								695
γF γR	*ccrC* [b]	*ccr* type 5			■		■		■		518

[a] Kondo *et al.* (2007) (primer sequences available in Appendix E).

[b] Present in SCC*mec* types V and VII (*ccr* gene complex), and in the staphylococcal cassette chromosome mercury (SCC*Hg*) component of SCC*mec* type III.

For the detection of *ccr* allotypes 1 and 5, reaction volumes of 50 µl were used with all components at the same final concentrations as those described for the *mec* complex detection assays, except for $MgCl_2$ (JMR Holdings, London, UK), which was included at 3.2 mM, and the template DNA [3.2.1], 10 ng of which was added. The degenerate primer βc, specific for *ccrB* allotypes 1 – 3, and primer α1 and the primer pair γF/ γR were included for the detection of *ccr* allotypes 1 and 5, respectively. All primers were synthesised at the Synthetic DNA Laboratory at the University of Cape Town (Cape Town, South Africa). Table 3.2 includes a summary of primer target sequences and expected product sizes, while the primer sequences are shown in Appendix E. *S. aureus* control strains COL (*ccr* allotype 1) and WIS (*ccr* allotype 5) were included in the individual assays as appropriate. The PCR amplification parameters were the same as those described for the *mec* complex detection assays, except that an annealing temperature of 60°C was used.

PCR products obtained from these assays were separated by conventional electrophoresis on 1.5 % agarose gels as previously described [3.2.2], except that 10 µl of the final volume (50 µl) of PCR product and 2 µl of 5X loading buffer (Bioline, London, UK) were loaded into the wells. Hyperladders I and IV were included as appropriate based on expected PCR product sizes (Table 3.2; Appendix C). Selected PCR products were purified for direct sequencing as previously described [3.2.3.2], except that amplicons of interest were separated on a 1.5 % agarose gel prior to purification.

3.2.4 Multiplex PCR assay for the subtyping of SCC*mec* type IV elements

The SCC*mec* type IV subtyping multiplex PCR assay developed by Milheiriço *et al.* (2007b) was used to further characterise the SCC*mec* content of local SCC*mec* type IV isolates. The assay includes primers for the detection of subtypes IVa – IVh based on differences in their respective J1 regions. Subtypes IVi and IVj had not been detected when the assay was developed; therefore, primers for the detection of these subtypes were not included. The subtypes detected by the multiplex assay, IVa – IVh, include 5 unique J1 regions as those of IVb and IVF, and IVc and IVE, are identical. Subtypes IVb and IVF differ in the J3 region: the J3 region of subtype IVb includes the *dcs* locus, whereas this locus is absent in subtype IVF. Similarly, subtype IVc carries a copy of Tn*4001* in the J3 region, whereas IVE does not.

Additional individual PCR assays including primers for the detection of *dcs* and Tn*4001* are recommended for the differentiation of subtypes IVb and IVF, and IVc and IVE, respectively.

Each PCR was carried out in a volume of 50 µl containing a final concentration of 1X PCR buffer, 1.5 mM $MgCl_2$ and 1.25 U of SuperTherm *Taq* polymerase (JMR Holdings, London, UK), as well as 160 µM dNTP mix (Thermo Scientific, Wilmington, DE, USA) and 5 ng of template DNA [3.2.1]. All primers were synthesised at the Synthetic DNA Laboratory at the University of Cape Town (Cape Town, South Africa) with the recommended final primer concentrations modified and added as presented in Table 3.3. The target sequences and expected product sizes of the primer pairs are also shown in Table 3.3, while the primer sequences are shown in full in Appendix E.

Table 3.3 Primers used in the multiplex PCR assay for the detection of SCC*mec* type IV subtypes IVa – IVh

Primer pair [a]	Final concentration of each primer used in the assay (µM)	Subtypes detected (SCC*mec* region containing complementary sequence[b])	Expected product size (bp)
J IVa F J IVa R	0.4	IVa (J1)	278
J IVb F J IVb R	0.8	IVb and IVF (J1)	336
J IVc F J IVc R	0.6	IVc and IVE (J1)	483
J IVd F J IVd R	0.8	IVd (J1)	575
J IVg F J IVg R	0.9	IVg (J1)	792
J IVh F J IVh R	2.2	IVh (J1)	663
ccrB2 F *ccrB2* R	0.8	IVa – IVh (*ccr* complex (*ccrB2*)) [c]	203

[a] Milheiriço *et al.* (2007b) (primer sequences available in Appendix E).

[b] J1, joining region 1.

[c] Internal positive control.

Amplification was carried out using an Applied Biosystems 2720 Thermocycler (Applied Biosystems, Carlsbad, CA, USA), including denaturation for 4 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 48°C for 30 s and extension at 72°C for 2 min, with a final extension step of 4 min at 72°C. Prototypic *S. aureus* strains JCSC4744 (IVa), JCSC2172 (IVb), DEN2949 (IVc), BK2529 (IVd), AR43/3330.1 (IVE), M03-68 (IVg) and HAR22 (IVh) were included in each assay. Control strains JCSC4744 and JCSC2172 were obtained from Professor Teruyo Ito (Department of Bacteriology, Juntendo University, Tokyo, Japan), while the remaining strains were obtained from Professor Hermínia de Lencastre (Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Oeiras, Portugal). It should be noted that, in the case of strain M03-68, a genomic DNA preparation sufficient for only a single reaction was provided; therefore, a PCR assay was carried out as for the multiplex PCR but included only the subtype IVg specific primers. In subsequent assays, 1 µl of PCR product was included as template DNA to control for the detection of the J1 region of subtype IVg. It was not possible to obtain a subtype IVF control strain; however, this was not essential given that the J1 region of subtype IVF is identical to that of IVb.

The PCR products were separated and visualised on 2 % agarose gels as described for the SCC*mec* typing multiplex PCR assay [3.2.3.1], except that the gels were prepared using 1X TAE buffer, and 20 µl PCR product and 4 µl of 5X loading buffer (Bioline, London, UK) were loaded into the wells.

3.3 Results

3.3.1 The classification of SCC*mec* types of local MRSA isolates

Using the SCC*mec* typing multiplex PCR described by Milheiriço *et al.* (2007a), PCR profiles were obtained for all of the MRSA isolates described in Chapter 2. A comparison of these profiles with the amplification patterns obtained from *S. aureus* control strains COL, BK2464, ANS46, MW2, WIS and HDE288, containing SCC*mec* types I – VI, respectively, determined the SCC*mec* types of 63 of the 100 MRSA isolates. Forty-six isolates had multiplex PCR profiles consistent with SCC*mec* type IV. SCC*mec* type II was detected in 12 isolates, while 4 and 1 isolates contained SCC*mec* types III and I, respectively. The PCR profiles obtained for the control strains and a subset of typable isolates are shown in Figure 3.3.

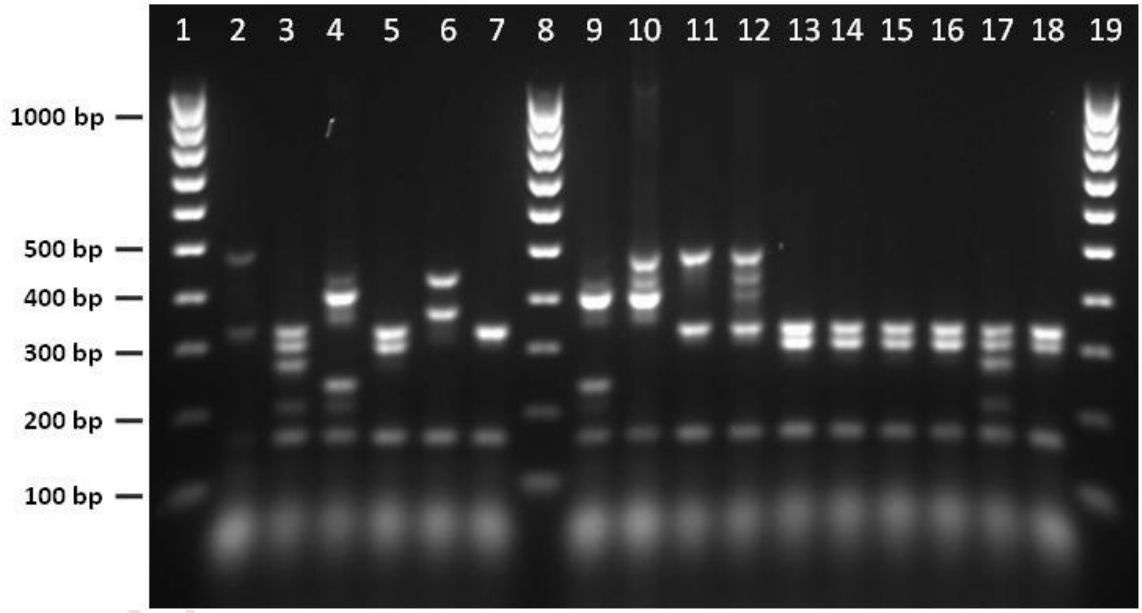


Figure 3.3 SCC*mec* typing multiplex PCR profiles obtained for methicillin-resistant *S. aureus* (MRSA) control strains and a representative selection of MRSA from hospitals in Cape Town. Lanes 2 – 7 contain MRSA control strains carrying SCC*mec* types I – VI; lanes 9 – 18 contain a selection of MRSA from hospitals in Cape Town. Lane 1, Hyperladder IV molecular weight marker; lane 2, COL (SCC*mec* type I); lane 3, BK2464 (SCC*mec* type II); lane 4, ANS46 (SCC*mec* type III); lane 5, MW2 (SCC*mec* type IV); lane 6, WIS (SCC*mec* type V); lane 7, HDE288 (SCC*mec* type VI); lane 8, Hyperladder IV molecular weight marker; lane 9, A1 (III); lane 10, S2 (unique atypical profile: 495 bp, 449 bp, 414 bp, 162 bp); lane 11, B1 (I); lane 12, C17 (atypical profile detected in 36 MRSA: 495 bp, 449 bp, 414 bp, 342 bp, 162 bp); lane 13, D4 (IV); lane 14, E4 (IV); lane 15, E5 (IV); lane 16, E32 (IV); lane 17, F8 (II) ; lane 18, S8 (IV); lane 19, Hyperladder IV molecular weight marker.

Atypical amplification patterns were obtained for 37 of the 100 isolates, necessitating additional investigations to determine the SCC*mec* content of these MRSA. The experimental approaches used in these investigations, and the results obtained, are summarised in Figure 3.4 and described in full below.

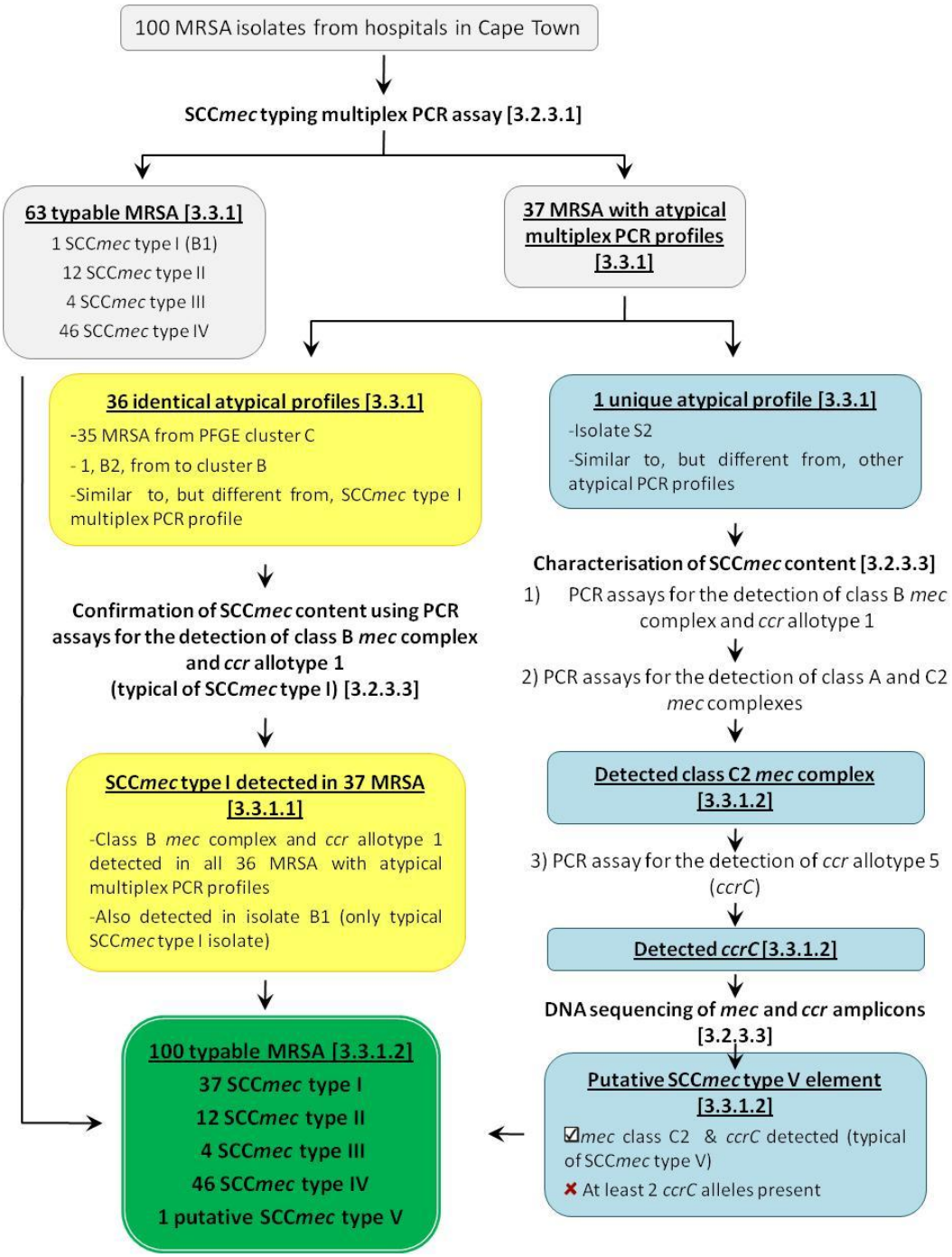


Figure 3.4 Flow-diagram representing the characterisation of the SCC*mec* content of 100 methicillin-resistant *S. aureus* isolates. Prototypic *S. aureus* strains containing SCC*mec* types I – VI were used to control the SCC*mec* typing multiplex PCR assay: COL (SCC*mec* type I (1B)) BK2464 (SCC*mec* type II (2A)) ANS46 (SCC*mec* type III (3A)) MW2 (SCC*mec* type IV (2B)) WIS (SCC*mec* type V (5C2)) HDE288 (SCC*mec* type VI (4B)) (Oliveira and de Lencastre, 2002; Milheiriço *et al.*, 2007a). PCR assays for the detection of class A, B and C2 *mec* complexes were controlled using ANS46, COL and WIS, respectively; those for the detection of *ccr* allotypes 1 and 5 were controlled using COL and WIS, respectively. ☑, result supporting SCC*mec* type assignment; ✘, result querying SCC*mec* type assignment.

*3.3.1.1 Characterisation of the SCC*mec *content of 36 MRSA isolates with atypical multiplex PCR profiles*

Thirty-seven MRSA isolates had atypical multiplex PCR profiles, 36 of which were similar to, but distinct from, the multiplex PCR profile observed for the SCC*mec* type I control strain, COL (Figure 3.3). Given the SCC*mec* type I-like profiles of the isolates, primer pairs described by Kondo *et al.*(2007) for the detection of the class B *mec* complex and *ccr* allotype 1 (the combination of *mec* and *ccr* complexes characteristic of SCC*mec* type I) were used in separate PCR assays to confirm the SCC*mec* content of these isolates (Figure 3.4). Products corresponding to 2827 bp and 695 bp regions of the class B *mec* complex and *ccr* allotype 1, respectively, were detected in all 36 MRSA, as shown for a selection of isolates in Figure 3.5. These products were also detected in isolate B1, the only local MRSA isolate with a typical SCC*mec* type I multiplex PCR profile, and in COL, the SCC*mec* type I control strain (Figure 3.5). Based on the detection of the class B *mec* complex and *ccr* allotype I, the 36 isolates with identical SCC*mec* type I-like multiplex PCR profiles were classified as SCC*mec* type I.

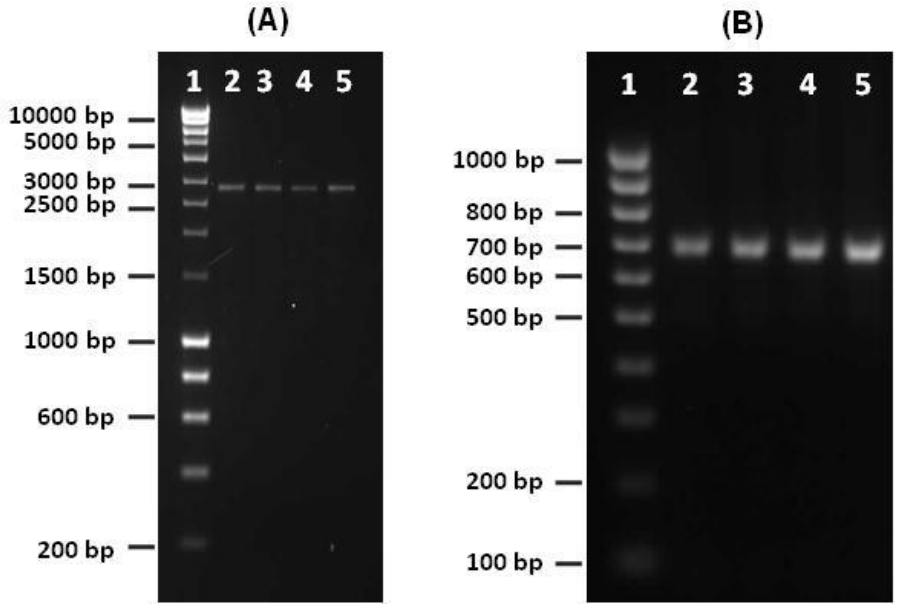


Figure 3.5 PCR products obtained following assays for the detection of the class B *mec* complex and *ccr* allotype 1 in SCC*mec* type I-like isolates. (A) PCR products detected following the amplification of a 2827 bp region of the class B *mec* complex. Lane 1, Hyperladder I molecular weight marker; lane 2, control strain COL (SCC*mec* type I; class B *mec* complex); lane 3, B2 (SCC*mec* type I-like); lane 4, B1 (SCC*mec* type I); lane 5, C17 (SCC*mec* type I-like). **(B)** PCR products detected following the amplification of a 695 bp region of *ccr* allotype 1. Lane 1, Hyperladder IV molecular weight marker; lane 2, control strain COL (SCC*mec* type I; *ccr* allotype 1); lane 3, B1 (SCC*mec* type I); lane 4, C2, (SCC*mec* type I-like); lane 5, C3 (SCC*mec* type I-like).

Inspection of the atypical multiplex PCR profiles obtained for the 36 SCC*mec* type I isolates revealed products of 495 bp, 342 bp and 162 bp that were consistent with those typical of SCC*mec* type I. Two additional products that were inconsistent with the multiplex PCR profile of SCC*mec* type I were also detected (Figure 3.3). The sizes of these products equated with the 449 bp and 414 bp amplicons emanating from primer pairs ccrC F2/ccrC R2 (for the detection of SCC*mec* type V) and RIF5 F10/RIF5 R13 (for the detection of SCC*mec* type III), respectively, as summarised in Figure 3.6 and Table 3.1.

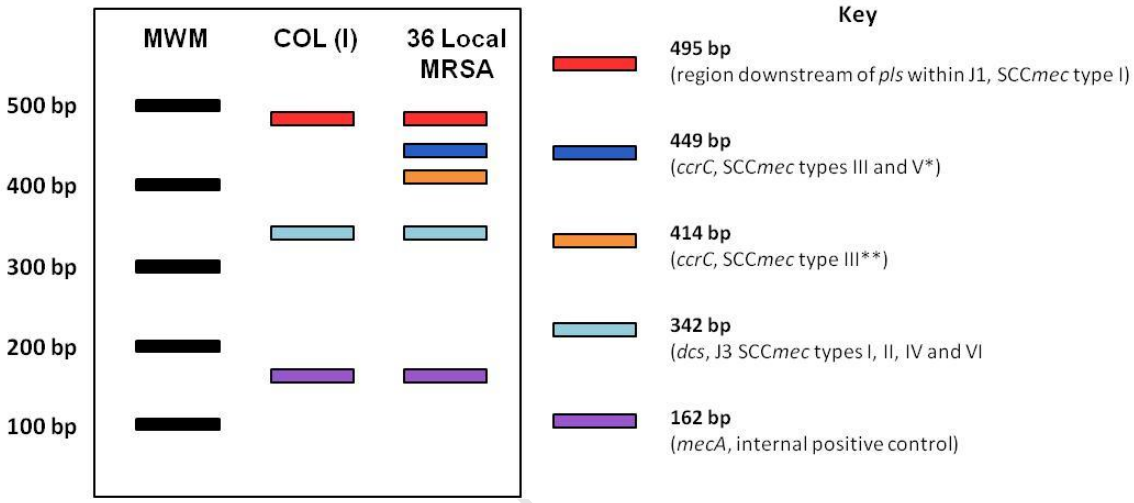


Figure 3.6 Schematic diagram representing SCC*mec* typing multiplex PCR profiles detected for SCC*mec* type I control strain COL and 36 atypical SCC*mec* type I isolates. Additional products detected for 36 SCC*mec* type I-like isolates equated with sizes of amplicons typically detected in SCC*mec* elements other than SCC*mec* type I. PCR assays including primer pairs for the amplification of the corresponding fragments, followed by sequencing where necessary, confirmed the identities of atypical products. J1, joining region 1; J3, joining region 3. *Emanating from primer pair ccrC2 F2/ccrC2 R2 (cross-complementary to *ccrC* present in SCC*mec* type V and in the SCC*Hg* component of SCC*mec* type III). **Emanating from RIF5 F10/RIF5 R13 (complementary to *ccrC* present in the SCC*Hg* component of SCC*mec* type III).

To investigate the possibility that ccrC F2/ccrC R2 and RIF5 F10/RIF5 were extended in the amplification of the additional products, PCR assays were carried out as for the SCC*mec* typing multiplex PCR assay, except that only the primer pair of interest was included in each individual assay. DNA from one of the 36 MRSA (isolate C17) and the local typical SCC*mec* type I isolate (isolate B1) was included in these assays, which were controlled using COL (SCC*mec* type I), WIS (SCC*mec* type V) and ANS46 (SCC*mec* type III).

In the PCR assay including only primer pair ccrC F2/ccrC R2, a 449 bp product corresponding to that amplified from WIS and ANS46 was detected in isolate C17, but not in isolate B1 or COL (Figure 3.7).

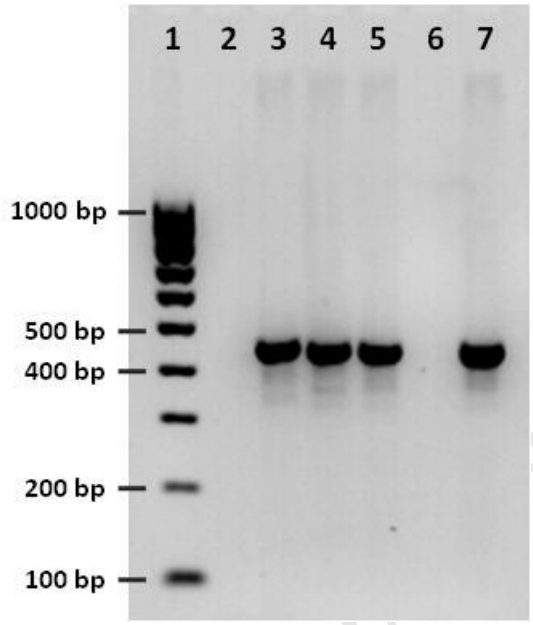


Figure 3.7 Agarose gel electrophoresis of amplicons obtained using the primer pair ccrC F2/ccrC R2 in PCR assays for the detection of *ccrC* in methicillin-resistant *S. aureus* control strains and local SCC*mec* type I isolates with typical and atypical multiplex PCR profiles. Lane 1, Hyperladder IV molecular weight marker; lane 2, control strain COL (SCC*mec* type I; *ccrC*-negative); lane 3, control strain ANS46 (SCC*mec* type III; *ccrC*-positive (SCC*Hg* component)); lane 4, control strain WIS (SCC*mec* type V; *ccrC*-positive (*ccr* complex)); lane 5, B2 (atypical SCC*mec* type I multiplex profile); lane 6, B1 (typical SCC*mec* type I multiplex profile); lane 7, C17 (atypical SCC*mec* type I multiplex profile).

The product detected in ANS46, the SCC*mec* type III control strain (Figure 3.7), was unexpected as Milheiriço *et al.* (2007a) described ccrC F2/ccrC R2 as complementary to only SCC*mec* type V. A BLAST query indicated that the copy of *ccrC* present in WIS (accession number AB121219) was in fact 91 % similar to a copy of *ccrC* present in the SCC*Hg* component of the prototypic SCC*mec* type III strain 85/2082 (accession number AB037671), which was used to develop the multiplex PCR assay. Subsequent analyses revealed that the primer pair ccrC2 F2/ccrC2 R2 is cross-complementary to *ccrC* sequences present in both SCC*mec* types V and III (Figure 3.6). Re-examination of the multiplex PCR profiles obtained for ANS46 and local SCC*mec* type III isolates revealed additional products

corresponding to 449 bp due to the cross-complementarity of this primer pair (Figure 3.3). The 449 bp product amplified from isolate C17 was sequenced, and a BLAST query indicated that it was 96 % similar to the *ccrC* sequence present in WIS (Appendix F). The *ccrC* gene is not typically present in SCC*mec* type I strains; therefore an individual PCR assay with the primer pair ccrC F2/ccrC R2 was carried out for all local SCC*mec* type I MRSA. The *ccrC* gene was detected in all of these isolates, but not in isolate B1.

In the PCR assay including primer pair RIF5 F10/RIF5 R13, a 414 bp product corresponding to that amplified from ANS46 was detected in isolate C17, but not in COL, WIS or isolate B1, as shown in Figure 3.8.

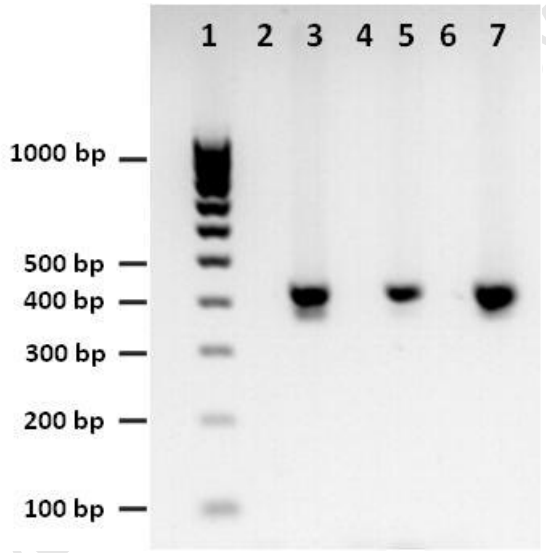


Figure 3.8 Agarose gel electrophoresis of PCR products obtained using the primer pair RIF5 F10/RIF5 R13 in PCR assays for the detection of a 414 bp product, described as unique to SCC*mec* type III isolates, in methicillin-resistant *S. aureus* control strains and local SCC*mec* type I isolates with typical and atypical multiplex PCR profiles. Lane 1, Hyperladder IV molecular weight marker; lane 2, control strain COL (SCC*mec* type I); lane 3, control strain ANS46 (SCC*mec* type III); lane 4, control strain WIS (SCC*mec* type V); lane 5, B2 (atypical SCC*mec* type I multiplex profile); lane 6, B1 (typical SCC*mec* type I multiplex profile); lane 7, C17 (atypical SCC*mec* type I multiplex profile).

The precise region of SCC*mec* type III amplified by RIF5 F10/RIF5 R13 was not specified by Oliveira and de Lencastre (2002) or Milheiriço *et al.* (2007a); however, examination of the nucleotide sequence of *S. aureus* strain 85/2082 (accession number AB037671), the prototypic SCC*mec* type III strain, revealed that

RIF5 F10/RIF5 R13 target a copy of *ccrC* present in the SCC*Hg* component of SCC*mec* type III. Although the *ccrC* genes in ANS46 and WIS are 91 % identical, alignment of RIF5 F10 and RIF5 R13 with the nucleotide sequences of WIS identified several mismatches between each of the primers and their complementary sequences. This explains the absence of a 414 bp product for the SCC*mec* type V control strain, WIS, in the individual PCR assay including RIF5 F10/RIF5 R13 (Figure 3.8).

*3.3.1.2 Characterisation of the SCC*mec *content of the remaining MRSA isolate with an atypical multiplex PCR profile*

The multiplex PCR profile of the remaining non-typable isolate, designated isolate S2 (Appendix A), was similar to, but distinct from, the 36 MRSA isolates described above. Four PCR products, including a 162 bp product corresponding to the *mecA* internal control, were detected in the multiplex PCR profile of S2 (Figure 3.3). As for the 36 atypical SCC*mec* type I isolates, 495 bp, 449 bp, 414 bp and 162 bp products were detected in S2; however, the 342 bp product, corresponding to the *dcs* locus present in the J3 region of SCC*mec* types I, II, IV and VI (Table 3.1), was absent in this isolate (Figure 3.3). Given the overall similarities between the multiplex profiles of the 36 SCC*mec* type I isolates and that of S2, PCR assays including primer pairs for the detection of the class B *mec* complex and *ccr* allotype 1 were also carried out for this isolate (Figure 3.4); however, products were only detected for the control strains in these assays (Figure 3.9).

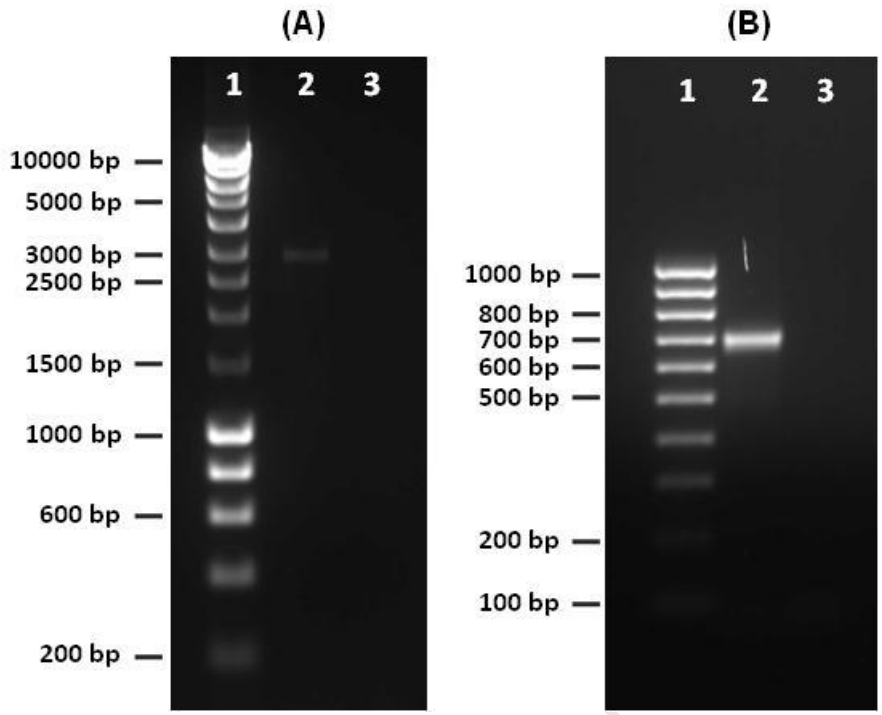


Figure 3.9 PCR products obtained following assays for the detection of the class B *mec* complex and *ccr* allotype 1, characteristic of SCC*mec* type I, in sporadic isolate S2 with a unique atypical multiplex PCR profile. (A) Amplicons obtained following PCR assay for the detection of a 2827 bp region of the class B *mec* complex. Lane 1, Hyperladder I molecular weight marker; lane 2, control strain COL (SCC*mec* type I; class B *mec* complex); lane 3, S2. **(B)** Amplicons obtained following PCR assay for the detection of a 695 bp region of *ccr* allotype 1. Lane 1, Hyperladder IV molecular weight marker; lane 2, control strain COL (SCC*mec* type I; *ccr* allotype 1); lane 3, S2.

In order to determine the SCC*mec* type of isolate S2, additional individual PCR assays, derived from the strategy described by Kondo *et al.* (2007), were carried out for the detection of the class A and C2 *mec* complexes. Based on the detection of an 804 bp region of the class C2 *mec* complex (Figure 3.10), an additional PCR assay for the detection of *ccr* allotype 5 was carried out using primers described by Kondo *et al.* (2007), as this *mec-ccr* combination is characteristic of SCC*mec* type V (Figure 3.4). The appropriate control strains were included in each assay (Figure 3.4). A PCR product corresponding to a 518 bp region of *ccr* allotype 5 (*ccrC*), was amplified from isolate S2 (Figure 3.10).

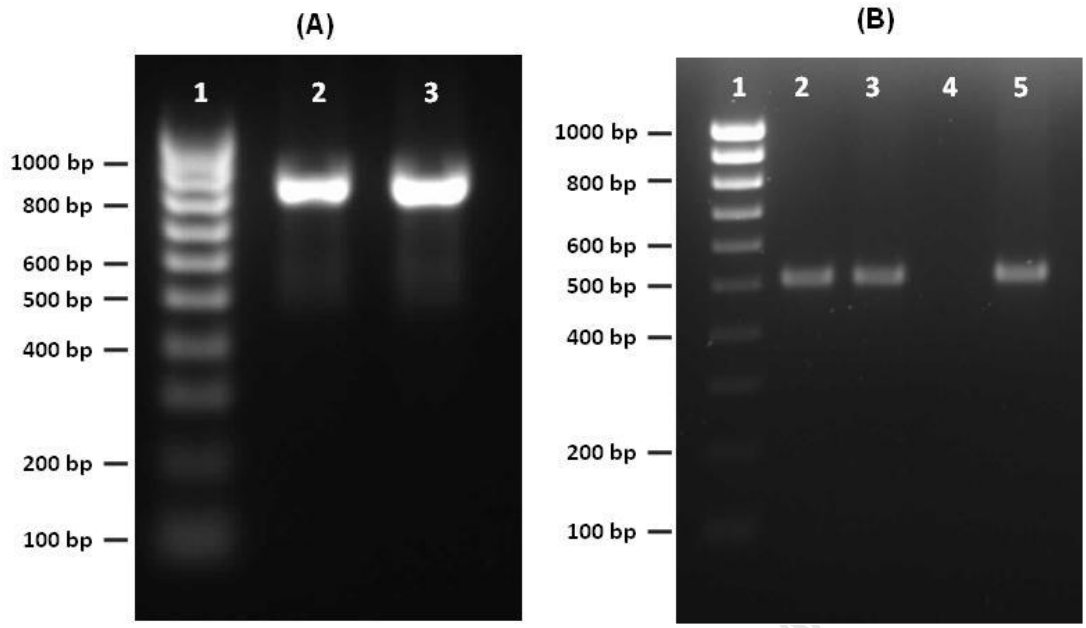


Figure 3.10 PCR products obtained following assays for the detection of the class C2 *mec* complex and *ccr* allotype 5, characteristic of SCC*mec* type V, in the sporadic isolate S2. (A) Amplicons detected following a PCR assay for the amplification of an 804 bp region of the class C2 *mec* complex. Lane 1, Hyperladder IV molecular weight marker; lane 2, control strain WIS (SCC*mec* type V; class C2 *mec* complex); lane 3, S2. (**B)** Amplicons detected following a PCR assay for the amplification of a 518 bp region of *ccr* allotype 5 (*ccrC*). Lane 1, Hyperladder IV molecular weight marker; lane 2, control strain ANS46 (SCC*mec* type III; *ccrC*-positive); lane 3, control strain WIS (SCC*mec* type V, *ccrC*-positive); lane 4, control strain HDE228 (SCC*mec* type VI, *ccrC*-negative); lane 5, isolate S2.

As the multiplex PCR profile of S2 was not consistent with that of WIS, the SCC*mec* type V control strain (Figure 3.3), the 804 bp and 518 bp putative *mec* class C2 and *ccrC* PCR products were sequenced directly. Analysis of the sequencing data obtained for the 804 bp class C2 *mec* complex product showed that it was 99 % similar to that present in WIS (Appendix F); however, the sequencing data obtained for the 518 bp product were inconclusive. The identities of nucleotide bases were ambiguous at several identical positions on both the forward and reverse sequences, as shown in Figure 3.11.

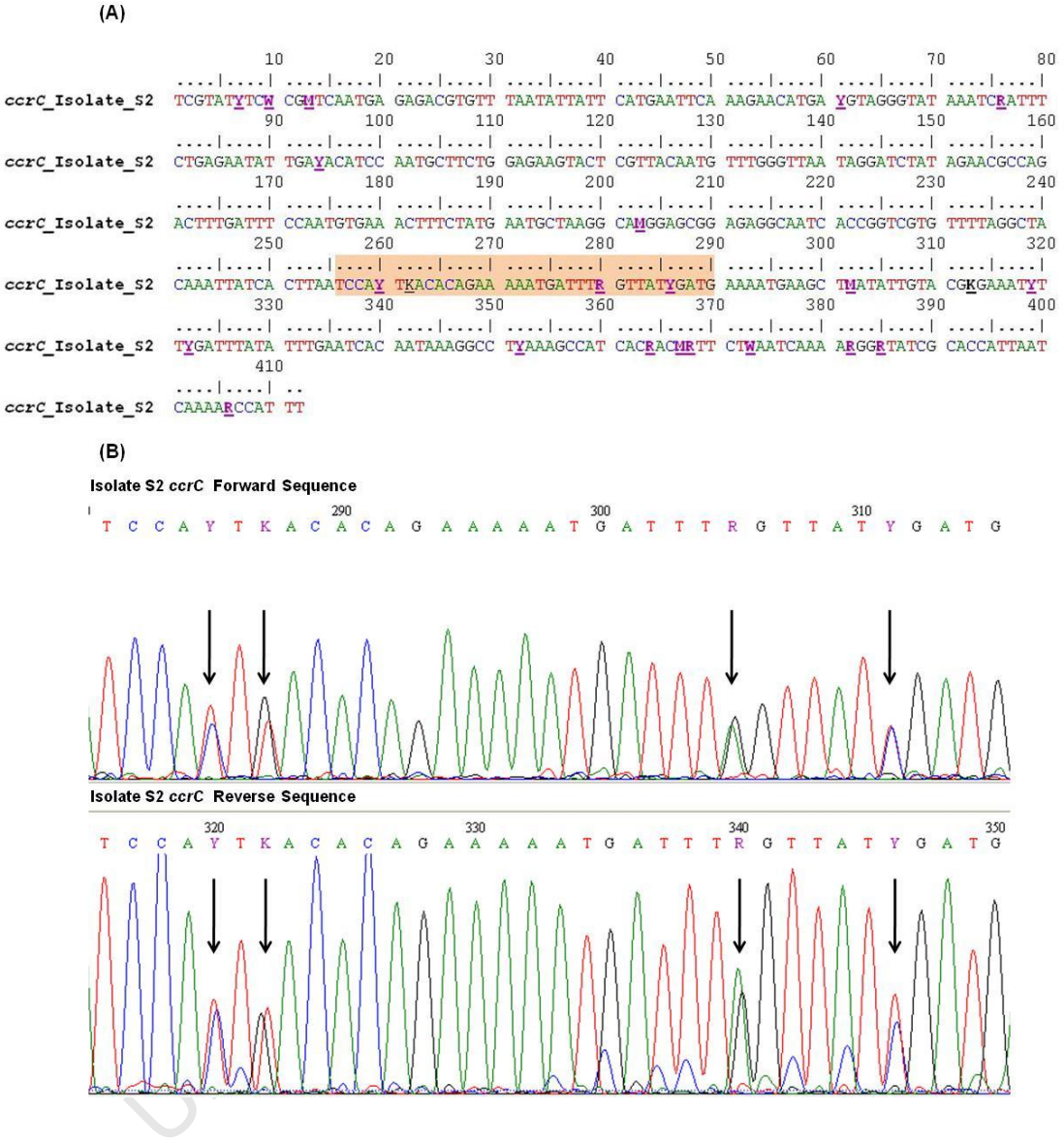


Figure 3.11 Nucleotide sequences of a portion of *ccrC* from sporadic isolate S2. (A) Trimmed nucleotide sequence generated from an alignment of the forward and reverse sequences of the 518 bp PCR product amplified from S2 using *ccrC*-specific primer pair γF/ γR. Identical ambiguous bases were detected on both the forward and reverse sequences (Y/K/R/M/W; shown in bold and underlined). **(B)** Ambiguous bases identified in (A) corresponded to identical double peaks at a single nucleotide position in the chromatograms of the forward and reverse sequences. This is illustrated for the portion of the sequence highlighted in orange in (A) with ambiguous bases indicated by downward arrows.

Scrutiny of the chromatograms revealed identical double peaks at corresponding nucleotide positions in the forward and reverse sequences, suggesting that the purified PCR products included at least two *ccrC* alleles (Figure 3.11). The detection of a class C2 *mec* complex in conjunction with *ccrC* suggested that S2 contained SCC*mec* type V; however, the assignment was tentative based on the detection of multiple copies of *ccrC* (Figure 3.4).

The typical multiplex PCR profile of SCC*mec* type V strains includes 449 bp, 377 bp and 162 bp products (Table 3.1); however, the amplification profile of S2 included only 2 of these products (449 bp and 162 bp), as well as 2 additional products (495 bp and 414 bp) not typically associated with SCC*mec* type V, as shown in Figures 3.3 and 3.12.

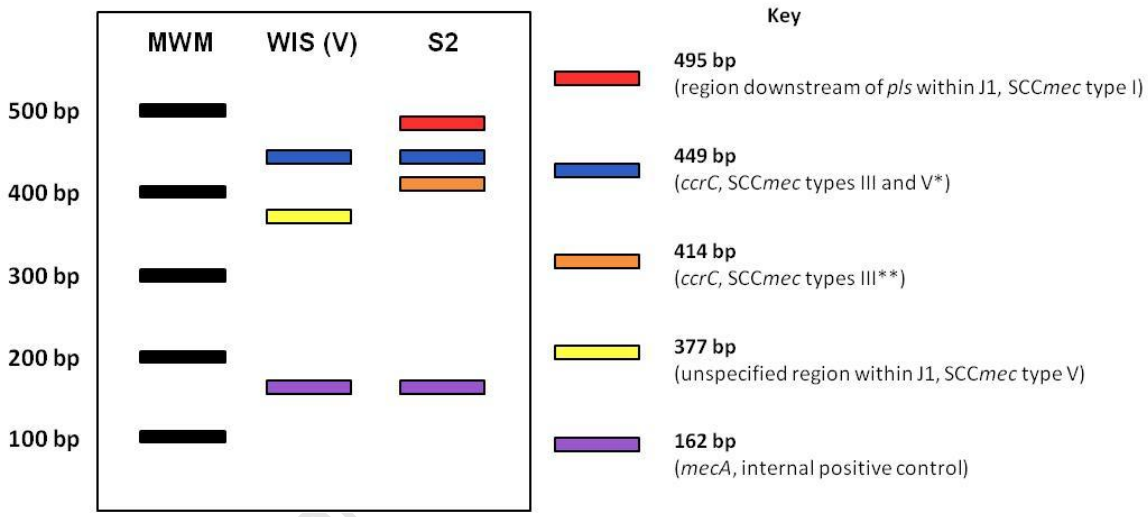


Figure 3.12 Schematic diagram depicting the atypical SCC*mec* typing multiplex PCR profile obtained for the putative SCC*mec* type V isolate, S2. The 377 bp amplicon typical of SCC*mec* type V was not detected in S2, and 2 additional amplicons that equated with the sizes of products typically amplified from SCC*mec* types other than V were present. Individual PCR assays including primers for the amplification of these corresponding products, followed by sequencing where necessary, confirmed the identities of atypical products, and also failed to detect a 377 bp fragment. *Emanating from primer pair ccrC2 F2/ccrC2 R2 (cross-complementary to *ccrC* present in SCC*mec* type V and in the SCC*Hg* component of SCC*mec* type III). **Emanating from RIF5 F10/RIF5 R13 (complementary to *ccrC* present in the SCC*Hg* component of SCC*mec* type III).

Given its atypical PCR profile, the amplicons generated for S2 during the SCC*mec* typing multiplex PCR were characterised further. Based on the detection of at least two *ccrC* alleles in S2, it was suspected that the 449 bp and 414 bp products emanated from the *ccrC*-specific primer pairs ccrC F2/ccrC R2 and RIF5 F10/RIF5 R13, respectively. This was confirmed using individual PCR assays that included these primer pairs, as shown in Figures 3.13 and 3.14).

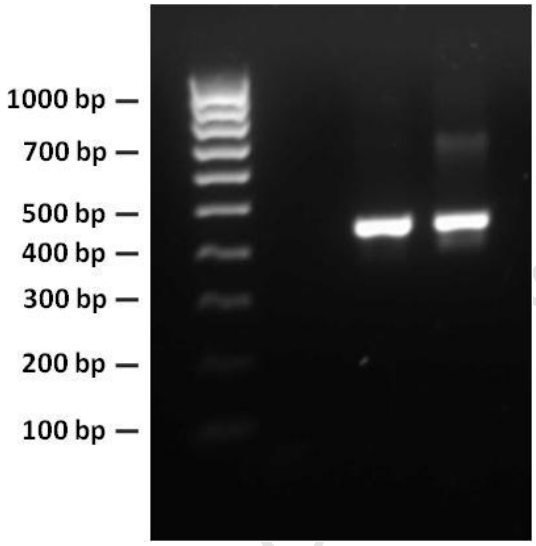


Figure 3.13 Agarose gel electrophoresis of amplicons obtained following PCR assays with the primer pair ccrC F2/ccrC R2 to confirm the origin of the 449 bp product detected in the multiplex PCR profile of the putative SCC*mec* type V isolate, S2. Lane 1, Hyperladder IV molecular weight marker; lane 2, COL (SCC*mec* type I; *ccrC*-negative); lane 3, WIS (SCC*mec* type V; *ccrC*-positive); lane 4, S2.

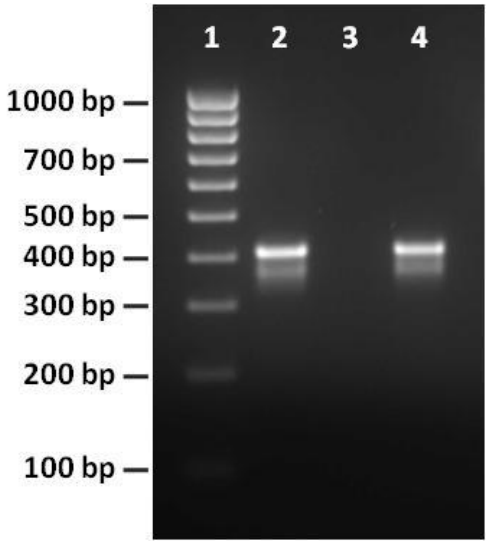


Figure 3.14 Agarose gel electrophoresis of amplicons obtained following PCR assays with the primer pair RIF5 F10/RIF5 R13 to confirm the origin of the 414 bp product detected in the multiplex PCR profile of the putative SCC*mec* type V isolate, S2. Lane 1, Hyperladder IV molecular weight marker; lane 2, ANS46 (SCC*mec* type III; *ccrC*-positive, detected by RIF5 F10/RIF5 R13); lane3, WIS (SCC*mec* type V; *ccrC*-positive, but not detected by RIF5 F10/RIF5 R13); lane 4, S2.

The 377 bp product typically amplified from the J1 region of SCC*mec* type V strains (Table 3.1; Figure 3.12) was not detected in the multiplex PCR profile of S2 (Figure 3.3). An individual PCR assay including only primer pair SCC*mec* V J1 F/SCC*mec* J1 R for the amplification of the 377 bp product was carried out; however, the 377 bp product was only amplified from the control strain, WIS, as indicated in Figure 3.15, suggesting that S2 may contain a modified J1 region.

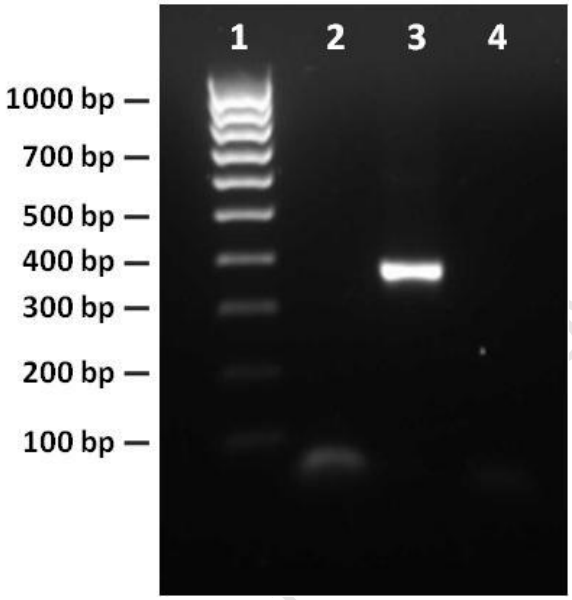


Figure 3.15 Agarose gel electrophoresis of amplicons obtained following PCR assays for the detection of a 377 bp portion of the SCC*mec* type V J1 region in the putative SCC*mec* type V isolate, S2. Lane 1, Hyperladder IV molecular weight marker; lane 2, control strain COL (SCC*mec* type I); lane 3, control strain WIS (SCC*mec* type V); lane 4, S2 (putative SCC*mec* type V).

The atypical 495 bp product detected in S2 corresponded to the expected size of the product amplified from the region downstream of *pls* in the J1 region of SCC*mec* type I (Figure 3.12; Table 3.1). An individual PCR assay including the primer pair CIF2 F2/CIF2 R2 complementary to this locus confirmed that the 495 bp product was indeed amplified from S2, but not from WIS, the SCC*mec* type V control strain (Figure 3.16). A BLAST query of the DNA sequence of the 495 bp product indicated a 96 – 100 % nucleotide identity with SCC*mec*-associated *pls* loci, suggesting the presence of a highly similar locus in S2.

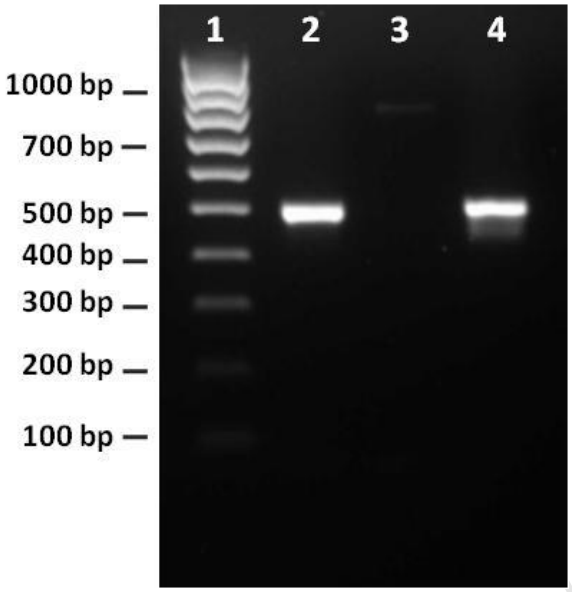


Figure 3.16 Agarose gel electrophoresis of amplicons obtained following PCR assays for the amplification of a 495 bp product, described as unique to SCC*mec* type I, in the putative SCC*mec* type V isolate, S2. Lane 1, Hyperladder IV molecular weight marker; lane 2, control strain COL (SCC*mec* type I); lane 3, control strain WIS (SCC*mec* type V); lane 4, S2.

In conclusion, using a number of PCR assays, including the multiplex method described by Milheiriço *et al.* (2007a) and the typing strategy of Kondo *et al.* (2007), SCC*mec* types were assigned to the 100 MRSA isolates. Forty-six isolates carried SCC*mec* type IV, 37 SCC*mec* type I, 12 SCC*mec* type II, 4 SCC*mec* type III and 1 carried a putative SCC*mec* type V element (Figure 3.4).

3.3.2 Subtyping of SCC*mec* type IV isolates

The multiplex PCR assay described by Milheiriço *et al.* (2007b) was used to subtype all SCC*mec* type IV isolates. The PCR profiles obtained for control strains IVa – IVE, IVg and IVh, and for a subset of local SCC*mec* type IV isolates, are shown in Figure 3.17. Forty-four of the 46 SCC*mec* type IV isolates contained subtype IVd. One of the two remaining MRSA isolates contained subtype IVh, while the PCR profile of the other isolate suggested the presence of subtype IVb or IVF. Based on the detection of the *dcs* locus in the SCC*mec* typing multiplex PCR, the subtype of the outstanding isolate was confirmed as IVb.

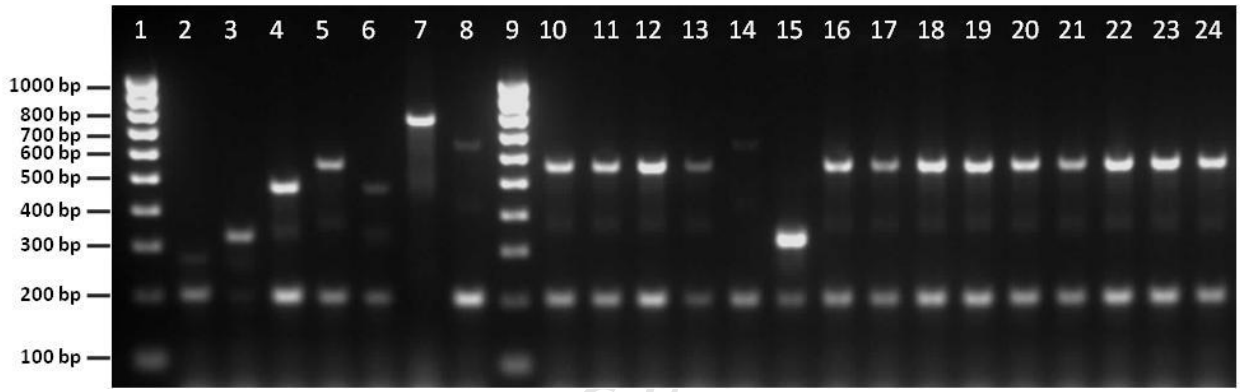


Figure 3.17 SCC*mec* type IV subtyping multiplex PCR profiles obtained for SCC*mec* type IV control strains and a representative selection of SCC*mec* type IV isolates from hospitals in Cape Town. Lanes 2 – 8 contain MRSA control strains with SCC*mec* type IV subtypes IVa – IVE, IVg and IVh; lanes 10 – 24 contain a selection of SCC*mec* type IV isolates obtained from hospitals in Cape Town. Lane 1, Hyperladder IV molecular weight marker; lane 2, JCSC4744 (subtype IVa); lane 3, JCSC2172 (subtype IVb); lane 4, DEN2949 (subtype IVc); lane 5, BK2529 (subtype IVd); lane 6, AR43/3330.1 (subtype IVE); lane 7, M03-68 (subtype IVg; 203 bp internal control amplicon absent because the 792 bp amplicon corresponding to a portion of the J1 region of IVg was used as template DNA); lane 8, HAR22 (subtype IVh); lane 9, Hyperladder IV molecular weight marker; lane 10, S1 (IVd); lane 11, S4 (IVd); lane 12, S5 (IVd); lane 13, S6 (IVd); lane 14, S7 (IVh); lane 15, S8 (IVb); lane 16, D1(IVd); lane 17, D2 (IVd) ; lane 18, D3 (IVd); lane 19, D4 (IVd); lane 20, D5 (IVd); lane 21, D6 (IVd); lane 22, D7 (IVd); lane 23, E1 (IVd); lane 24, E2 (IVd).

3.4 Discussion

Multiplex PCR assays are popular worldwide for SCC*mec* typing because they enable the rapid determination of SCC*mec* types in both the diagnostic and research environments (de Lencastre *et al.*, 2007); however, these assays are not without their pitfalls (Deurenberg and Stobberingh, 2008). Previous studies have incorrectly classified SCC*mec* elements due to primer cross-complementarity (Jansen *et al.*, 2009), which was also problematic in the current study, and the identification of atypical amplification patterns frequently forces researchers to report isolates as non-typable. The investigations required to resolve atypical multiplex PCR profiles are intricate, often necessitating long-range PCR or the creation of cosmid or fosmid libraries in order to determine the underlying genetic structure of the element (Shore *et al.*, 2005; Heusser *et al.*, 2007; Berglund *et al.*, 2008; Berglund *et al.*, 2009).

A further shortcoming of the majority of existing SCC*mec* typing methods is that they lack the capacity to characterise novel SCC*mec* types and subtypes. The most comprehensive typing strategy currently available is that of Kondo *et al.* (2007), which allows for the detection of novel combinations of *mec* complex and *ccr* allotype. The method includes six multiplex PCR assays, and, as a result, it is not practical for routine use (de Lencastre *et al.*, 2007; Deurenberg and Stobberingh, 2008); however, as shown in the current study, the method has value in the research setting. While multiplex strategies such as that of Kondo *et al.* (2007) may detect new combinations of *mec* complex and *ccr* allotype, the IWG-SCC does not deem this sufficient for the description of novel SCC*mec* types. The IWG-SCC currently recommends complete nucleotide sequencing of any putative novel SCC*mec* type or subtype inferred from multiplex profiles because PCR products represent only a small portion of the element, and are not absolute indicators of underlying nucleotide sequence identities (Ito *et al.*, 2009).

In the current study the SCC*mec* types of the 100 MRSA isolates were determined using two popular multiplex PCR assays (Kondo *et al.*, 2007; Milheiriço *et al.*, 2007a) and DNA sequencing. SCC*mec* types IV and I were most prevalent among local MRSA, accounting for forty-six and thirty-seven isolates, respectively. Twelve and four isolates contained SCC*mec* types II and III, respectively, while the remaining isolate carried a putative SCC*mec* type V element. It was unsurprising that SCC*mec*

types I, II and III collectively accounted for the majority of the isolates given that these elements have frequently been described in association with HA-MRSA isolates worldwide (de Lencastre *et al.*, 2007; Deurenberg and Stobberingh, 2008; Ratnaraja and Hawkey, 2008). Similarly, SCC*mec* types I, II and III also accounted for the collective majority of isolates included in the recent molecular characterisation of MRSA from public and private microbiology laboratories from throughout South Africa (Moodley *et al.*, 2010).

Examination of the SCC*mec* content of the MRSA in conjunction with the PFGE data indicated an absolute correlation between PFGE cluster and SCC*mec* type (Appendix A). The SCC*mec* type IV isolates were distributed across clusters D and E, and also included six sporadic isolates (S1, S4, S5, S6, S7 and S8). Clusters B and C contained all SCC*mec* type I isolates, while SCC*mec* type II and III isolates were assigned to clusters F and A, respectively (Appendix A). The remaining sporadic isolates, S2 and S3, contained the putative SCC*mec* type V element and SCC*mec* type II, respectively. Associations between PFGE clusters and particular SCC*mec* types were also reported by Moodley *et al.* (2010) following their recent study on the molecular epidemiology of MRSA from South Africa. In contrast, multiple SCC*mec* types have previously been reported within a single PFGE cluster (Laplana *et al.*, 2007). In the local context, a recent study on the molecular characterisation of MRSA from the KwaZulu-Natal province of South Africa reported up to four SCC*mec* types within a single PFGE profile (Shittu *et al.*, 2009). Whether these differences in associations between PFGE clusters and SCC*mec* types are due to the excision and subsequent replacement of SCC*mec* elements within particular MRSA lineages, or to variations in PFGE protocols and methods of data analysis remains to be determined.

Since the first description of SCC*mec* type IV in CA-MRSA isolated from children in a Chicago hospital (Daum *et al.*, 2002; Ma *et al.*, 2002), this SCC*mec* type has been described frequently in both communities and hospitals worldwide (McDougal *et al.*, 2003; Donnio *et al.*, 2004; Ma *et al.*, 2006; Taneike *et al.*, 2006; de Lencastre *et al.*, 2007; Laplana *et al.*, 2007; Mimica *et al.*, 2009; Shittu *et al.*, 2009; Strandén *et al.*, 2009). Perhaps unsurprisingly, therefore, SCC*mec* type IV was the dominant SCC*mec* element identified in the current study accounting for 46 % of the isolates, which accords with data from other regions of South Africa. Moodley *et al.* (2010)

reported that the national prevalence of SCC*mec* type IV was 38 %, and showed that the element was distributed throughout the country, while Shittu *et al.* (2009) and Essa *et al.* (2009) reported a prevalence of 62.3 % and 79 %, respectively, among MRSA from the KwaZulu-Natal province. The low frequency of SCC*mec* type IV (4 %) among MRSA from the Steve Biko Academic Hospital in Pretoria, in the Gauteng province of South Africa, was the only inconsistency in the literature (Makgotlho *et al.*, 2009).

SCC*mec* type IVd accounted for forty-four of the forty-six SCC*mec* type IV isolates (Appendix A), which included all isolates from PFGE clusters D and E. Internationally, SCC*mec* type IVd was common among MRSA obtained from Japanese hospitals during the early 1980s (Ma *et al.*, 2006), and has since been described in the epidemic USA500 clone (Zhang *et al.*, 2008), at a low frequency in MRSA from Israel (Maor *et al.*, 2009), in association with CC22 isolates from England and Wales (Boakes *et al.*, 2010), and also in MRSA isolated from horses and veterinary staff in the UK and Europe (Cuny *et al.*, 2006; Williams, 2008; Clegg, 2010). In South Africa, SCC*mec* type IV subtyping remains uncommon, with only the report of Makgotlho *et al.* (2009) including information obtained using this technique. Interestingly, that study reported that all four SCC*mec* type IV isolates detected among MRSA from the Steve Biko Academic Hospital, Pretoria, contained subtype IVd. The four isolates were classified as CA-MRSA based on the presence of SCC*mec* type IV and *pvl*, both of which are currently considered molecular markers of CA-MRSA (Millar *et al.*, 2007). This raised the question whether or not the SCC*mec* type IV isolates from Cape Town might also carry *pvl*, and may have originated in the community, which will be considered further in Chapter 4.

The two remaining SCC*mec* type IV isolates, S7 and S8 (Appendix A), contained subtypes IVh and IVb, respectively. Subtype IVb was first described in CA-MRSA from the USA (Ma *et al.*, 2002), but has rarely been reported since. In addition to containing the uncommon SCC*mec* type IVb, S8 was also the PFGE outlier and the only isolate pan-susceptible to non-β-lactam antibiotics, which collectively suggested that this isolate may have had a different origin to the other study isolates. The same may be true of S7, which contained subtype IVh. This subtype has been commonly described in association with the pandemic clone EMRSA-15 (Milheiriço *et al.*, 2007b), which was first reported in UK hospitals, and has since been identified in

several countries in HA-MRSA, CA-MRSA and MRSA from companion animals (Faria *et al.*, 2005; Moodley *et al.*, 2006; Strommenger *et al.*, 2006b; de Lencastre *et al.*, 2007; Boakes *et al.*, 2010).

The reasons for the prevalence of MRSA isolates carrying SCC*mec* type IV in Cape Town and other regions of South Africa are likely to be complex. It is possible that the prevalence of this element is related to the fact that SCC*mec* type IV is the most frequently transferred SCC*mec* element, and is thought to be associated with a lower fitness cost than the larger SCC*mec* elements (Ma *et al.*, 2002; Okuma *et al.*, 2002; Robinson and Enright, 2003). In light of the fact that 65.32 % of SCC*mec* type IV isolates obtained from public and private microbiology laboratories across South Africa belonged to two PFGE clusters (Moodley *et al.*, 2010), it is interesting to consider the possibility that the SCC*mec* type IV isolates from Cape Town may be related to those from other regions of the country; however, a complete molecular characterisation, incorporating sequence-based typing techniques, is required to resolve this issue, as will be discussed further in subsequent chapters of this thesis.

In terms of SCC*mec* epidemiology, one of the major differences observed in the present study was the relatively low prevalence of isolates containing SCC*mec* types II (12 %) and III (4 %) in hospitals in Cape Town compared to the nationwide prevalence rates of 25.94 % and 25.00 %, respectively (Moodley *et al.*, 2010). Closer examination of two regional studies indicated that, although SCC*mec* type II comprised a small proportion of MRSA from healthcare institutions in KwaZulu-Natal (Essa *et al.*, 2009; Shittu *et al.*; 2009), SCC*mec* type III was more prevalent in that province than in Cape Town, accounting for 26.5 % (Shittu *et al.*, 2009) and 13 % (Essa *et al.*, 2009) of the isolates. In contrast, SCC*mec* type II accounted for the majority (67 %) of the MRSA from the Steve Biko Academic Hospital in Pretoria, while SCC*mec* type III was second-most prevalent accounting for 14 % of the isolates (Makgotlho *et al.* 2009).

Perhaps the most striking difference between SCC*mec* epidemiology in Cape Town and the remainder of South Africa was the markedly varied frequencies of SCC*mec* type I isolates throughout the country. SCC*mec* type I was the second-most prevalent SCC*mec* element among MRSA from hospitals in Cape Town accounting

for 37 % of the isolates. This contrasted sharply with the work of Essa *et al.* (2009) and Makgotlho *et al.* (2009) who did not detect SCC*mec* type I isolates among MRSA from KwaZulu-Natal and Pretoria, respectively. Similarly, Shittu *et al.* (2009) only detected a single SCC*mec* type I isolate comprising 1.6 % of MRSA from healthcare institutions in KwaZulu-Natal. Based on the recent report by Moodley *et al.* (2010), the nationwide prevalence of SCC*mec* type I was only 10.3 %. Additionally, that study showed an uneven geographical distribution of isolates from PFGE clusters associated with SCC*mec* type I. Isolates carrying this SCC*mec* type were obtained from laboratories in all provinces except Gauteng and the Northern Cape, hinting at regional differences in SCC*mec* epidemiology (see Appendix G for a provincial map of South Africa). It should be noted that Moodley *et al.* (2010) did not indicate the proportion of isolates obtained from each province, and it is possible that the uneven distribution of MRSA clones observed in that study may reflect uneven provincial contributions. However, assuming that the contributions from each province were equal, these findings may suggest that MRSA lineages carrying SCC*mec* type I have not have been disseminated throughout South Africa. Alternatively, these lineages may have enjoyed a limited success, or may have been usurped by other clones in certain regions of the country.

The amplification of *ccrC* in thirty-six of the otherwise typical SCC*mec* type I isolates was unexpected as the gene is not commonly described in strains carrying this SCC*mec* element. A portion of the gene was amplified with primers specific for the *ccrC* allele present in SCC*Hg* of SCC*mec* type III, but not that present in SCC*mec* type V; therefore, it is possible to suggest that the *ccrC* allele present in local SCC*mec* type I isolates may be more similar to that present in SCC*Hg*. To the best of the author's knowledge this is the first report of SCC*mec* type I isolates carrying *ccrC* in South Africa as previous studies have not detected the gene, or reported SCC*mec* type I isolates with atypical multiplex PCR profiles. In the global context, to the best of the author's knowledge, the amplification of *ccrC* from SCC*mec* type I strains has only been reported in two isolates, one each from England and Egypt (Kondo *et al.*, 2007); however mosaic or composite elements including multiple *ccr* complexes are being described with increasing frequency in *S. aureus* and CNS (Heusser *et al.*, 2007; Higuchi *et al.*, 2008; Ito *et al.*, 2009; Chlebowicz *et al.*, 2010; Coombs *et al.*, 2010; Garza-González *et al.*, 2010).

Mosaic or composite SCC*mec* types consist of combinations of SCC(*mec*) elements, and occur in two similar but distinct forms. In the first, two SCC elements, both carrying *ccr* complexes, but typically only one carrying a *mec* complex, integrate in tandem into the *S. aureus* chromosome. These composite elements are defined by the presence of the characteristic 15 bp terminal direct repeat sequence at their internal junction, as illustrated by SCC*mec* type III, which consists of tandem copies of SCC*Hg* and SCC*mec* III. Alternatively, the two SCC elements may fuse to form a composite element in which no direct repeat sequence can be detected at the internal junction, as seen in SCC*mec* type VII (Ito *et al.*, 2009). It is possible that local SCC*mec* type I isolates in fact contain one of these two types of composite elements including SCC*mec* type I, and another *ccrC*-driven SCC element; however, the possibility that *ccrC* is located elsewhere on the bacterial chromosome cannot be excluded at present.

It is interesting to speculate on the absence of *ccrC* in isolate B1, the only typical SCC*mec* type I isolate, in the context of its relatedness to *ccrC*-positive B2. Isolates B1 and B2 were defined as a two-member PFGE cluster due to their similarity level of 80.01 %. It is possible that B1, or its recent ancestors, contained *ccrC*, but subsequently lost the region, which would explain the presence of *ccrC* in isolate B2. On the other hand, the similarity level defining cluster B is only marginally higher than the recommended 80 % cluster threshold (Murchan *et al.*, 2003), and there are marked differences between the macrorestriction profiles of B1 and B2; therefore, it is interesting to consider the possibility that these isolates may in fact be less similar than estimated by PFGE. Although it is unusual to overestimate the relatedness of isolates using PFGE, it is possible that the similarities in the macrorestriction profiles of B1 and B2 did not reveal underlying diversity in the DNA sequences. If this is the case, *ccrC* may only have been acquired after B1 and the *ccrC*-positive SCC*mec* type I isolates diverged from a common ancestor. The additional investigations required to determine the precise location of *ccrC*, characterise the potential composite element, and explain the absence of a corresponding amplicon in isolate B1 would necessitate sequencing of the entire SCC*mec* element and its flanking chromosomal regions, which was not possible given the time and funding available for this project.

One isolate, S2, was tentatively classified as containing SCC*mec* type V, based on the detection of the class C2 *mec* complex in conjunction with *ccr* allotype 5 (*ccrC*). SCC*mec* type V is commonly associated with CA-MRSA (de Lencastre *et al.*, 2007; Tristan *et al.*, 2007b; Deurenberg and Stobberingh, 2008; Ratnaraja and Hawkey, 2008), and, to the best of the author's knowledge, has not been previously described in South Africa (Essa *et al.*, 2009; Shittu *et al.*, 2009; Makgotlho *et al.*, 2009; Moodley *et al.*, 2010). The detection of at least two copies of *ccrC* in S2 was not wholly unexpected as there have recently been several reports of SCC*mec* type V isolates carrying multiple *ccrC* alleles (Higuchi *et al.*, 2008; Chlebowicz *et al.*, 2010; Coombs *et al.*, 2010). On the other hand, it is also possible that S2 may contain a novel composite SCC*mec* element with multiple *ccrC* alleles.

As recommended by the IWG-SCC (Ito *et al.*, 2009), S2 was classified based on the combination of *mec* complex and *ccr* allotype detected, but queries regarding the composition and identity of this SCC*mec* element were raised based on the results of investigations into its atypical multiplex PCR profile. Additional PCR assays for the detection of a portion of the SCC*mec* type V J1 region did not detect this region in S2; therefore, it is possible to suggest that S2 contains a variant of SCC*mec* type V with a modified J1 region. Alternatively, it is possible that the SCC*mec* element present in S2 does not correspond to SCC*mec* type V. The detection of the *pls* locus, characteristic of SCC*mec* type I, was also unexpected (Oliveira and de Lencastre, 2002; Milheiriço *et al.*, 2007a; Werbick *et al.*, 2007); this locus has been detected in an SCC*mec* type IV isolate (Werbick *et al.*, 2007), and also in SCCmec type II and III isolates (Deschamps *et al.*, 2003) but, to the best of the author's knowledge, has not been described in association with SCC*mec* type V. As recommended by the IWG-SCC (Ito *et al.*, 2009), sequencing of the entire SCC*mec* element and its flanking regions is necessary to definitively determine the type of the SCC*mec* element present in isolate S2 and, as previously discussed, these experiments were beyond the scope of this thesis.

CHAPTER 4

The Identification of Community- and Hospital-Acquired Methicillin-Resistant *S. aureus*

4.1 Introduction

Community-associated MRSA (CA-MRSA) was first reported in the early 1990s in Western Australia. Those infected were previously healthy members of indigenous populations living in remote communities who had no known risk factors for MRSA infection (DeLeo *et al.*, 2010). In the past decade, there have been increasingly frequent reports of CA-MRSA infections worldwide, particularly in the USA, Europe and Oceania (Millar *et al.*, 2007; Deurenberg and Stobberingh, 2008; Witte, 2009; DeLeo *et al.*, 2010). CA-MRSA primarily tends to cause skin and soft tissue infections, but has also been implicated in more severe systemic infections such as sepsis, pneumonia and necrotising fasciitis (Millar *et al.*, 2007; Witte, 2009; Cooke and Brown, 2010). The additional virulence factors present in many CA-MRSA lineages cause these strains to be more virulent than their HA-MRSA counterparts, resulting in severe disease (Deurenberg and Stobberingh, 2008; DeLeo *et al.*, 2010).

Studies on the molecular evolution of CA-MRSA have indicated that these lineages are distinct from HA-MRSA lineages, and are also more diverse (Groom *et al.*, 2001; Naimi *et al.*, 2001; Enright *et al.*, 2002; Okuma *et al.*, 2002; Vandenesch *et al.*, 2003; Tristan *et al.*, 2007b; Deurenberg and Stobberingh, 2008; Elston and Barlow, 2009). Certain CA-MRSA clones belong to unique lineages which have not given rise to HA-MRSA, such as ST59, ST152 and ST80, while others appear to have emerged independently in lineages known to contain HA-MRSA clones, such as ST1, ST5, ST8, ST22 and ST30 (Deurenberg and Stobberingh, 2008; Witte, 2009; Lindsay, 2010). Table 4.1 provides an overview of the major CA-MRSA clones and their geographical distributions.

Table 4.1 An overview of important community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) clones[a]

Clone[b]	Clonal complex	Panton-Valentine leukocidin	Common names	Geographical distribution[c]
ST1-MRSA-IV	1	Positive/ negative	USA400/ WA-MRSA-1	Ast, Can, Fin, Fra, Ger, Sin, Swi, Uru, USA
ST8-MRSA-IV	8	Positive	USA300	Ast, Can, Fin, Fra, Ger, Sin, Swi, Uru, USA
ST30-MRSA-IV	30	Positive	Southwest Pacific/ USA1100	Ast, Aus, Bel, Bra, Chn, Den, Fin, Egy, Ger, Ire, Jap, Lat, Net, Nor, NZ, Rus, Sin, Spa, Swe, Swi, UK, Uru, USA
ST59-MRSA-IV/V	59	Positive	USA1000	Ast, Chn, Fra, Ger, Net, Nor, Sin, Swe, Tai, USA
ST80-MRSA-IV	80	Positive	European	Alg, Ast, Aus, Bel, Bul, Cro, Den, Fin, Fra, Ger, Gre, Ire, Lib, Net, Nor, Rom, Sin, Slo, Spa, Swe, Swi, Tun, UK, Yug

[a] Adapted from Deurenberg and Stobberingh (2008); Elston and Barlow (2009); DeLeo *et al.* (2010).

[b] Clone name as determined using the universal nomenclature proposed by Enright *et al.* (2002) following the form (Sequence Type-methicillin resistance phenotype-SCC*mec* type).

[c] Alg, Algeria; Ast, Australia; Aus, Austria; Bel, Belgium; Bra, Brazil; Bul, Bulgaria; Can, Canada; Chn, China; Cro, Croatia; Den, Denmark; Egy, Egypt; Fin, Finland; Fra, France; Ger, Germany; Gre, Greece; Ire, Ireland; Isr, Israel; Jap, Japan; Kor, Korea; Lat, Latvia; Lib, Libya; Net, Netherlands; NZ, New Zealand; Nor, Norway; Rom, Romania; Rus, Russia; Sin, Singapore; Spa, Spain; Swe, Sweden; Swi, Switzerland; Tai, Taiwan; Tun, Tunisia; UK, United Kingdom; Uru, Uruguay; USA, United States of America; Yug, Yugoslavia.

Several molecular markers have traditionally been associated with CA-MRSA, and have been used to distinguish these strains from their HA-MRSA counterparts (Millar *et al.*, 2007). CA-MRSA have typically been associated with the smaller SCC*mec* elements, such as SCC*mec* types IV, V and VII, while HA-MRSA generally carry SCC*mec* types I – III (Table 4.1) (Deurenberg and Stobbering, 2008; Cooke and Brown, 2010; DeLeo *et al.*, 2010; Nastaly *et al.*, 2010). CA-MRSA isolates have frequently been described as susceptible to non-β-lactam antibiotics, while HA-MRSA isolates are often resistant to several classes of antimicrobial agents (Millar *et al.*, 2007; Deurenberg and Stobbering, 2008; Cooke and Brown, 2010; Nastaly *et al.*, 2010). Additionally, the Panton-Valentine leukocidin (PVL) toxin has been described in frequent association with major CA-MRSA clones (Table 4.1) (Vandenesch *et al.*,

2003; Millar *et al.*, 2007; Tristan *et al.*, 2007a; Tristan *et al.*, 2007b; Deurenberg and Stobbering, 2008; Diep and Otto, 2008; Cooke and Brown, 2010; DeLeo *et al.*, 2010).

PVL is encoded by the *lukPV* operon (also known as *pvl*), which is carried by several prophages (Diep and Otto, 2008; Malachowa and DeLeo, 2010). The *pvl* operon consists of the co-transcribed *lukS-PV* and *lukF-PV* genes, which encode the two subunits of the pore-forming toxin (Boyle-Vavra and Daum, 2007; Nastaly *et al.*, 2010). The LukS-PV and LukF-PV subunits are secreted by the bacterial cell, bind to each other, and then assemble into heptamers, which localise to the membranes of polymorphonuclear leukocytes (PMNs) (Nastaly *et al.*, 2010). Once bound to the cell membrane, PVL induces pore formation which, at low PVL concentrations, causes apoptosis or, at high PVL concentrations, cell lysis (Boyle-Vavra and Daum, 2007; Nastaly *et al.*, 2010). It is thought that PVL-mediated apoptosis and lysis of PMNs, enables CA-MRSA to evade the host's innate immune response; however, the precise role of PVL in the pathogenesis of CA-MRSA infections is not yet fully understood (Boyle-Vavra and Daum, 2007). It has been suggested that sepsis and tissue necrosis are indirectly mediated either by an inflammatory cascade induced by apoptosis or lysis of immune cells, or by cytotoxic elements released by lysed components of the immune system (Kaneko and Kamio, 2004).

The contribution of PVL to *S. aureus* pathogenesis remains controversial as several studies have reported conflicting results (Deurenberg and Stobberingh, 2008). A study carried out using isogenic *pvl* MRSA strains in an acute pneumonia mouse model showed that only mice infected with *pvl*-positive MRSA developed necrotising pneumonia (Labandeira-Rey, 2007). This contrasted with studies reported by Saïd-Salim *et al.* (2005) and Voyich *et al.* (2006). Saïd-Salim *et al.* (2005) found no difference in the ability of *pvl*-positive and *pvl*-negative clinical CA-MRSA to lyse human PMNs. Similarly, Voyich *et al.* (2006) found no difference in the survival of mice infected with *pvl*-positive and *pvl*-negative MRSA in a mouse sepsis model, and, in a mouse abscess model, abscesses caused by *pvl*-negative strains were in fact slightly larger than those caused by *pvl*-positive strains. In the same study, the authors reported no difference in the ability of isogenic *pvl* strains of the epidemic CA-MRSA clones USA300 and USA400 to lyse PMNs. Studies have also provided evidence that the virulence of CA-MRSA can be attributed to factors other than *pvl* (Deurenberg and Stobberingh, 2008; Gordon and Lowy, 2008). These factors

include the arginine catabolic mobile element (ACME), other toxins such as the α-toxin, and secreted phenol-soluble modulins able to lyse human neutrophils, which have been found in epidemic CA-MRSA clones such as USA300 and USA400 (Deurenberg and Stobberingh, 2008; Gordon and Lowy, 2008). It has also been suggested that differential expression of virulence determinants may play a role in the pathogenesis of certain CA-MRSA strains (Li *et al.*, 2009).

While the contribution of PVL to the virulence of CA-MRSA is currently under debate, numerous studies have provided epidemiological data suggesting an association between PVL and CA-MRSA. Diep and Otto (2008) carried out a PubMed search for articles on *pvl* published between 2002 and 2007, and found over 250 articles describing an association between *pvl* and CA-MRSA outbreaks. Although there is strong epidemiological evidence supporting an association between CA-MRSA and *pvl*, recent reports suggest that the association is not universal as was initially assumed, with *pvl*-negative CA-MRSA described in Australia, Japan, Korea, Madagascar and parts of Europe (Coombs *et al.*, 2004; Deurenberg and Stobberingh, 2008; Nimmo and Coombs, 2008; DeLeo *et al.*, 2010).

It is currently recommended that isolates be defined as CA- or HA-MRSA based on a combination of their epidemiological origin, which is most commonly ascertained using criteria developed by the Centers for Disease Control and Prevention (CDC) (Figure 4.1), and the presence of distinguishing molecular characteristics typical of these two groups (Millar *et al.*, 2007; Elston and Barlow, 2009). The classification of CA- and HA-MRSA has become increasingly complicated in recent years as the distinctions between these groups have started to blur (Millar *et al.*, 2007; Deurenberg and Stobberingh, 2008). Strains with molecular characteristics associated with CA-MRSA have been reported in the healthcare setting, and, conversely, strains with characteristics typical of HA-MRSA have become prevalent in communities, revealing inadequacies in the existing terminology (Millar *et al.*, 2007; Deurenberg and Stobberingh, 2008; Nimmo and Coombs, 2008; Ratnaraja and Hawkey, 2008; Elston and Barlow, 2009; Witte, 2009). In this thesis, CA-MRSA and HA-MRSA will only be used to denote strains known to have evolved in the community and healthcare settings, respectively. As described by Witte (2009) and summarised in Figure 4.1, an additional set of abbreviations may be used to indicate the epidemiological origin of MRSA isolates, regardless of their evolutionary origins.

For the purposes of this thesis, ca- and ha-MRSA will denote community- and hospital-acquired MRSA, respectively, regardless of their molecular characteristics. In this context, ha-MRSA will be used to describe nosocomial infections, as well as community-onset infections in patients with one or more of the risk factors for nosocomial infection.

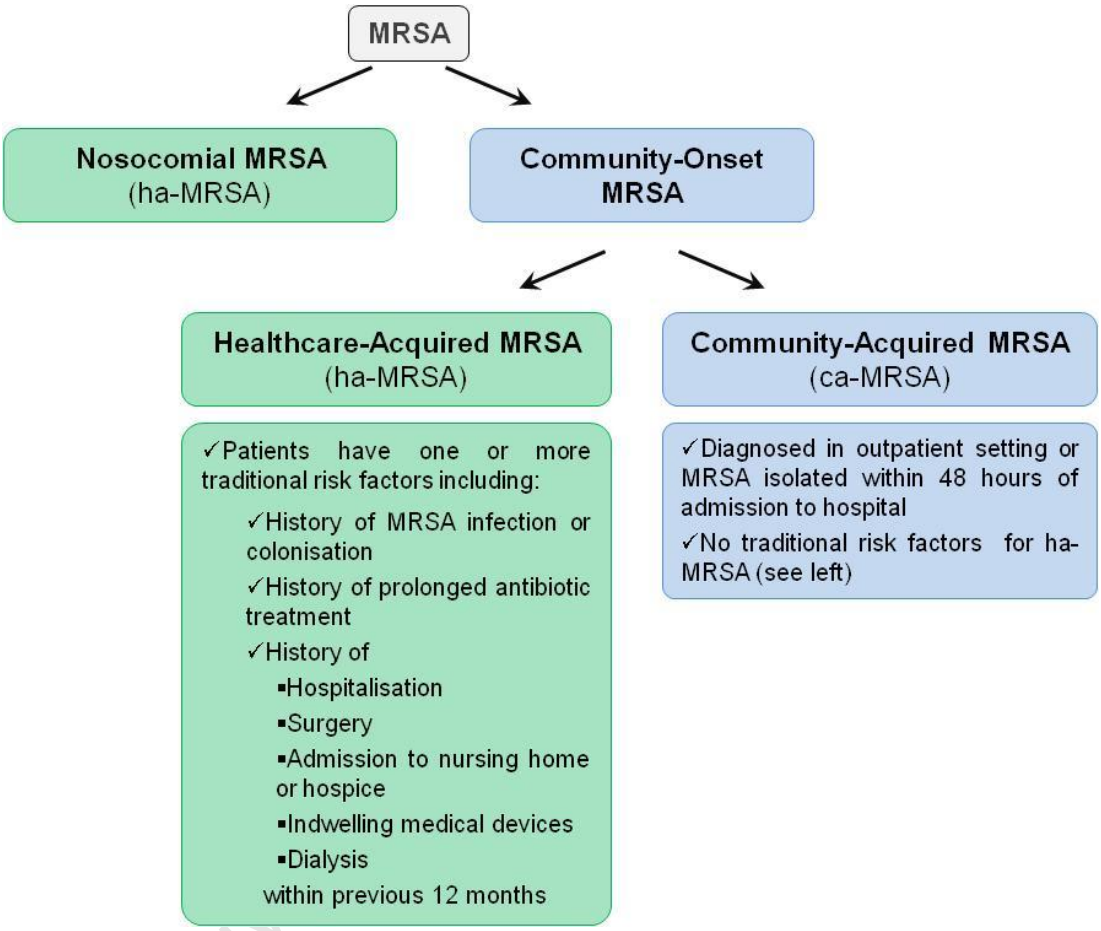


Figure 4.1 Definitions and Centers for Disease Control and Prevention (CDC) criteria used to distinguish methicillin-resistant *S. aureus* (MRSA) with different epidemiological origins. In this thesis, the term ha-MRSA will be used to indicate both nosocomial MRSA and community-onset MRSA isolated from patients with one or more of the traditional risk factors for healthcare-acquired MRSA. Adapted from Millar *et al.* (2007) and Witte (2009).

The SCC*mec* types detected in Chapter 3 raised questions regarding the origins of several isolates. Of particular interest was the large number of isolates with SCC*mec* type IV, given that this SCC*mec* element is considered a common molecular marker of CA-MRSA (Millar *et al.*, 2007). SCC*mec* type IVd accounted for the majority of the SCC*mec* type IV isolates and, interestingly, all SCC*mec* type IVd isolates described

to date in South Africa have contained *pvl*, (Makgotlho *et al.*, 2009), which is also considered a common molecular marker of CA-MRSA (Millar *et al.*, 2007). Although drawing epidemiological distinctions between local ca- and ha-MRSA was not the focus of this study, available admissions data were used to classify the MRSA isolates as ca-MRSA or ha-MRSA. Additionally, all isolates were screened for the presence of *pvl*.

4.2 Experimental Protocol

4.2.1 Identification of community- and hospital-acquired MRSA based on admissions data

The CDC defines ca-MRSA as MRSA isolated within 48 hours of admission to hospital, or in an outpatient setting, from patients who have not been hospitalised, admitted to a nursing home or hospice, or undergone dialysis within the previous 12 months. Additionally, the patient should not have a medical history of MRSA infection or colonisation, and should not have permanent medical devices that pass through the skin (Millar *et al.*, 2007). As indicated, it was not the aim of this study to compare ca- and ha-MRSA; nevertheless a rudimentary approach was used to define isolates based on electronic admission records.

The electronic admission records were reviewed and isolates defined as either ca- or ha-MRSA by Dr Andrew Whitelaw (consultant microbiologist in the NHLS laboratory, GSH). In accordance with the CDC definition, MRSA isolated within 48 hours of hospital admission from patients who had not been hospitalised or treated in an outpatient clinic within the previous 12 months, was classified as ca-MRSA, and all other isolates were classified as ha-MRSA. It should be noted that the electronic admission records only detail admission data and outpatient visits related to state healthcare facilities in Cape Town, and thus do not include a history of admission to private healthcare facilities, nursing homes or healthcare facilities outside of Cape Town. Likewise, data regarding the presence of indwelling medical devices was not available; however, one can assume that a patient with no admissions or outpatient visits within the previous 12 months was probably unlikely to have had an indwelling medical device. Additionally, patients should not have been colonised with MRSA but, as patients are not screened on admission to hospitals in Cape Town, a history of colonisation would not have been detected.

4.2.2 Detection of *pvl*

The PCR assay for the detection of *pvl* described by Lina *et al.* (1999) was used in this study. The method was optimised by Dr Sumayya Haffejee who screened 45 of the study isolates. The remaining MRSA isolates were screened by the author. The PCR was carried out in a volume of 50 µl with a final concentration of 1 X PCR

buffer, 1.5 mM $MgCl_2$, 1.25 U of SuperTherm *Taq* polymerase (JMR Holdings, London, UK), and 200 µM dNTP mix (Thermo Scientific, Wilmington, DE, USA). The primers *luk-PV-1* and *luk-PV-2* (Appendix E) were included at a final concentration of 0.8 µM each for the co-amplification of *lukS-PV* and *lukF-PV*. Thereafter, 5 ng of template DNA were added to the reaction volume. PCR amplification was carried out in an Applied Biosystems 2720 Thermocycler (Applied Biosystems, Carlsbad, CA, USA) and the parameters were as follows: an initial denaturation step for 3 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C, and a final extension step of 1 min at 72°C. The *S. aureus* control strains ATCC49775 and ATCC43300 were included in each experiment as positive and negative controls respectively. PCR products were visualised using UV transillumination and photographed after electrophoresis on a 2 % agarose gel [3.2.2].

4.3 Results

4.3.1 Identification of community- and hospital-acquired MRSA based on admissions data

Using the criteria described in section 4.2.1, 10 isolates were identified as putative ca-MRSA (Table 4.2). These isolates were obtained from patients at RCCH (*n* = 6), GSH (*n* = 3) and MMH (*n* = 1) and were cultured from pus and pus swabs (*n* = 6), urine (*n* = 3) and sputum (*n* = 1). Available collection data for the putative ca-MRSA isolates are shown in Table 4.2, with full details shown in Appendix A.

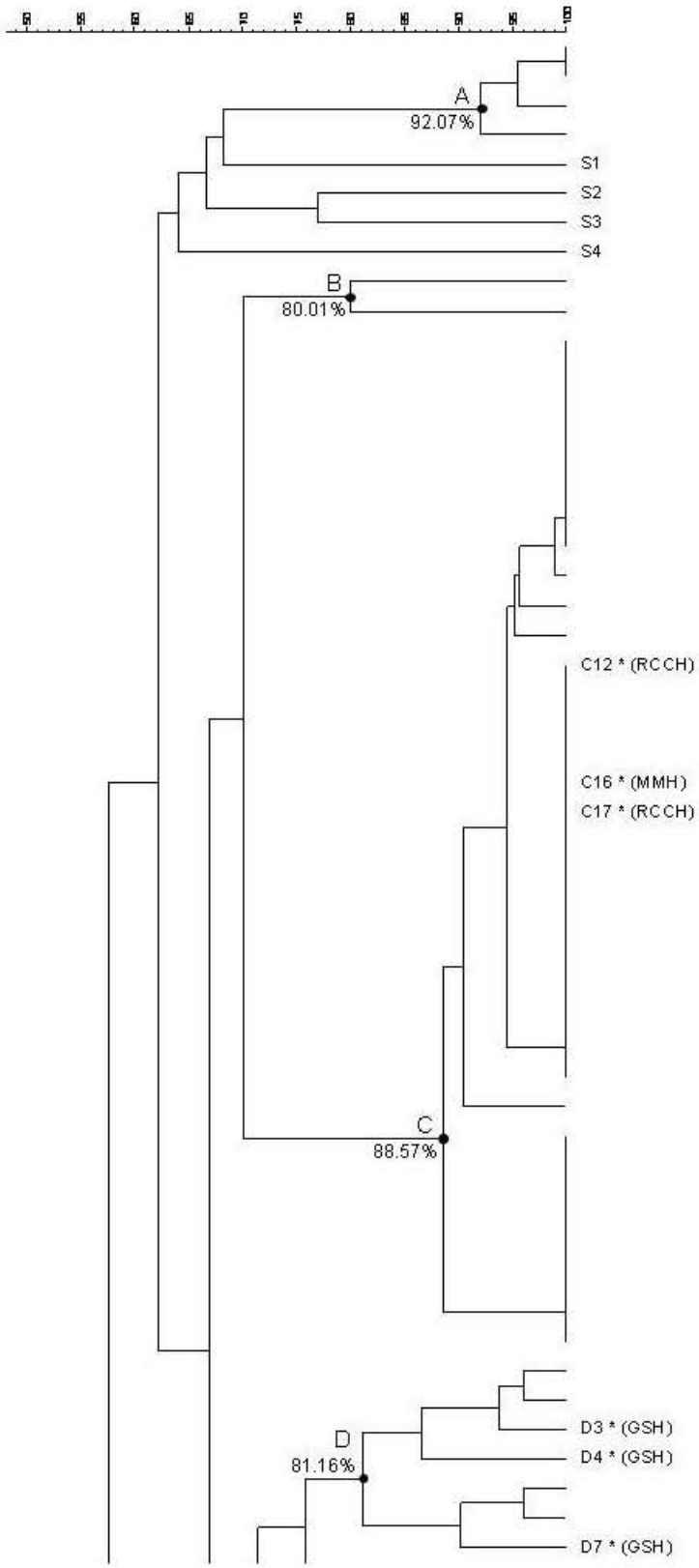
Table 4.2. Molecular typing and laboratory data for putative community-acquired methicillin-resistant *S. aureus*

MRSA Isolate [a]	PFGE Cluster; (SCC*mec* type) [b]	Hospital	Ward	Specimen Type
C12	C (I)	RCCH	Data not provided	Pus Swab
C16	C (I)	MMH	Gynaecology and Obstetrics	Urine
C17	C (I)	RCCH	Outpatient Clinic	Urine
D3	D (IVd)	GSH	Medical	Pus Swab
D4	D (IVd)	GSH	Emergency Services	Sputum
D7	D (IVd)	GSH	Medical	Pus Swab
E1	E (IVd)	RCCH	ICU	Pus Swab
E6	E (IVd)	RCCH	ICU	Pus
E22	E (IVd)	RCCH	Emergency Services	Urine
E25	E (IVd)	RCCH	Surgical	Pus Swab

[a] Strain designations as introduced in Chapter 2.

[b] PFGE, pulsed-field gel electrophoresis; SCC*mec*, staphylococcal cassette chromosome *mec*.

The ca-MRSA isolates were assigned to PFGE clusters C (*n* = 3), D (*n* = 3) and E (*n* = 4), and the distribution of these MRSA across PFGE clusters is indicated in Figure 4.2. It was noted that all ca-MRSA isolates assigned to cluster D were obtained from patients at GSH, and were susceptible to ciprofloxacin, whereas those assigned to cluster E were from patients at RCCH, and all but one were ciprofloxacin-resistant (Appendix A).


A
92.07%
S1
S2
S3
S4
B
80.01%
C12 * (RCCH)
C16 * (MMH)
C17 * (RCCH)
C
88.57%
D3 * (GSH)
D
81.16%
D4 * (GSH)
D7 * (GSH)

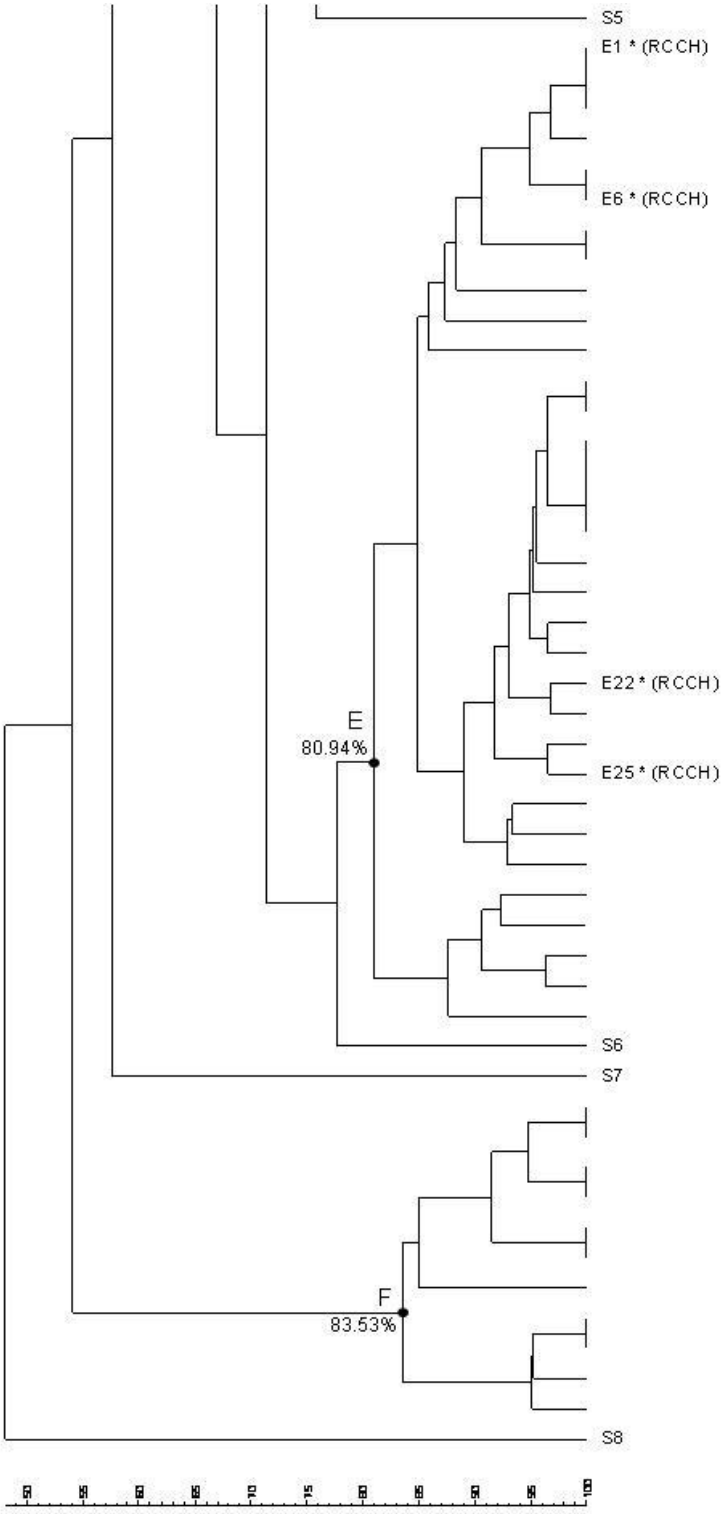


Figure 4.2 Dendrogram indicating the levels of genetic similarity between putative community- and healthcare-acquired methicillin-resistant *S. aureus* identified among 100 MRSA isolated from patients in hospitals in Cape Town. Nodes and similarity levels of PFGE clusters A – F are marked on the dendrogram, and sporadic isolates are marked using strain designations introduced in Chapter 2. Scale bars above and below the dendrogram indicate levels of similarity (%) between isolates. Strain designations of the 10 putative community-acquired MRSA isolates are marked with an asterisk, and the hospital of origin indicated in brackets.

4.3.2 Detection of *pvl*

The 433 bp region of the *pvl* locus was not detected in any of the 100 isolates. A 433 bp product was obtained from the *pvl*-positive control strain but not from the *pvl*-negative control strain. The integrity of the template DNA was confirmed by the PCR amplification of SCC*mec* components [Chapter 3]. These controls served to corroborate the negative PCR results.

4.4 Discussion

It was not the aim of this study to investigate the differences between local ca- and ha-MRSA; however, the fact that SCC*mec* types IV and V are considered molecular markers of CA-MRSA suggested that a large proportion of the study isolates may have been acquired in the community (Millar *et al.*, 2007). Alternatively, if the isolates were true ha-MRSA, a CA-MRSA clone may have taken hold in local hospitals. The classification of isolates as ca- or ha-MRSA, albeit rudimentary, identified only ten putative ca-MRSA isolates, suggesting that most of the study isolates were in fact ha-MRSA.

The *pvl* gene was not detected in any of the study isolates, including the ca-MRSA. While there have been few studies describing the molecular characteristics of MRSA from South Africa and the rest of Africa, this gene has been described in ca-MRSA from Algeria (Tristan *et al.*, 2007b), Nigeria (Ghebremedhin *et al.*, 2009) and Egypt (Enany *et al.*, 2010), in ca- and ha-MRSA from Senegal (Breurec *et al.*, 2010), and also infrequently in MRSA of unknown epidemiological origins from South Africa (Makgotlho *et al.*, 2009; Moodley *et al.*, 2010). Although the presence of *pvl*, SCC*mec* type IV and a multiply-susceptible antimicrobial profile have been used as markers of CA-MRSA, studies suggest that they are at best only guides to the origins of MRSA isolates. Firstly, there have been reports of *pvl*-negative CA-MRSA (O'Brien *et al.*, 2004; Park *et al.*, 2007a; Rossney *et al.*, 2007; DeLeo *et al.*, 2010) and, to further dispute the definition, there have been reports of *pvl*-positive HA-MRSA (Taneike *et al.*, 2006; Deurenberg and Stobberingh, 2008; Ratnaraja and Hawkey, 2008). The larger SCC*mec* elements, including SCC*mec* type I, identified in three local putative ca-MRSA isolates (Table 4.2), and SCC*mec* types II and III, were considered markers of HA-MRSA; however they have also been described in CA-MRSA (Deurenberg and Stobberingh, 2008). Conversely, SCC*mec* type IV, identified in seven of the local putative ca-MRSA isolates (Table 4.2), does not appear restricted to CA-MRSA as it has also been reported in the hospital setting (Donnio *et al.*, 2004; Ma *et al.*, 2006; Taneike *et al.*, 2006; de Lencastre *et al.*, 2007; Mimica *et al.*, 2009; Shittu *et al.*, 2009; Strandén *et al.*, 2009). Studies have also shown that antimicrobial susceptibility profiles of CA-MRSA may vary according to geographic origin (Millar *et al.*, 2007). These data indicate that the distinctions between HA- and CA-MRSA have started to blur (Deurenberg and Stobberingh, 2008). As suggested by Millar *et al.* (2007), ca- and ha-MRSA should not be

classified on the basis of molecular characteristics alone, but in conjunction with epidemiological criteria, which stresses the importance of a comprehensive clinical history.

It was perhaps surprising that the ten putative CA-MRSA isolates did not include any of the sporadic isolates (Figure 4.2). The relatively low levels of genetic similarity between the sporadic and clustered isolates suggested that the sporadic isolates may have had a different origin, possibly in the community. S8 and S2 were the obvious examples. S8 was the PFGE outlier, contained SCC*mec* type IV and was pan-susceptible to non-β-lactam antibiotics; S2 contained a putative SCC*mec* type V element, which is commonly associated with CA-MRSA and, with the exception of erythromycin and clindamycin, was susceptible to the non-β-lactam antibiotics. Taken together, these characteristics suggested that S8 and S2 might well have been acquired in the community. Rather, the designated community-acquired isolates were distributed across PFGE clusters C, D and E, suggesting that ca- and ha-MRSA from Cape Town are highly similar with respect to SCC*mec* content and overall genetic identity. The mathematical modeling study of D'Agata *et al.* (2009) predicted that CA-MRSA will gradually replace HA-MRSA in hospitals in the USA, and this will likely also prove true in other countries. This prediction seems to be supported by recent studies that have reported the introduction of CA-MRSA clones into healthcare facilities (Seybold *et al.*, 2006; Millar *et al.*, 2007; Nimmo and Coombs, 2008; Popovich *et al.*, 2008; Elston and Barlow, 2009; Witte, 2009); similarly, HA-MRSA clones have also been reported in the community (Coombs *et al.*, 2004; Elston and Barlow, 2009; Witte, 2009). It is possible that "cross-pollination" of MRSA between hospitals and communities in Cape Town may have resulted in the high levels of genetic similarity observed between ca- and ha-MRSA isolates in this study.

In spite of the possible equivocal classification of the ca-MRSA, it was instructive to analyse the PFGE results in conjunction with the collection data. With respect to the seven ca-MRSA containing SCC*mec* type IV, three belonged to cluster D and were isolated from adult patients at GSH, whereas the remaining four belonged to cluster E and were isolated from children at RCCH (Table 4.2). The cluster D isolates were susceptible to ciprofloxacin, while those from cluster E were resistant to this antibiotic (Appendix A). Relatively few studies have been carried out to investigate the

differences in epidemiology of ca-MRSA from adults and children but, in one such study, David *et al.* (2006) described differences in antimicrobial susceptibility profiles of adult and paediatric ca-MRSA, showing that paediatric isolates were more likely to be susceptible to non-β-lactam antibiotics. The results of the current study may hint at differences in the epidemiology of adult and paediatric ca-MRSA from Cape Town, but should be interpreted with caution given that only a few ca-MRSA isolates were characterised.

This study was not designed to investigate the differences between local ca- and ha-MRSA, or to describe the differences in molecular epidemiology and evolution of CA- and HA-MRSA in this setting; therefore, future larger prospective studies are required to provide more accurate information regarding the epidemiology of ca-MRSA from Cape Town. This information may clarify the differences between local CA- and HA-MRSA clones, which could prove informative when identifying molecular markers suitable for the differentiation of ca- and ha-MRSA in the local setting. Additionally, future studies should further the investigation of any possible differences in epidemiology between adult and paediatric CA-MRSA from the region.

CHAPTER 5

Local Methicillin-Resistant *S. aureus* in the Global Context

5.1 Introduction

While PFGE remains the gold standard for *S. aureus* typing, the method has been criticised for being too discriminatory, failing to detect underlying relationships between distantly related isolates (Maiden *et al.*, 1998; Spratt, 1999). As a result, PFGE is not well suited to studying the epidemiology of isolates obtained over long periods of time or geographical distances (Spratt, 1999; Cookson *et al.*, 2007; van Belkum *et al.*, 2009). Aside from being labour intensive and technically demanding, a major drawback of PFGE has been its limited portability between laboratories (Spratt *et al.*, 1999; Cookson *et al.*, 2007; Hallin *et al.*, 2007). By the late 1990s, the shortcomings of PFGE and other band-based typing strategies left epidemiologists wanting techniques suited to global epidemiology that were also less subjective and more portable between laboratories than the existing typing methods. Over the past decade, a combination of advances in molecular biology, the development of software packages for data analysis, and the dawn of the Internet era has led to the development of several sequence-based typing techniques, including multilocus sequence typing, *spa* typing and *dru* typing (Deurenberg and Stobberingh, 2008; Goering *et al.*, 2008a).

Multilocus sequence typing (MLST) was developed for the identification of hyper-virulent *Neisseria meningitidis* clones (Maiden *et al.*, 1998), and has since been adapted for many other pathogens, including *S. aureus* (Enright *et al.*, 2000; Feil and Enright, 2004). As depicted in Figure 5.1, MLST for *S. aureus* entails the PCR amplification and direct sequencing of internal fragments of seven housekeeping genes. The sequence of each fragment is then compared to the corresponding sequences present in the online *S. aureus* MLST database (http://www.mlst.net) and, for each locus, each unique allele is assigned a particular number. The series of numbers representing the seven loci, corresponding to the housekeeping genes taken in alphabetical order, comprises the isolate's allelic profile, which can be compared to those present in the database and summarised as a Sequence Type, or

ST, that 'marks' that particular *S. aureus* clone (Figure 5.1) (Enright *et al.*, 2000; Feil and Enright, 2004; Aanensen and Spratt, 2005).

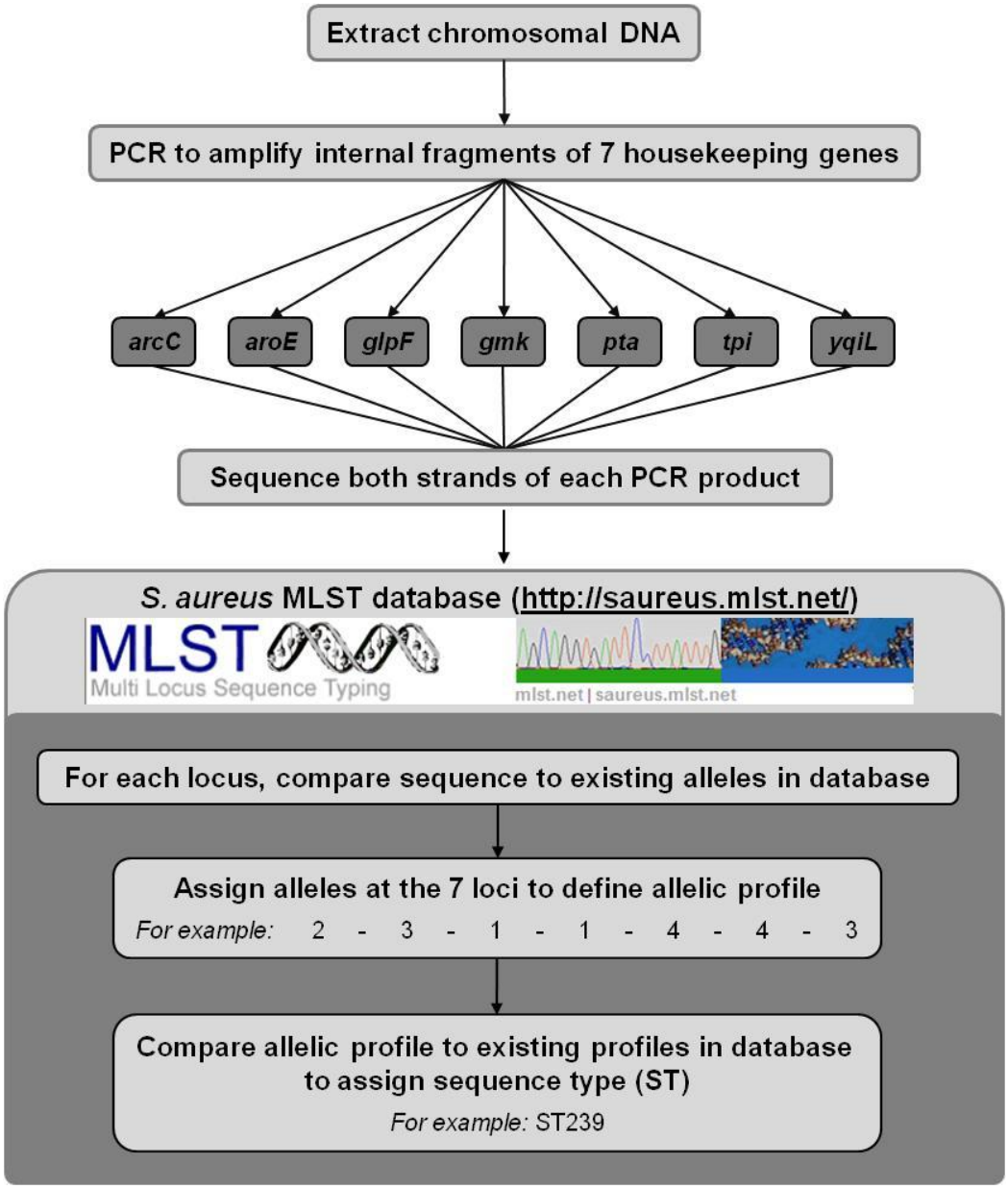


Figure 5.1 Multilocus sequence typing workflow. *arcC*, carbamate kinase; *aroE*, shikimate dehydrogenase; *glpF*, glycerol kinase; *gmk*, guanlyate kinase; *pta*, phosphate acetyltransferase; *tpi*, triosephosphate isomerase; *yqiL*, acetyl co-enzyme A acetyltransferase. Adapted from Spratt (1999) and Enright *et al.* (2000).

As MLST detects genetic variation that accumulates slowly in housekeeping genes, it is able to detect genetic relationships between isolates obtained over extended periods of time and geographical distances (Spratt, 1999; Enright *et al.*, 2000). As a result, the method is widely used to describe the global epidemiology of *S. aureus* (Deurenberg and Stobberingh, 2008). As described in Chapter 1 [1.6.3; 1.7], MLST and the eBURST algorithm have been used to provide a preliminary overview of the *S. aureus* population structure that is gradually being refined as studies examining larger proportions of the genome become routine (Enright *et al.*, 2002; Robinson and Enright, 2003; Feil *et al.*, 2004; Robinson and Enright, 2004b; Nübel *et al.*, 2008; Smyth *et al.*, 2009; Harris *et al.*, 2010; Nübel *et al.*, 2010).

Several seminal studies on the evolution of MRSA were facilitated by MLST used in conjunction with the eBURST algorithm. Enright *et al.* (2002) used these tools to dispel the single clone theory, showing that MRSA lineages emerged from successful MSSA clones on several occasions [1.7.2]. Further, the fact that extant MRSA isolates were assigned to a limited number of *S. aureus* CCs suggested that not all *S. aureus* lineages have the capacity to acquire methicillin-resistance (Enright *et al.*, 2002; Katayama *et al.*, 2005). MLST-based studies also provided insights into the underlying relationships between major epidemic clones prevalent in distinct geographical regions, often over extended time periods, which provided the basis for the development of a more informative universal nomenclature [1.7.2] (Enright *et al.*, 2002; Robinson and Enright, 2003; Robinson and Enright, 2004b).

A shortcoming of MLST is that it is less than ideal for routine surveillance because it is relatively time consuming and expensive (Deurenberg and Stobberingh, 2008; Cookson *et al.*, 2007). Instead, *spa* typing has become increasingly popular for routine surveillance (Hallin *et al.*, 2007; Deurenberg and Stobbering, 2008; Strommenger *et al.*, 2008; van Belkum *et al.*, 2009). Frénay *et al.* (1996) developed *spa* typing as an alternative to the phenotypic or band-based typing methods that were in use at the time. This technique indexes variation that arises within *spa*, which is located on the *S. aureus* chromosome. The *spa* gene encodes protein A, which binds to the Fc region of immunoglobulin G to prevent opsonisation, thereby contributing to *S. aureus* pathogenesis (Winn *et al.*, 2006).

As presented in Figure 5.2, the basic structure of *spa* includes a hyper-variable region, known as the X-region, which is comprised of 24 bp repeats flanked by conserved sequences (Frénay *et al.*, 1996; Shopsin *et al.*, 1999). For *spa* typing, the X-region is amplified by PCR using primers complementary to the 3 prime and 5 prime conserved flanking sequences; the resulting amplicons are sequenced and these data determine the *spa* type of the isolate (Harmsen *et al.*, 2003). Although relatively simple compared to MLST, the technique was initially not widely used, primarily because it was not a portable typing tool as there was no uniform system for the classification of novel repeats and *spa* types (Harmsen *et al.*, 2003; Deurenberg and Stobberingh, 2008). The method was also further hampered by the lack of software packages for data analysis (Harmsen *et al.*, 2003).

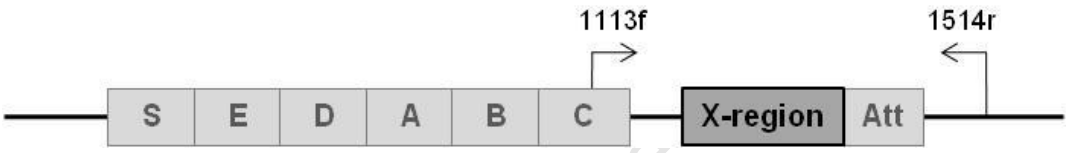


Figure 5.2 Schematic representation of the structure of *spa*. The hyper-variable X-region is shown in dark grey. Conserved flanking sequences are shown in light grey. A – D encode immunoglobulin G-binding regions; E is homologous to A – D; S encodes the signal sequence; and Att encodes the cell wall attachment sequence. The positions of sequences complementary to the forward (1113f) and reverse (1514r) primers used to amplify and sequence the X-region are indicated. Adapted from Shopsin *et al.* (1999) and Harmsen *et al.* (2003).

In 2003, the introduction of Ridom StaphType, a novel software package for the analysis of *spa* sequences, contributed to an increase in the popularity of *spa* typing (Harmsen *et al.*, 2003; Deurenberg and Stobberingh, 2008). Ridom StaphType has simplified *spa* typing as the program automatically assesses the quality of the DNA sequences, identifies repeats in the X-region, and determines the corresponding *spa* type. A numeric code is used to define *spa* repeats (prefixed by "r") and types (prefixed by "t") and, when a novel repeat or type is identified, it is classified through the online *spa* server (http://spaserver.ridom.de/), which ensures that the universal nomenclature is maintained (Harmsen *et al.*, 2003). As described in Chapter 1 [1.6.4], the BURP algorithm available in Ridom StaphType can be used to assess the relatedness of different *spa* types (Mellmann *et al.*, 2008). Ridom StaphType dramatically reduces the time taken to characterise an isolate, adding to its appeal as a tool for outbreak investigations and routine surveillance. The *spa* types listed on

the *spa* server can also be converted to the older Kreiswirth nomenclature, which is useful when comparing *spa* types determined using Ridom StaphType to those previously described in the literature (Harmsen *et al.*, 2003).

Although a licence is required for Ridom StaphType, the data deposited in the *spa* server (*spa* repeats and types, global *spa* type frequencies, and any additional epidemiological data provided by users) are freely available. The creation of a bespoke *spa* typing software package that encourages data sharing through the online *spa* server has resulted in the development of one of the largest *S. aureus* databases: as of December 2010, the *spa* server included 7589 *spa* types identified in over 150 000 *S. aureus* strains from 78 countries (http://spaserver.ridom.de/).

Research has indicated that *spa* typing has a discriminatory power between that of PFGE and MLST and, as a result, is useful for both local and global epidemiology (Deurenberg and Stobberingh, 2008). Several studies have demonstrated the utility of *spa* typing for investigating the local epidemiology of *S. aureus*, particularly when used in conjunction with additional techniques such as PFGE, SCC*mec* typing, antimicrobial susceptibility testing and virulence gene profiling (Shopsin *et al.*, 1999; Harmsen *et al.*, 2003; Aires-de-Sousa *et al.*, 2006; Strommenger *et al.*, 2006a; Cookson *et al.*, 2007; Hallin *et al.*, 2007; Faria *et al.*, 2008; Strommenger *et al.*, 2008; Khandavilli *et al.*, 2009). Ridom GmbH has also incorporated an electronic advanced warning system into Ridom StaphType, which can be used to detect potential *S. aureus* outbreaks in regions where *spa* typing forms part of routine surveillance strategies (Mellmann *et al.*, 2006). In the context of *S. aureus* global epidemiology and evolution, Robinson and Enright (2003) used *spa* typing to further delineate the CCs identified by Enright *et al.* (2002). The study detailed the acquisition of SCC*mec* by CC5, CC8, CC22, CC30 and CC45, and was the first to suggest multiple acquisitions of certain SCC*mec* types within particular STs, highlighting the transmissibility of SCC*mec* type IV. More recently, Grundmann *et al.* (2010) detected regional clustering of MRSA lineages based on *spa* typing data collected by laboratories across Europe, thereby providing a greater understanding of patterns of transmission across that continent.

Even when using a combination of MLST, *spa* typing and PFGE, it can be impossible to differentiate MRSA belonging to certain highly homogeneous clones. This is well illustrated by ST22-MRSA-IV and ST36-MRSA-II isolates from hospitals in Scotland and Ireland, large proportions of which are indistinguishable by PFGE (Goering *et al.*, 2008a; Shore *et al.*, 2010). The differentiation of isolates belonging to homogeneous clones is vital when investigating nosocomial outbreaks and tracing the dissemination of MRSA within and between hospitals (Shore *et al.*, 2010). The need for additional high-resolution sequence-based typing techniques has renewed interests in variable-number tandem repeat methods, one of which is *dru* typing (Goering *et al.*, 2008a; Pourcel *et al.*, 2009; Schouls *et al.*, 2009).

The *dru* locus is present in most MRSA isolates and consists of a series of SCC*mec*-associated direct repeat units (*dru*) (Ryffel *et al.*, 1991). When present, the *dru* region is stable and found adjacent to the copy of IS*431* that is situated on the border of the *mec* gene complex and the J3 region, regardless of SCC*mec* type (Figure 5.3) [1.5.3.1] (Ryffel *et al.*, 1991; Goering *et al.*, 2008a). The imperfect direct repeat units are 40 bp in length and, as in the X-region in *spa*, variation at the *dru* locus accumulates due to point mutations, as well as duplications and deletions of repeats (Goering *et al.*, 2008a).

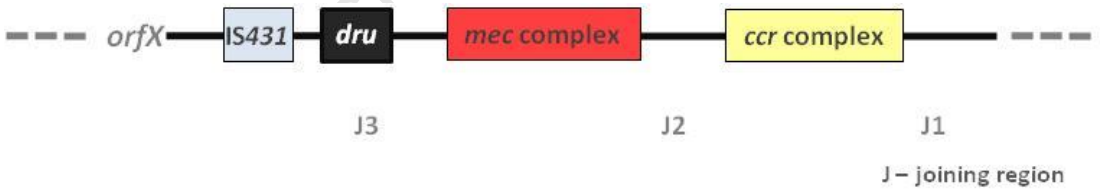


Figure 5.3 Schematic representation of the basic structure of SCC*mec* indicating the position of the *dru* locus.

For *dru* typing, the *dru* locus is amplified by PCR, and sequenced directly using primers complementary to the conserved 5 prime and 3 prime flanking sequences (Goering *et al.*, 2008a). In order to determine the isolate's *dru* type, the sequencing data are analysed using DruID, an application developed by Dr Davida Smyth and Mal McKay that is freely available from the *dru* typing website (http://www.dru-typing.org/). The universal *dru* typing nomenclature is based on a numeric code similar to that used for *spa* typing where the prefixes "dr" and "dt" denote *dru* repeats

and types, respectively (Goering *et al.*, 2008a). The *dru* typing website is currently under construction, but the database is functional and is updated when users submit new *dru* repeats or types. The *dru*-typing database has the potential to develop and expand over time, providing the scientific community with useful epidemiological data in much the same way that the *spa* server has.

Although still in its infancy, *dru* typing appears to have potential as a tool for subtyping homogeneous isolates that are indistinguishable by PFGE. The technique has been used successfully to subtype isolates belonging to the ST36-MRSA-II and ST22-MRSA-IV clones that were submitted to Scottish MRSA Reference Laboratories over an eight year period (Goering *et al.*, 2008a), as well as isolates belonging to the latter clone that were obtained from a single hospital in Ireland over one year (Shore *et al.*, 2010). The results of Shore *et al.* (2010) suggested that *dru* typing, in conjunction with *spa* typing and PFGE, may prove useful for investigating outbreaks and tracing intra- and inter-hospital dissemination of homogeneous MRSA clones. Interestingly, although *dru* typing has been used for short-term or local epidemiology to date, a recent study on the population structure of ST239-MRSA-III indicated that *dru* typing was phylogenetically informative for that clone, most likely due to the stability of SCC*mec* type III within the lineage (Smyth *et al.*, 2009); therefore, it is possible that *dru* typing may prove phylogenetically informative for other MRSA clones with stable SCC*mec* content.

MLST, *spa* typing and *dru* typing are suited to investigating different aspects of *S. aureus* epidemiology and, when used in conjunction with techniques such as SCC*mec* typing and PFGE, they provide information pertinent to both global and local epidemiology (Deurenberg and Stobberingh, 2008; Goering *et al.*, 2008a; van Belkum *et al.*, 2009; Shore *et al.*, 2010). While the three sequence-based techniques examine different aspects of *S. aureus* epidemiology, they have a number of common features that have resulted in their widespread use:

i) they provide unbiased sequence-based data

ii) due to the development of software packages and web-based applications, data analysis is rapid and accurate

iii) results are reproducible and easily compared between laboratories

iv) universal nomenclatures have been developed

v) they are all associated with freely available online databases, which enables users from around the world to compare data

In this study, MLST, *spa* typing and *dru* typing were used to obtain an understanding of the relatedness of isolates belonging to the six PFGE clusters, and to investigate the clonal composition of local MRSA within the context of global *S. aureus* epidemiology.

5.2 Experimental Approach

5.2.1 Selection of representative isolates for *spa* typing, multilocus sequence typing and *dru* typing

As PFGE can, at times, fail to detect underlying relationships between *S. aureus* isolates (Spratt, 1999), *spa* typing was included in this study to further investigate the relatedness of MRSA from hospitals in Cape Town. Twenty-six isolates were selected for *spa* typing and included 18 representatives of PFGE clusters A – F, while the remaining MRSA corresponded to the 8 sporadic isolates. The distribution of the representative isolates across the PFGE clusters can be seen in Appendix A. Multiple isolates were selected from the larger clusters in order to include MRSA with varying levels of genetic similarity and different antimicrobial susceptibility profiles. Accordingly, 3 isolates were selected from clusters C (C5, C16 and C31) and D (D2, D4, D6), while 7 isolates were selected from cluster E (E3, E4, E5, E8, E19 and E32). The 3 isolates from cluster C were selected to include a representative of each of the 3 subgroups containing MRSA indistinguishable by PFGE [2.3]. The remaining representative isolates corresponded to 2 indistinguishable MRSA from cluster F (F8 and F9), both members of cluster B (B1 and B2), and a single member of cluster A (A1).

Although *spa* typing provided insights into aspects of the local and global epidemiology of MRSA from hospitals in Cape Town, in accordance with current international conventions, MLST was required, in conjunction with SCC*mec* typing, to determine the identities of local clones (Enright *et al.*, 2002). Given the costs associated with this technique, it was necessary to select a subset of the 26 isolates described above for characterisation by MLST. Nineteen of the 26 isolates were selected after reviewing the PFGE dendrogram, antimicrobial susceptibility profiles and *spa* typing data. These isolates included 11 diverse representatives of PFGE clusters A – F: A1; B1 and B2; C5, C16 and C31; D4; E3, E4 and E32; and F8 (Appendix A). The sporadic isolates comprised the remaining 8 MRSA selected for MLST.

A number of isolates were indistinguishable by *spa* typing and MLST, or by PFGE; therefore *dru* typing, which appears promising for the differentiation of isolates belonging to homogeneous MRSA clones (Goering *et al.*, 2008a; Shore *et al.*, 2010), was used to further characterise the 26 isolates selected for *spa* typing.

5.2.2 *spa* typing

The revised *spa* typing protocol of Shopsin *et al.* (1999) and Harmsen *et al.* (2003) available on the Ridom GmbH website (version 1.1, June 2004) was followed in this study (http://www3.ridom.de/doc/Ridom_spa_sequencing.pdf). The PCR for the amplification of the X-region was carried out as described in the Ridom StaphType protocol. Reactions were carried out in a final volume of 50 µl with a final concentration of 1X PCR buffer, 1.5 mM $MgCl_2$, 1.25 U of *Taq* polymerase (JMR Holdings, London, UK), 400 µM dNTP mix (Thermo Scientific, Wilmington, DE, USA), and 10 pmol each of the primers spa-1113f and spa-1514r (Synthetic DNA Laboratory, University of Cape Town, Cape Town, South Africa) (Appendix E). Five microlitres of template DNA [3.2.1] was added to each reaction. Amplification was carried out in an Applied Biosystems 2720 Thermocycler (Applied Biosystems, Carlsbad, CA, USA). The PCR amplification parameters included an initial denaturation step at 80°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 45 s and extension at 72°C for 90 s, with a final extension step of 10 min at 72°C.

The PCR products were visualised on 2 % agarose gels as described in Chapter 3 [3.2.2]. The PCR products were separated on 2 % agarose gels and purified as previously described [3.2.3.2]. Direct sequencing of both strands of the purified products was carried out at the Central Analytical Facility at the University of Stellenbosch using the cycle sequencing parameters recommended by Ridom GmbH (http://www3.ridom.de/doc/Ridom_spa_sequencing.pdf). The resulting sequences were analysed using Ridom StaphType (version 2.1.1) (Ridom GmbH, Würzburg, Germany). Novel *spa* types were assigned by submitting the data to the *spa* server (http://spaserver.ridom.de/).

The BURP algorithm available in Ridom StaphType was used to detect clustering among the isolates selected for *spa* typing. When using the BURP algorithm, the user may define the clustering cost, which represents the number of genetic differences between *spa* types that will be included in a particular cluster (Mellmann *et al.*, 2008). As recommended in the Ridom StaphType user-guide (version 2.1.1), a cost of 6 was applied. Additionally, given that the evolutionary history of *spa* types with less than 5 repeats cannot be reliably deduced, *spa* types containing 4 repeats or less were excluded from the analysis (Mellmann *et al.*, 2008).

5.2.3 Multilocus sequence typing

MLST was carried out as described by Enright *et al.* (2000), with minor modifications. Internal fragments of approximately 500 bp were amplified from 7 housekeeping genes. All PCR assays were carried out in 50 µl volumes including final concentrations of 1X PCR buffer, 1.5 mM $MgCl_2$ (JMR Holdings, London, UK), 0.4 mM dNTP mix and 1 U of *Taq* polymerase (JMR Holdings, London, UK). Primers synthesised by the Synthetic DNA Laboratory at the University of Cape Town (Cape Town, South Africa) were added at a final concentration of 0.5 µM each in the individual assays for the amplification of 7 housekeeping genes, including *arcC* (carbamate kinase), *aroE* (shikimate dehydrogenase), *glpF* (glycerol kinase), *gmk* (guanlyate kinase), *pta* (phosphate acetyltransferase), *tpi* (triosephosphate isomerase), and *yqiL* (acetyl co-enzyme A acetyltransferase). The primer sequences are shown in Appendix E, while the primer names and target sequences are shown in Table 5.1. The amount of genomic DNA [3.2.1] added to each reaction was decreased to 100 ng, except in assays for the amplification of *tpi*, which contained 200 ng.

Table 5.1 Primers for the amplification of 7 housekeeping genes for multilocus sequence typing

Primer Pair[a]	Housekeeping gene targeted
arcC-Up *arcC*-Dn	Carbamate kinase (*arcC*)
aroE-Up *aroE*-Dn	Shikimate dehydrogenase (*aroE*)
glpF-Up *glpF*-Dn	Glycerol kinase (*glpF*)
gmk-Up gmk-Dn	Guanlyate kinase (*gmk*)
pta-Up *pta*-Dn	Phosphate acetyltransferase (*pta*)
tpi-Up *tpi*-Dn	Triosephosphate isomerase (*tpi*)
yqiL-Up *yqiL*-Dn	Acetyl co-enzyme A acetyltransferase (*yqiL*)

[a] Enright *et al.* (2000) (primer sequences available in Appendix E)

All PCRs were carried out using an Applied Biosystems 2720 Thermocycler (Applied Biosystems, Carlsbad, CA, USA) and amplification parameters for all 7 assays were as follows: denaturation for 5 min at 95°C, followed by 30 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 55°C, elongation for 1 min at 72°C, and a final extension step of 5 min at 72°C.

The resulting amplicons were visualised on 2 % agarose gels, and purified as described in Chapter 3 [3.2.3.2]. Both strands of the purified products were sequenced directly as described in Chapter 3 [3.2.3.2]. The chromatograms were examined in BioEdit Sequence Alignment Editor (version 7.0.5.2) (Hall, 1999), and any ambiguous bases were corrected. The edited sequences were aligned to template sequences obtained from the MLST website (http://saureus.mlst.net/misc/info.asp). The template sequences are internal to the amplified region; therefore trimming query sequences to the length of the corresponding template sequences excluded unreliable data from the final analysis. Allelic profiles and STs were assigned to each isolate by comparing the trimmed sequences of the 7 housekeeping genes to those present in the *S. aureus* MLST database (http://saureus.mlst.net/). The STs were assigned to *S. aureus* CCs with

eBURST (version 3) (http://saureus.mlst.net/eburst/), using the stringent default parameters.

5.2.4 *dru* typing

The *dru* typing method of Goering *et al.* (2008a) was modified for use in this study. PCRs were carried out in a volume of 50 µl with a final concentration of 1X PCR buffer, 1.5 mM MgCl2 and 400 µM dNTP mix. The primers *dru*1 and *dru*2 (Appendix E) were included at a final concentration of 0.5 µM each, and each reaction also contained 100 ng of template DNA [3.2.1] and 1.25 U of *Taq* polymerase. The cycling conditions for each assay included an initial denaturation step of 2 min at 94°C, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min and annealing at 72°C for 1 min, with a final extension step of 2 min at 72°C. Amplification was carried out using an Applied Biosystems 2720 Thermocycler (Applied Biosystems, Carlsbad, CA, USA).

The PCR products were visualised, purified and sequenced as described for MLST [5.2.3]. The raw sequencing data files were copied into DruID, which automatically determined the *dru* types of most isolates. In the case of isolates where *dru* types were not assigned automatically, the quality of the sequencing data was assessed by examining the chromatograms in BioEdit Sequence Alignment Editor (version 7.0.5.2) (Hall, 1999), and ambiguous bases were edited as necessary. The edited sequencing files were then entered into DruID and a *dru* type was determined. Novel *dru* types were assigned by submitting the sequencing data to the curators of the *dru* typing website (http://www.dru-typing.org/).

5.3 Results

5.3.1 *spa* typing

Using Ridom StaphType, *spa* types were assigned to all 26 isolates selected for sequence-based characterisation. In total, 12 *spa* types were assigned to the 26 MRSA, as shown in Table 5.2 in conjunction with the PFGE cluster assignment, SCC*mec* type and antimicrobial susceptibility profile of each isolate.

Table 5.2 Properties of methicillin-resistant *S. aureus* isolates selected for *spa* typing, *dru* typing and multilocus sequence typing[a].

MRSA isolate[b]	PFGE cluster (no. isolates)	SCC*mec* type	*spa* type (*spa*-CC)	*dru* type	ST (CC)	Antimicrobial resistance profile
A1	A (4)	III	t037 (021)	dt14e [c]	239 (8)	PEN, OXA, ERY, CLI, SXT, CIP, GEN
B1	B (2)	I	t7185[c] (045/002)	dt10ar [c]	5 (5)	PEN, OXA, ERY, CLI, CIP
B2	B (2)	I	t045 (045/002)	dt10a	5 (5)	PEN, OXA, ERY, CLI, GEN
C5	C (35)	I	t045 (045/002)	dt10a	5 (5)	PEN, OXA, ERY, CLI
C16	C (35)	I	t045 (045/002)	dt10a	5 (5)	PEN, OXA
C31	C(35)	I	t045 (045/002)	dt10a	5 (5)	PEN, OXA, ERY, CLI, RIF
D2	D (7)	IVd	t064 (064)	dt10i	ND	PEN, OXA, ERY, CLI, RIF, SXT, GEN
D4	D (7)	IVd	t064 (064)	dt10i	612 (8)	PEN, OXA, ERY, CLI, RIF, SXT, GEN
D6	D (7)	IVd	t064 (064)	dt10i	ND	PEN, OXA, ERY, CLI, RIF, SXT
E3	E (33)	IVd	t1443 (064)	dt10i	612 (8)	PEN, OXA, RIF, SXT, CIP, GEN
E4	E (33)	IVd	t2196 (Excluded[d])	dt10ap [c]	612 (8)	PEN, OXA, ERY, CLI, RIF, SXT, CIP, GEN
E5	E (33)	IVd	t1443 (064)	dt9ac [c]	ND	PEN, OXA, RIF, SXT, CIP, GEN
E8	E (33)	IVd	t064 (064)	dt9ad [c]	ND	PEN, OXA, ERY, CLI, RIF, SXT, CIP, GEN
E19	E (33)	IVd	t1443 (064)	dt10o	ND	PEN, OXA, RIF, SXT, CIP, GEN

E26	E (33)	IVd	t1443 (064)	dt10i	ND	PEN, OXA, RIF, SXT, CIP, GEN
E32	E (33)	IVd	t1443 (064)	dt8v [c]	612 (8)	PEN, OXA, RIF, SXT, CIP, GEN, FUS[e]
F8	F (11)	II	t012 (021)	dt9a	36 (30)	PEN, OXA, ERY, CLI, CIP
F9	F (11)	II	t021 (021)	dt7s	ND	PEN, OXA, ERY, CLI, CIP
S1	Sporadic isolate	IVd	t1443 (064)	dt10aq [c]	612 (8)	PEN, OXA, RIF, SXT, CIP, GEN
S2	Sporadic isolate	V	t3092 (Singleton)	dt11a	72 (8)	PEN, OXA, ERY, CLI
S3	Sporadic isolate	II	t021 (021)	dt4c [c]	36 (30)	PEN, OXA, ERY, CLI, CIP, GEN
S4	Sporadic isolate	IVd	t1257 (064)	dt10i	612 (8)	PEN, OXA, ERY, CLI, RIF, SXT, CIP, GEN
S5	Sporadic isolate	IVd	t064 (064)	dt10i	612 (8)	PEN, OXA, RIF, SXT, GEN
S6	Sporadic isolate	IVd	t064 (064)	dt10i	612 (8)	PEN, OXA, RIF, SXT, GEN
S7	Sporadic isolate	IVh	t032 (Singleton)	dt9j	22 (22)	PEN, OXA, ERY, CLI, CIP, FUS[e]
S8	Sporadic isolate	IVb	t002 (045/002)	dt10t [c]	ST650 (5)	PEN, OXA

[a] *spa*-CC, *spa* clonal complex; ST, sequence type; CC, clonal complex (based on multilocus sequence typing); PEN, penicillin; OXA, cloxacillin; ERY, erythromycin; CLI, clindamycin; RIF, rifampicin; SXT, co-trimoxazole; CIP, ciprofloxacin; GEN, gentamicin; FUS, fusidic acid; ND, not determined (STs inferred from representatives of PFGE cluster included for MLST).

[b] Strain designations introduced in Chapter 2.

[c] Novel types.

[d] Excluded from BURP (based-upon repeat pattern) analysis as the *spa* sequence included less that 5 repeats.

[e] Intermediate level of resistance to fusidic acid.

The 12 *spa* types were unevenly distributed among the 26 isolates with 3 *spa* types (t045, t064 and t1443) accounting for 16 isolates. The isolates corresponding to t045 (*n* = 4) included all 3 representatives of cluster C, and isolate B2. The remaining member of cluster B, isolate B1, corresponded to t7185, which was the only novel *spa* type identified in this study. The Ridom StaphType alignment of t045 and t7185 detected 4 differences in their repeat sequences, as illustrated in Figure 5.4, and was assigned a genetic cost of 6 based on the BURP algorithm. Scrutiny of the underlying sequences of the mismatched repeats r26 and r135 revealed a single nucleotide difference (Appendix H). The remaining differences corresponded to the presence of additional repeats in t7185, which were absent in t045 (Figure 5.4). These repeats (r20-r17-r12) may represent a duplication event as two copies of the series are present in t7185, while only a single copy is present in t045 (Figure 5.4).

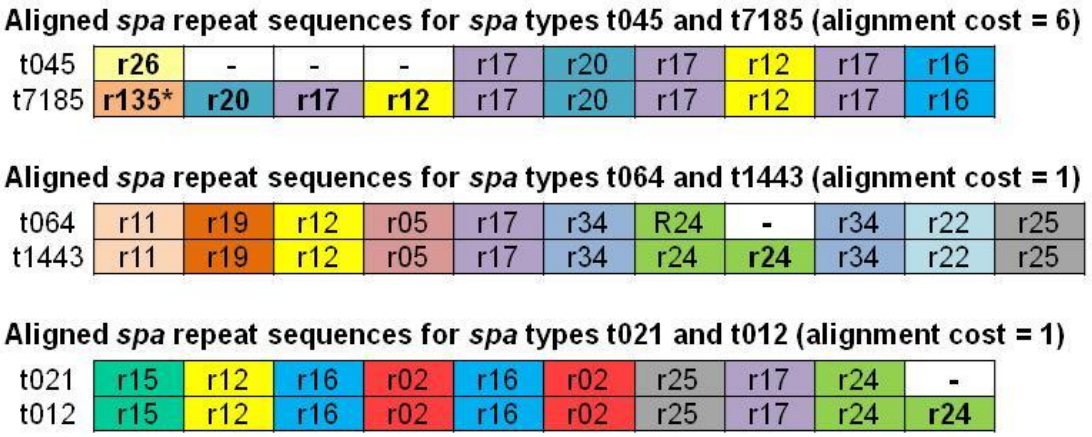


Figure 5.4 Schematic diagram representing *spa* repeat sequence alignments of selected *spa* types. The *spa* repeat sequences of interest were aligned using the *spa* type alignment function in Ridom StaphType. Unique repeats are represented by different colours; differences are marked in bold; gaps are indicated by (-); single nucleotide differences are indicated by (*). Alignment costs as calculated by the BURP (based upon repeat pattern) algorithm in Ridom StaphType.

The other major *spa* types identified in this study, t064 and t1443, were co-dominant, accounting for 6 isolates each, which comprised almost half of the selected MRSA. The isolates consistent with t064 included all 3 representatives of cluster D, as well as a single representative of cluster E and 2 sporadic isolates (Table 5.2). The co-dominant t1443 was identified in 5 out of 7 representative isolates from cluster E, as well as in the sporadic isolate S1 (Table 5.2). The alignment of the repeat sequences of the co-dominant *spa* types was assigned a cost of 1, and revealed only

a single difference: t1443 contains an additional copy of r24 compared to t064 (Figure 5.4). The detection of subtly different *spa* types within a single PFGE cluster was not unique to cluster E. Although isolates F8 and F9 were indistinguishable by PFGE, the former was consistent with t012, while the latter corresponded to t021, as did the sporadic isolate S3. An alignment of the repeat sequences of these two *spa* types was assigned a cost of 1, and indicated that t012 contains an additional copy of r24 that is not present in t021 (Figure 5.4). The remaining representative isolates (A1 and E4), as well as sporadic isolates S2, S4, S7 and S8, corresponded to *spa* types detected in single isolates (Table 5.2).

Although the number of *spa* types detected in this study was small, it remained instructive to carry out a BURP analysis to infer the relatedness of the included isolates. In accordance with the BURP parameters recommended by Ridom GmbH, isolate E4 was excluded from the analysis because it contained only 4 repeats. The 25 isolates included in the BURP analysis segregated into 3 *spa* clonal complexes (*spa*-CCs) and 2 singleton *spa* types, as shown in Figure 5.5.

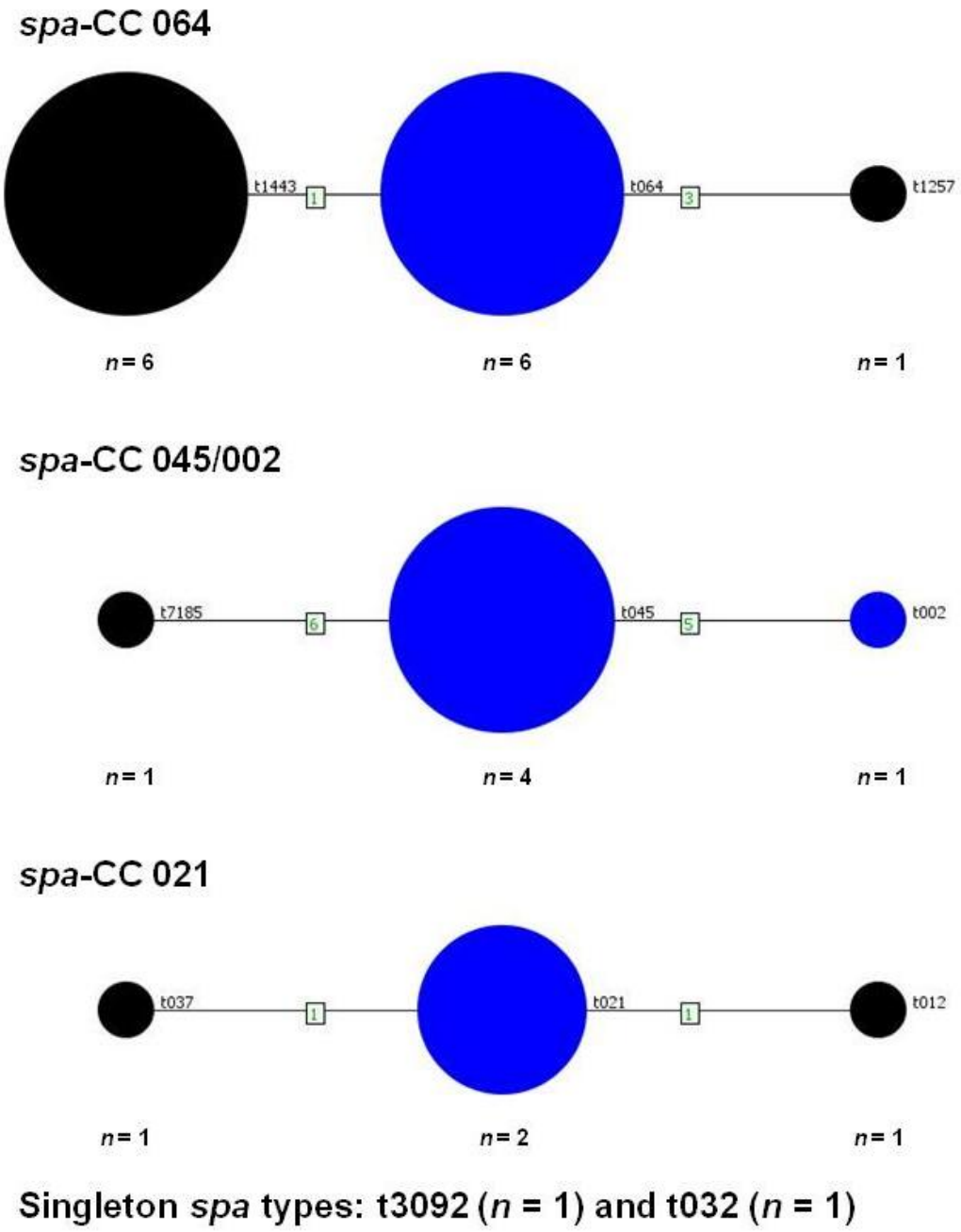


Figure 5.5 The clonal relatedness of 25 methicillin-resistant *S. aureus* isolates included in a BURP analysis of the *spa* typing data. The parameters used in the BURP analysis corresponded to those recommended by Ridom GmbH, including a cluster cost of 6 or less and the exclusion of *spa* types with less than 5 repeats. Each circle or node represents a unique *spa* type. Node size is proportional to the number of isolates corresponding to that particular *spa* type (number of isolates indicated below each node). Blue nodes indicate putative founding *spa* types of each *spa*-CC. The costs between *spa* types, as determined by the BURP algorithm, are shown in green text in boxes between nodes.

The largest clonal complex was *spa*-CC064 (*n* = 13), named after its putative founding *spa* type, t064 (Figure 5.5). This *spa*-CC consisted of 3 *spa* types (t064, t1443 and t1257), and included representative isolates from clusters D and E, as well as 3 sporadic isolates (Table 5.2). The second largest clonal complex was *spa*-CC045/002 (*n* = 6), which was assigned 2 putative founding *spa* types, t045 and t002 (Figure 5.5). This *spa*-CC included 3 *spa* types (t045, t002 and t7185) that were identified in representatives of clusters B and C, and the sporadic isolate S8 (Table 5.2). The remaining clonal complex, *spa*-CC021 (*n* = 4), also included 3 *spa* types (t021, t021 and t037), corresponding to representative isolates from clusters A and F, and the sporadic isolate S3.

5.3.2 Multilocus sequence typing

Using the online *S. aureus* MLST database, STs were assigned to all 19 isolates selected for complete molecular characterisation. The STs were then assigned to *S. aureus* CCs using eBURST (version 3) (http://saureus.mlst.net/eburst/). The STs and corresponding CCs of the isolates are shown in Table 5.2.

In accordance with current international conventions, the clonal types of the isolates were determined based on the combination of ST and SCC*mec* type. Analysis of the MLST and SCC*mec* typing data identified 7 clones among the 19 isolates (Table 5.2). Examination of these data in conjunction with the PFGE results revealed that the representatives of the 6 PFGE clusters corresponded to 4 major clones. Isolate A1 corresponded to ST239-MRSA-III, while the representative of cluster F, F8, corresponded to ST36-MRSA-II, as did the sporadic isolate S3. The second-most prevalent clone identified among the MRSA was ST5-MRSA-I (*n* = 5), which corresponded to all representative isolates from clusters B and C (Table 5.2). The dominant clone identified among the representative isolates was ST612-MRSA-IVd (*n* = 8), which included all representatives of clusters D and E, as well as 4 sporadic isolates. The three remaining sporadic isolates were not consistent with any of the major clonal types identified in the 6 PFGE clusters. Instead, these isolates corresponded to 3 sporadic clones: ST72-MRSA-V, ST22-MRSA-IVh and ST650-MRSA-IVb (Table 5.2).

5.3.3 *dru* typing

The utility of *dru* typing for the sequence-based differentiation of isolates belonging to the same clonal type, or corresponding to identical PFGE profiles, was also investigated in this study. Using DruID, *dru* types were assigned to all 26 isolates. In total, 16 *dru* types were identified among the 26 MRSA, 9 of which were novel (Table 5.2). The *dru* repeat sequences of isolates assigned to ST612-MRSA-IVd, ST5-MRSA-I and ST36-MRSA-II [5.3.2] were aligned manually to examine variation within the *dru* loci of these clonal types (Figure 5.6).

ST5-MRSA-I (PFGE clusters B and C)

dt10a	5a	2d	4a	0	2d	5b	3a	2g	3b	4e
dt10ar	5a	2d	3c*	2g**	2c***	5b	3a	2g	3b	4e

ST612-MRSA-IVd (PFGE clusters D and E and sporadic isolates S1, S4, S5 and S6)

dt10i	5a	2d	4a	0	2d	4f	3a	2g	3b	4e
dt10ap	5a	2d	4a	0	2d	4f	3a	2g	4l***	4e
dt10aq	5a	2d	4a	1b*	2d	4f	3a	2g	3b	4e
dt10o	5a	2d	4a	0	2d	4f	3a	2g	2c***	4e
dt9ac	5a	2d	4a	0	2d	4f	-	4g**	3b	4e
dt9ad	5a	2d	4a	0	-	4f	3a	2g	3b	4e
dt8v	5a	2d	4a	0	2d	4f	**4h***	-	-	4e

ST36-MRSA-II (PFGE cluster F and sporadic isolate S3)

dt9a	5a	2d	2d	4a	0	2g	3b	4e	3e
dt7s	5a	2d	2d	4a	0	2g	-	-	3e
dt4c	5a	2d	2d	-	-	-	3b	-	-

Figure 5.6 Schematic diagram representing aligned *dru* repeat sequences of isolates corresponding to ST5-MRSA-I, ST612-MRSA-IVd and ST36-MRSA-II. For ST5-MRSA-I and ST612-MRSA-IVd isolates, *dru* repeat sequences were aligned to the most prevalent *dru* type (dt10a and dt10i, respectively); in the case of ST36-MRSA-II isolates, *dru* repeat sequences were aligned to dt9a. Unique repeats are represented by different colours; repeat units containing mutations are underlined; (*) indicates one nucleotide difference; (-) indicates gaps.

The alignments of *dru* types corresponding to ST5-MRSA-I and ST612-MRSA-IVd isolates were particularly interesting. All ST5-MRSA-I isolates representative of cluster C shared dt10a, which was the second-most prevalent *dru* type identified in the current study (*n* = 4). This *dru* type was also identified in isolate B2

(ST5-MRSA-I); however, the remaining ST5-MRSA-I representative of cluster B, B1, corresponded to dt10ar, one of the novel *dru* types identified in this study. Alignment of the repeat sequences of dt10a and dt10ar revealed a difference of 3 repeats (Figure 5.6). Examination of the underlying sequences of the 3 discrepant repeats detected a total of 6 nucleotide differences between dt10a and dt10ar (Figure 5.6).

All ST612-MRSA-IVd isolates representative of cluster D corresponded to dt10i, which was the most prevalent *dru* type identified in the current study (*n* = 8). This *dru* type was also identified in 2 representatives of cluster E, and in 3 sporadic isolates that corresponded to ST612-MRSA-IVd (Table 5.2). The remaining 5 ST612-MRSA-IVd isolates representative of cluster E, in addition to the remaining ST612-MRSA-IVd sporadic isolate S1, comprised a variety of *dru* types, including 5 novel *dru* types (Table 5.2). Alignment of the *dru* repeat sequences obtained for ST612-MRSA-IVd isolates with dt10i revealed that the underlying sequences are very similar with variation ranging from a single point mutation in dt10aq (S1) to differences in 3 repeats in dt8v (E32) (Figure 5.6; Table 5.2).

5.4 Discussion

Seven clonal types, defined by ST and SCC*mec* type, were identified among nineteen representatives of the one hundred MRSA isolates included in this study. Examination of the distribution of clonal types across the PFGE dendrogram revealed that four clones accounted for the majority of the isolates, including all six PFGE clusters. MLST was supplemented with *spa* typing and *dru* typing to obtain a more comprehensive characterisation of selected isolates, resulting in a greater understanding of the local and global epidemiology of MRSA from hospitals in Cape Town.

The smallest PFGE cluster, A, corresponded to the multidrug-resistant ST239-MRSA-III, t037, clone. This HA-MRSA clone forms a major subgroup of CC8, one of the oldest and most diversified *S. aureus* clonal complexes (Figure 5.7) (Enright *et al.*, 2002; Robinson and Enright, 2003). Robinson and Enright (2003) used a combination of MLST, SCC*mec* typing, *spa* typing and *sas* typing to show that ST239-MRSA-III corresponds to several pandemic and epidemic clones, including those formerly known as the Brazilian Hungarian, Portuguese and Viennese clones, as well as EMRSA-1, -4, -7, -9 and -11 (Enright *et al.*, 2002; Robinson and Enright, 2003; Deurenberg and Stobberingh, 2008). ST239-MRSA-III was first described as a cause of hospital epidemics in the UK, USA and Australia during the 1970s and 1980s, and then, during the 1990s, as a pandemic clone prevalent in Europe and South America (Smyth *et al.*, 2009). This clone has been reported on every continent, and Africa is no exception as ST239-MRSA-III is predominant in major cities in Algeria, Morocco, Niger, Senegal, and South Africa (Deurenberg and Stobberingh, 2008; Shittu *et al.*, 2009; Breurec *et al.*, 2010; Moodley *et al.*, 2010).

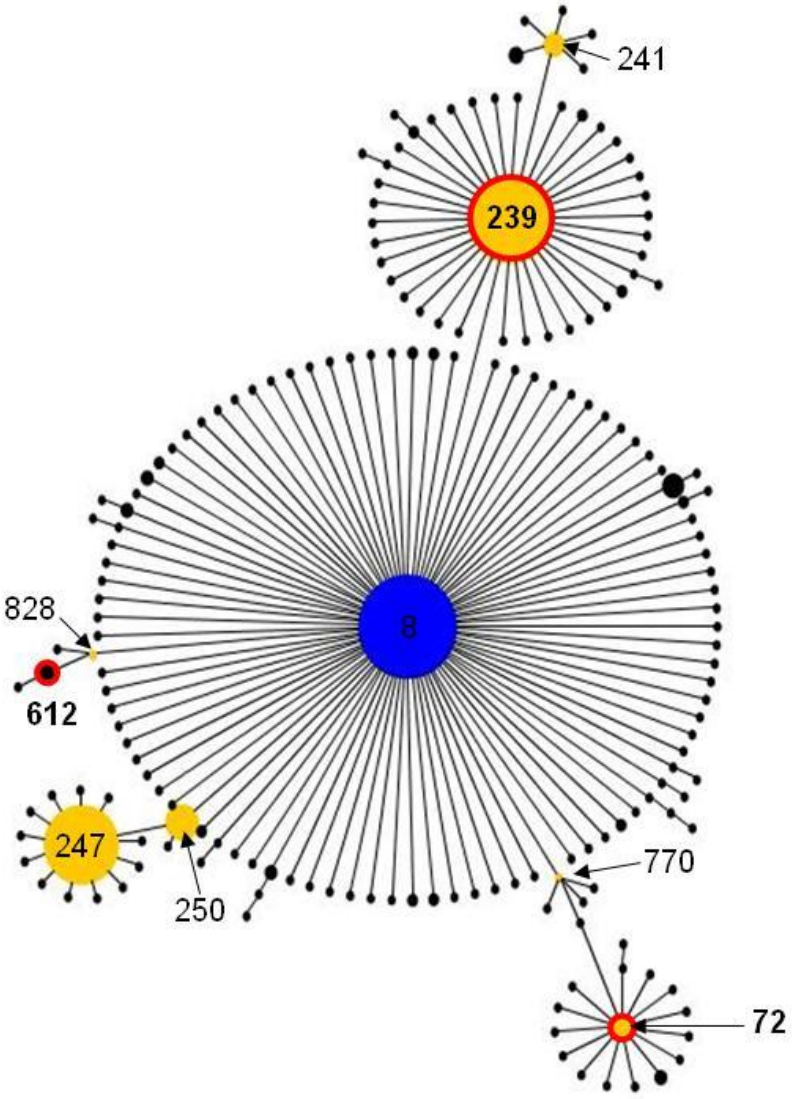


Figure 5.7 Graphical representation of patterns of descent among members of *S. aureus* clonal complex 8 as determined using the eBURST algorithm. The predicted founder of clonal complex (CC) 8, ST8, is indicated by the blue circle. Descendants of ST8 are indicated by yellow (subgroups) and black circles with sequential nodes indicating the progressive accumulation of mutations in different housekeeping genes monitored by multilocus sequence typing (MLST). For clarity, only the STs of subgroups are indicated. STs identified in the current study are shown in bold and the nodes are circled in red. The relative sizes of the nodes are proportional to the corresponding number of isolates reported in the MLST database.

In spite of its global success, ST239-MRSA-III represented the smallest clonal type identified in the current study, accounting for only four MRSA; however, this clone appears to be far more prevalent in other regions of South Africa. A study on the epidemiology of *S. aureus* obtained from patients at Tygerberg Hospital (in the Western Cape province, approximately 20km from GSH) between 2008 and 2009 indicated that 27.27 % of MRSA isolates corresponded to t037 (Salaam-Dreyer, 2010). That t037 is thought to represent the ancestral ST239-MRSA-III *spa* type (Harris *et al.*, 2010) suggests this clone is predominant at Tygerberg Hospital; therefore the prevalence of ST239-MRSA-III may be variable within the Western Cape. ST239-MRSA-III also seems to be more prevalent in the KwaZulu-Natal province of South Africa than in central Cape Town. Shittu *et al.* (2009) and Essa *et al.* (2009) both identified ST239-MRSA-III as the second-most prevalent clone present in healthcare institutions in KwaZulu-Natal, reporting prevalence rates of 16.4 % and 13 %, respectively. In the national context ST239-MRSA-III, t037, appears to represent a major clone, accounting for 21.61 % of MRSA obtained from public and private diagnostic laboratories across all provinces except the Western Cape, which suggests an uneven distribution of this clone across South Africa (Moodley *et al.*, 2010). However, Moodley *et al.* (2010) did not indicate the proportion of isolates obtained from each province, and unequal provincial contributions may have resulted in the uneven distribution of MRSA clones observed in that study.

It is interesting to consider the evolution of ST239-MRSA-III isolates from hospitals in Cape Town. Of all the major MRSA clones, the population structure and evolution of ST239-MRSA-III is perhaps best understood. A recent study utilising high-throughput genomics has revealed that the widespread prevalence of this clone is due to its global dissemination, rather than its frequent emergence from ST239-MSSA ancestors (Harris *et al.*, 2010). In the light of these studies, it is highly likely that ST239-MRSA-III has been imported into South Africa.

Another extensive study on the population structure of a collection of ST239-MRSA-III from 29 countries detected geographical clustering among the isolates, identifying three major clades corresponding to European, Asian and South American isolates, respectively (Smyth *et al.* 2009). The same study found that the *dru* locus is phylogenetically informative for ST239-MRSA-III, with geographically clustered

isolates sharing similar *dru* types, most likely due to the stability of SCC*mec* type III within the lineage. With this in mind, it was of interest that dt14e, the novel *dru* type identified in A1, the ST239-MRSA-III representative isolate, was most similar to dt15b, which was detected in isolates obtained from China, Vietnam and Korea (Figure 5.8) (Smyth *et al.,* 2009). As the *dru* locus is phylogenetically informative for this clone, it is possible to suggest that ST239-MRSA-III from Cape Town may be closely related to those from Asia.

Figure 5.8 Aligned *dru* repeat sequences of ST239-MRSA-III isolates from Cape Town and Asia. Alignment of *dru* sequences identified in ST239-MRSA-III from Cape Town and Asia (Smyth *et al.*, 2009) detected a single repeat difference (highlighted in bold and underlined). Countries of origin are indicated below the *dru* type. RSA, Republic of South Africa.

Local and global dissemination has been proposed to play a major role in shaping the population structure of ST239-MRSA-III (Smyth *et al.*, 2009; Harris *et al.*, 2010), and several other important EMRSA clones (Enright *et al.*, 2002; Robinson and Enright, 2003); therefore it is worthwhile pausing to consider mechanisms of MRSA dissemination. Over the past century, humans have become more mobile, resulting in increasingly frequent exchanges of individuals between distant regions. Travel, including migration and tourism within and between countries, has been suggested as important factor in shaping bacterial populations (MacPherson *et al.*, 2009; Gray *et al.*, 2010; Nübel *et al.*, 2010; Zanger *et al.*, 2010). In South Africa, migration and tourism, both domestic and international, are common, and may have influenced the composition of the local MRSA population. The history of travel between South Africa and Asia may explain the possible relationship between ST239-MRSA-III isolates from Cape Town and Asia; however, the evolutionary history of ST239-MRSA-III, and other MRSA clones from South Africa, will only be resolved when local isolates are included in studies similar to those carried out by Smyth *et al.* (2009) and Harris *et al.* (2010).

The other minor clonal type corresponding to multiple isolates in the present study was ST36-MRSA-II, which accounted for PFGE cluster F and the sporadic isolate S3. Robinson and Enright (2003) identified ST36-MRSA-II as an important subgroup within CC30, one of the major MRSA CCs (Figure 5.9). ST36-MRSA-II, also known as EMRSA-16 (Enright *et al.*, 2002), emerged during the 1990s as an epidemic clone in hospitals in the UK (Johnson *et al.*, 2005), and has since been reported worldwide [1.7.2] (Deurenberg and Stobberingh, 2008). In the present study, representatives of ST36-MRSA-II (CC30) and ST239-MRSA-III (CC8) were all assigned to *spa*-CC021, suggesting an underlying genetic relationship between the isolates; however, clustering of these *spa* types was unsurprising given that ST239-MRSA-III represents a hybrid *S. aureus* lineage with 80 % of its genome donated by an ST8 parent strain, and the remaining 20 %, including the *spa* locus, donated by an ST30 (CC30) parent strain (Robinson and Enright, 2004a).

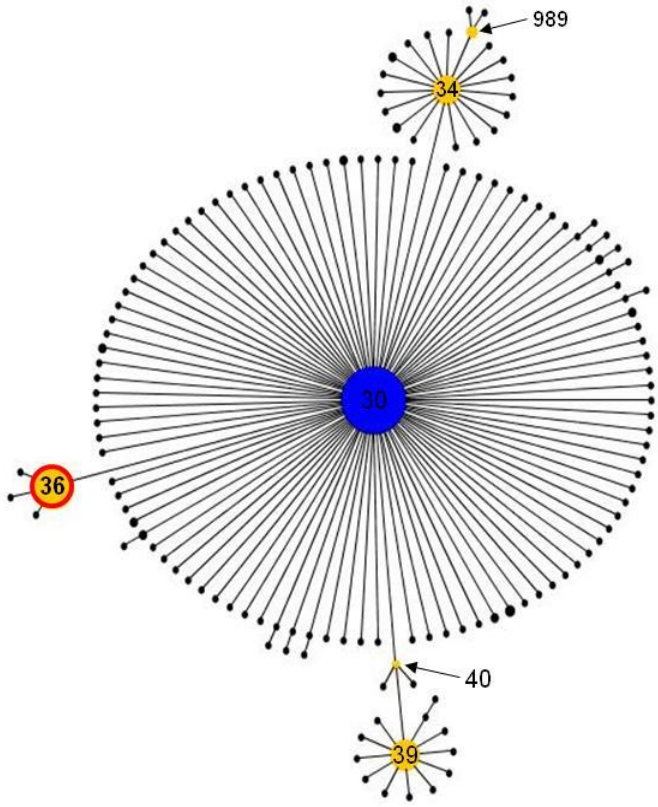


Figure 5.9 Graphical representation of patterns of descent among members of *S. aureus* clonal complex 30 as determined using the eBURST algorithm. The predicted founder of clonal complex (CC) 30, ST30, is indicated by the blue circle. Descendants of ST30 are indicated by black and yellow circles. Subgroups that have given rise to their own variants are indicated by yellow circles. For clarity, only the STs of subgroups are indicated. STs identified in the current study are shown in bold and the nodes are circled in red. The relative sizes of the nodes are proportional to the corresponding number of isolates reported in the MLST database.

ST36-MRSA-II isolates are frequently highly homogeneous and indistinguishable by PFGE (Goering *et al.*, 2008a). Similarly, the PFGE macrorestriction profiles of three pairs of local ST36-MRSA-II isolates were indistinguishable [2.3], including isolates F8 and F9, which were selected for sequence-based characterisation. Homogeneous ST36-MRSA-II isolates from Scotland have been differentiated using *dru* typing (Goering *et al.*, 2008a), and, based on the unique combinations of similar *spa* and *dru* types observed in F8 and F9, it is possible that these techniques hold promise for the differentiation of local isolates belonging to this clone.

In addition to differentiating ST36-MRSA-II isolates with identical macrorestriction profiles, *dru* typing proved useful for examining the relationship between isolates from cluster F and the sporadic isolate, S3. Although S3 was consistent with ST36-MRSA-II, and corresponded to the same *spa* type as F9 (t021), the *dru* type of this isolate was markedly different to those detected in cluster F (Table 5.2; Figure 5.6). A review of the available laboratory data indicated that, while isolates from cluster F were obtained from adults at GSH and UCTPH, the sporadic isolate was obtained from a child at RCCH. Collectively, these data suggest a different epidemiological origin for S3 compared to isolates from cluster F, supporting the PFGE clustering.

While ST36-MRSA-II represented a minor clone in hospitals in Cape Town, accounting for 12 % of the isolates, the prevalence of this clonal type differed in other regions of South Africa. In total, 18.75 % of MRSA isolates from Tygerberg Hospital were consistent with t012 or t021, but the STs and SCC*mec* types of these isolates were not determined (Salaam-Dreyer, 2010). As these *spa* types were detected among ST36-MRSA-II isolates in the present study, it is possible that t021 and t021 isolates at Tygerberg Hospital were also consistent with ST36-MRSA-II. This would suggest slight differences in the prevalence of this clone in the Western Cape; however, MLST and SCC*mec* typing are required to confirm the clonal types of the t012 and t021 MRSA because these *spa* types have also been described in ST30 isolates (http://spaserver.ridom.de/). As described in Chapter 3 [3.4], SCC*mec* type II was most prevalent among MRSA obtained from the Steve Biko Academic Hospital in Pretoria, Gauteng, accounting for 64 % of the isolates (Makgotlho *et al.*, 2009); however, whether these MRSA corresponded to ST36-MRSA-II remains to be determined as no sequence-based typing methods were used to characterise the

isolates. In contrast with the results of the current study and that of Moodley *et al.* (2010), ST36-MRSA-II was not detected during two investigations into the epidemiology of MRSA from healthcare institutions in the KwaZulu-Natal province (Essa *et al.*, 2009; Shittu *et al.*, 2009). In the national context, ST36-MRSA-II has been reported as the second-most prevalent MRSA clone (24.52 %), and has been identified in all provinces, except the Northern Cape and the North West provinces (see Appendix G for a provincial map of South Africa) (Moodley *et al.*, 2010).

Although the population structure and evolution of ST36-MRSA-II has not been analysed as rigorously as ST239-MRSA-III, studies have suggested that the worldwide prevalence of this clone is also likely due to dissemination (Enright *et al.*, 2002; Robinson and Enright, 2003). All ST36 isolates reported to date have been MRSA associated with SCC*mec* type II (Feil and Enright, 2004), suggesting that the SCC*mec* element is stable within this lineage; therefore, it is possible that the *dru* locus may be phylogenetically informative for ST36-MRSA-II strains as observed for ST239-MRSA-III (Smyth *et al.*, 2009). The *dru* type of F8 (dt9a) corresponded to the second-most prevalent *dru* type identified among ST36-MRSA-II isolates from seven cities or towns in Scotland (Goering *et al.*, 2008a). Although the *dru* type of F9 (dt7s) was not detected among ST36-MRSA-II isolates from Scotland (Goering *et al.*, 2008a), it differs from that of F8 by just two repeats (Figure 5.6). ST36-MRSA-II isolates from Cape Town were also resistant to erythromycin, clindamycin and ciprofloxacin, which is common of UK isolates corresponding to this clonal type (Johnson, 1998; Murchan *et al.*, 2004; Gould *et al.*, 2008). Collectively, these data suggest that ST36-MRSA-II isolates from hospitals in Cape Town may be related to EMRSA-16 isolates from the UK. Travel has previously been proposed as a driver of the dissemination of EMRSA-16 from the UK to Malta and the Canary Islands, both of which are popular tourist destinations for UK citizens (Montesinos *et al.*, 2006; Gould *et al.*, 2008; Scicluna *et al.*, 2009); therefore, given the history of migration and tourism between South Africa and the UK, it is possible that international travel introduced ST36-MRSA-II into this country.

The clonal types of three sporadic isolates, S2, S7 and S8, were not consistent with those of the six PFGE clusters, or any of the other sporadic MRSA. Rather, these isolates were consistent with three unique or sporadic clones. The sporadic isolate

S7 was consistent with ST22-MRSA-IVh, t032, which belongs to CC22, one of the major MRSA lineages (Figure 5.10) (Robinson and Enright, 2003).

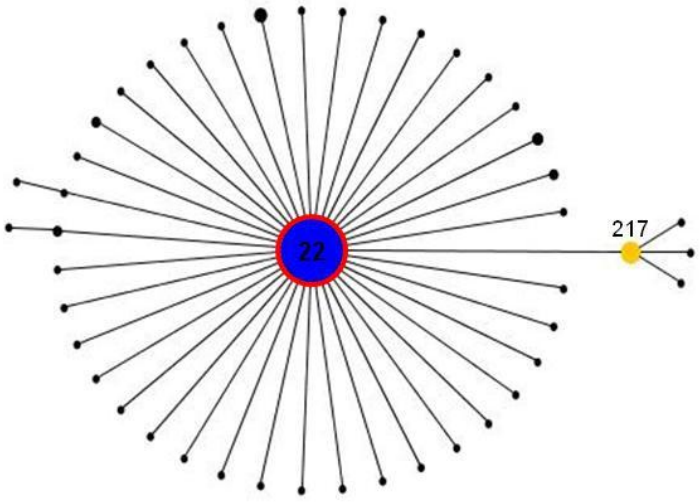


Figure 5.10 Graphical representation of patterns of descent among members of *S. aureus* clonal complex 22 as determined using the eBURST algorithm. The predicted founder of clonal complex (CC) 22, ST22, is indicated by the blue circle. As indicated by the red circle, ST22 was the only member of CC22 identified in the current study. Descendants of ST22 are indicated by black circles with the yellow circle representing the only known subgroup of CC22 (ST217). For clarity, no other STs are indicated. The relative sizes of the nodes are proportional to the corresponding number of isolates reported in the MLST database.

ST22-MRSA-IV corresponds to the pandemic HA-MRSA clone known as EMRSA-15, which emerged during the 1990s in UK hospitals (Johnson *et al.*, 2005), and has since been described throughout Europe, and also in Canada, the Middle East, New Zealand and Australia (Deurenberg and Stobberingh, 2008). In addition to being described as a cause of ha-MRSA infections, ST22-MRSA-IV has more recently emerged as a cause of ca-MRSA infections, and has also been isolated from companion animals (Faria *et al.*, 2005; Moodley *et al.*, 2006; Strommenger *et al.*, 2006b; de Lencastre *et al.*, 2007; Boakes *et al.*, 2010). A review of the body of work that formed the basis of the report by Moodley *et al.* (2010) revealed that the *spa*-CC including t032 (detected in S7) comprised just 1.78 % of the MRSA, with isolates obtained from only the Western Cape, Eastern Cape and KwaZulu-Natal (Oosthuysen, 2007). Combined with the results of the current study, these data suggest that ST22-MRSA-IV has not been particularly successful in South Africa.

Isolate S7 exhibited several molecular characteristics typical of the UK epidemic clone EMRSA-15. S7 corresponded to t032, the *spa* type most frequently associated with EMRSA-15 (Deurenberg and Stobberingh; 2008), and dt9j, a common *dru* type among ST22-MRSA-IVh isolates from Scotland and Ireland (Goering *et al.*, 2008a; Shore *et al.*, 2010). Additionally, the antimicrobial susceptibility profile of S7 was similar to that described for EMRSA-15 isolates (Johnson, 1998; Livermore, 2000; Johnson *et al.*, 2005). Travel between the UK and Malta and the Azores archipelago has been proposed as one possible route of entry of EMRSA-15 into hospitals on those islands (Gould *et al.*, 2008; Scicluna *et al.*, 2009; Conceição *et al.*, 2010). Similarly, it is possible that travel has resulted in the introduction of ST22-MRSA-IVh into hospitals in South Africa, given its history of frequent exchanges with the UK.

The remaining sporadic clones identified in this study, ST72-MRSA-V and ST650-MRSA-IVb, have not, to the best of the author's knowledge, been previously described in South Africa. Isolate S2 was consistent with the sporadic clone ST72-MRSA-V, which belongs to a successful subgroup of CC8 that has diversified, producing several SLVs and a DLV (Figure 5.7). This isolate represents an unusual clone in that ST72 has typically been described in association with SCC*mec* type IV (McDougal *et al.*, 2003; Kim *et al.*, 2007; Park *et al.*, 2007b; Bae *et al.*, 2010; Lim *et al.*, 2010; Tavares *et al.*, 2010) and, on rare occasions, SCC*mec* type II (Peck *et al.*, 2009). As suggested in Chapter 3 [3.4], S2 may contain a variant of SCC*mec* type V or, alternatively a composite SCC*mec* element, neither of which have been described in ST72-MRSA. Isolate S2 and previously described ST72 isolates did, however, share similar antimicrobial susceptibility profiles, with members of this lineage typically susceptible to the non-β-lactam antibiotics, except erythromycin and clindamycin (McDougal *et al.*, 2003; Lim *et al.*, 2010; Tavares *et al.*, 2010).

Although this is the first report of ST72-MRSA in South Africa, ST72-MRSA-IV has been reported as a major MRSA lineage in Korea (Kim *et al.*, 2007; Park *et al.*, 2007b; Peck *et al.*, 2009; Bae *et al.*, 2010; Lim *et al.*, 2010), and as one of the eight major epidemic strain types (USA700) in the USA (McDougal *et al.*, 2003). In both of these countries, ST72-MRSA-IV has been described as a cause of both ca- and ha-MRSA (McDougal *et al.*, 2003; Kim *et al.*, 2007; Park *et al.*, 2007b; Tenover *et al.*, 2008; Peck *et al.*, 2009; Bae *et al.*, 2010) infections and, in Korea, the clone has also been isolated from animal products (Lim *et al.*, 2010). Outside of Korea and the

USA, there have only been isolated reports of ST72-MRSA-IV, including ca-MRSA isolates from Portugal (Tavares *et al.*, 2010) and ha-MRSA isolates from Brazil (Scheunck *et al.*, 2009) and Germany (Luedicke *et al.*, 2010). Although the definition of isolates as ca- and ha-MRSA in the current study was rudimentary, S2 was classified as ha-MRSA. The low prevalence of ST72-MRSA-V in hospitals in Cape Town, and in the rest of South Africa, suggests that this clone may not have taken hold in local hospitals.

Following a BURP analysis using the default parameters described by Ridom StaphType [5.2.2], the *spa* types of isolate S2 and ST72-MRSA from Korea, Portugal and the USA (McDougal *et al.*, 2003; Peck *et al.*, 2009; Bae *et al.*, 2010; Tavares *et al.*, 2010) were assigned to the same *spa*-CC (Figure 5.11).

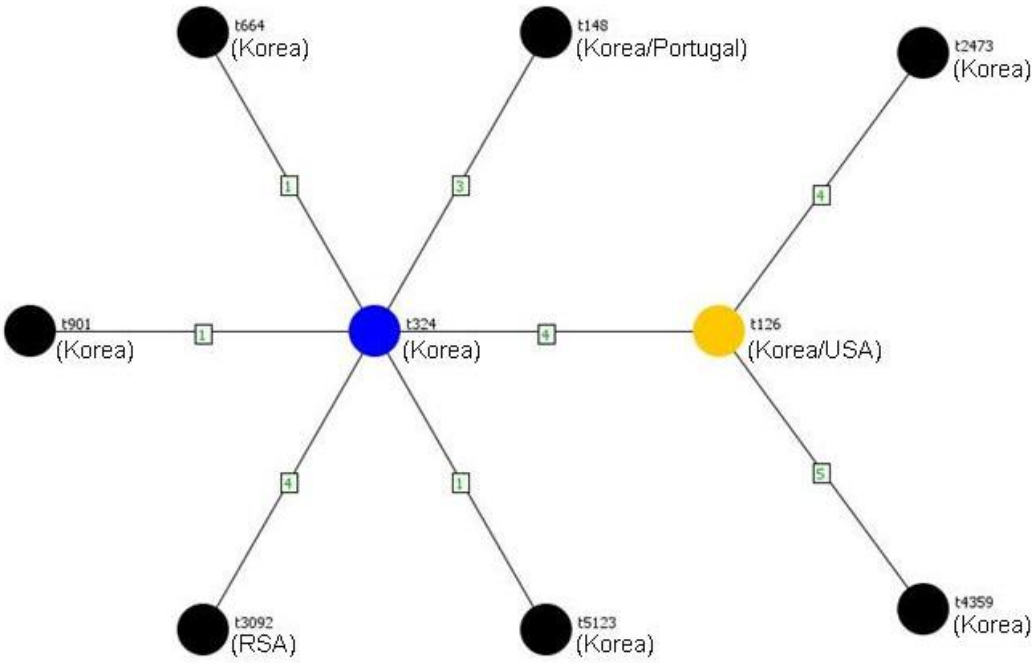


Figure 5.11 Graphical representation of clustering among *spa* types of ST72-MRSA isolates as determined by a BURP analysis. The blue circle indicates the putative founding *spa* type, and the yellow circle the founder of a subgroup. The costs between *spa* types, as determined by the BURP algorithm, are shown in green text in boxes between nodes. Countries in which the *spa* types have been previously described are indicated in brackets alongside each node (RSA, Republic of South Africa). (McDougal *et al.*, 2003; Peck *et al.*, 2009; Bae *et al.*, 2010; Tavares *et al.*, 2010)

Further, individual alignments of these *spa* types indicated that t3092 of S2 was more similar to *spa* types commonly identified in ST72-MRSA clones described in Korea and Portugal (Peck *et al.*, 2009; Bae *et al.*, 2010; Tavares *et al.*, 2010), than those reported in the USA (McDougal *et al.*, 2003) (Figure 5.12). Given that this clone is most prevalent in Korea, and that SCC*mec* type V has not been reported previously in South Africa, it is likely that ST72-MRSA-V was imported into South Africa as a result of travel, which commonly occurs between these two countries. Future studies will be required to obtain a greater understanding of the evolution of ST72-MRSA-V.

t3092 (RSA)	r07	r23	r12	r21	r12	r17	r20	r17	r12	-	r17	-
t126 (USA; 6)	r07	r23	r12	r21	-	-	-	r17	r12	r12	r17	-
t148 (K/P; 1)	r07	r23	r12	r21	r12	r17	r20	r17	r12	r12	r17	-
t664 (K; 3)*	r07	r23	r12	-	r12	r17	r20	r17	r12	-	r17	-
t324 (K; 4)*	r07	r23	r12	-	r12	r17	r20	r17	r12	r12	r17	-
t901 (K; 5)	r07	r23	-	-	r12	r17	r20	r17	r12	r12	r17	-
t2473 (K; 6)	r07	r23	r12	r21	-	-	-	-	r12	-	r17	-
t4359 (K; 7)	r07	r23	r12	-	-	-	-	-	r12	-	r17	-
t5123 (K; 5)	r07	r23	r12	-	r12	r17	r20	r17	r12	r12	r17	r17

Figure 5.12 Aligned *spa* repeat sequences for *spa* types of ST72-MRSA isolates described in South Africa, Korea, the USA and Portugal. Pairwise alignments were carried out in Ridom StaphType to compare all *spa* repeat sequences to t3092 (isolate S2 from Cape Town). Unique repeats are represented by different colours and gaps indicated by (-). Countries of origin and alignment costs as calculated by Ridom StaphType are indicated in parentheses adjacent to the *spa* type (country; cost). RSA, Republic of South Africa; USA, United States of America; K, Korea. (*) indicates predominant *spa* types for ST72-MRSA (McDougal *et al.*, 2003; Peck *et al.*, 2009; Bae *et al.*, 2010; Tavares *et al.*, 2010).

The only other sporadic clone, corresponding to isolate S8, was consistent with ST650-MRSA-IVb. ST650 (allelic profile 81-4-1-4-12-1-10) is a SLV of ST5 (1-4-1-4-12-1-10), varying at the *arcC* locus, which indicates that it is a member of one of the oldest and most diverse *S. aureus* lineages, CC5 (Figure 5.13). ST650 appears to be a rare genotype and has been reported only once previously (personal communication, Daniel Godoy, curator of *S. aureus* MLST database).

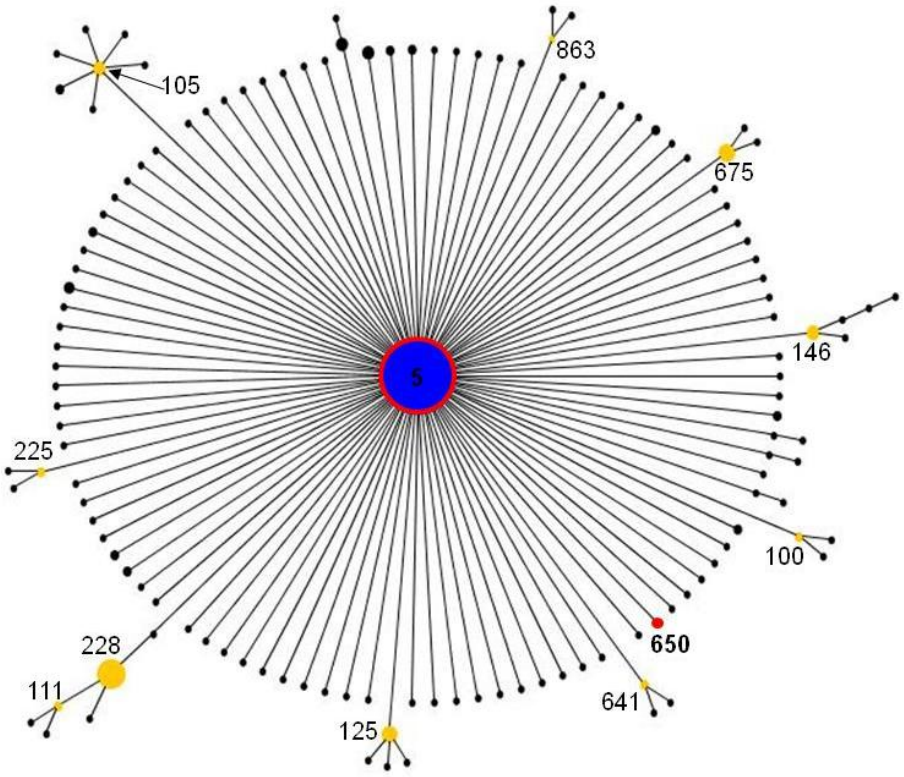


Figure 5.13 Graphical representation of patterns of descent among members of *S. aureus* clonal complex 5 as determined using the eBURST algorithm. The predicted founder of clonal complex (CC) 5, ST5, is indicated by the blue circle. Descendants of ST5 are indicated by black and yellow circles. Subgroups that have given rise to their own variants are indicated by yellow circles. For clarity, only the STs of subgroups are indicated. STs identified in the current study are shown in bold and the nodes are circled in red. The relative sizes of the nodes are proportional to the corresponding number of isolates reported in the MLST database.

Isolate S8 contained SCC*mec* type IVb, was susceptible to non-β-lactam antibiotics, corresponded to an unusual ST uncommon among HA-MRSA, and was the PFGE outlier, all of which are characteristics typical of CA-MRSA (Millar *et al.*, 2007). It was, therefore, surprising that this isolate was classified as ha-MRSA. This may suggest that ST650-MRSA-IVb has not been successful in hospitals in South Africa, regardless of whether it evolved in this setting or was imported from the community.

The evolution and origin of ST650-MRSA-IVb in South Africa is unclear. Previous reports of ST5-MRSA from South Africa have indicated that these isolates typically contain SCC*mec* type I (Moodley *et al.*, 2010), or, in some cases SCC*mec* type III (Shittu *et al.*, 2009), and correspond to t045, not t002 as in S8. To the best of the

author's knowledge, the only ST5-MRSA-IV reported in South Africa to date include two isolates from the KwaZulu-Natal province (Essa *et al.*, 2009); however, the *spa* types and SCC*mec* subtypes of these isolates are unknown. It seems unlikely that ST650-MRSA-IVb arose from ST5-MRSA-I/III following the acquisition of a mutation in *arcC*, and the replacement of SCC*mec* type I/III with IVb. Instead, it seems more plausible that ST650-MRSA-IVb emerged locally from an ST8-MRSA-IV ancestor. Interestingly, although the epidemiology of MSSA in South Africa is poorly understood, the recent characterisation of *S. aureus* isolates from Tygerberg hospital reported that t002 accounted for approximately 8.70 % of MSSA isolates, suggesting that the *S. aureus* population in the Western Cape may include ST5-MSSA (Salaam-Dreyer, 2010). This observation makes it possible to suggest that ST650-MRSA-IVb arose locally from ST5-MSSA following the acquisition of SCC*mec* type IVb and a mutation in the *arcC* gene, either of which may have occurred first; however, the possibility that ST650-MRSA-IVb was imported from another region cannot be excluded at present.

The second-most prevalent clone identified among isolates from hospitals in Cape Town was ST5-MRSA-I. ST5-MRSA-I corresponded to a pandemic HA-MRSA clone assigned to CC5 (Figure 5.13), which is also known as EMRSA-3. EMRSA-3 emerged in the UK (Johnson *et al.*, 2005) and is now prevalent worldwide (Deurenberg and Stobberingh). In the current study, the identification of three putative ca-MRSA isolates among the ST5-MRSA-I suggests that this clone is also present in communities in Cape Town (Appendix A). The similarities between the ca- and ha-MRSA corresponding to ST5-MRSA-I suggest transmission between hospitals and communities or *vice versa*, highlighting a need for further studies to obtain a better understanding of the dissemination of MRSA between hospitals and communities in Cape Town.

Epidemiological studies from South Africa suggest that ST5-MRSA-I, t045, is far more prevalent in hospitals in Cape Town (37 %) than in other regions of the country. The *spa* types of MRSA collected from Tygerberg Hospital did not include t045 or any related *spa* types, suggesting that ST5-MRSA-I, t045, was not present in that hospital (Salaam-Dreyer, 2010). Similarly, no isolates corresponding to ST5-MRSA-I were described in studies recently carried out in the KwaZulu-Natal (Essa *et al.*, 2009; Shittu *et al.*, 2009). Instead Shittu *et al.* (2009) described ST5-MRSA-III as a

minor clone in KwaZulu-Natal, accounting for 9.8 % of the isolates, while Essa *et al.* (2009) described ST5-MRSA-IV as a minor clone, accounting for 8.33 % of the isolates in that province. SCC*mec* type I was not detected among MRSA from the Steve Biko Academic Hospital in Pretoria, suggesting that the ST5-MRSA-I clone was absent in that institution (Makgotlho *et al.*, 2009); however, the prevalence of ST5-MRSA carrying other SCC*mec* elements remains to be determined as the isolates were not characterised using sequence-based typing methods. In contrast to these regional studies, Moodley *et al.* (2010) reported that ST5-MRSA-I represented one of five major MRSA clones within the South African context, accounting for 6.77 % of isolates. As observed for the other common clonal types identified in this study, the national distribution of ST5-MRSA-I was uneven, as this clone was not detected in the KwaZulu-Natal, Northern Cape and Gauteng provinces (Moodley *et al.*, 2010). Whether the apparently uneven national distribution of ST5-MRSA-I (and ST36-MRSA-II and ST239-MRSA-III) was simply due to the sampling strategy of Moodley *et al.* (2010), or perhaps due to differences in clonal dissemination, or local infection control practices and antibiotic prescription policies, is yet to be determined.

While it is possible that ST5-MRSA-I isolates present in Cape Town evolved from an imported ancestral clone, it is interesting to consider its evolution in the light of the study reported by Nübel *et al.* (2008). Nübel *et al.* (2008) found that, at least in the case of ST5-MRSA, the acquisition of SCC*mec* has been grossly underestimated, and the global prevalence of ST5-MRSA is in fact due to the frequent emergence of methicillin-resistant lineages from ST5-MSSA in distinct geographic locations. The detection of *ccrC* in local ST5-MRSA-I isolates was surprising. The SCC*mec* content of these strains appears to differ from ST5-MRSA-I isolates previously described in South Africa, and indeed from their counterparts from the rest of the world; to the best of the author's knowledge, *ccrC* has only been previously described in two SCC*mec* type I isolates (Kondo *et al.*, 2007). When considered in conjunction with the results of Nübel *et al.* (2008), these data suggest that the *ccrC*-positive ST5-MRSA-I present in Cape Town may have emerged locally when an MSSA ancestor acquired an unusual SCC*mec* element. Alternatively, if *ccrC* is present within a mobile genetic element distinct from SCC*mec* type I, this element may have been acquired locally by an ST5-MRSA-I ancestor.

As described in Chapter 3 [3.3 and 3.4], isolate B1 corresponded to the only local representative of ST5-MRSA-I that did not contain the *ccrC* gene. At that point, it was suggested that B1 and B2 may not be as closely related as estimated by PFGE. The *spa* typing and *dru* typing data support this suggestion: B1 corresponded to the similar yet distinct t7185 and dt10ar, while all the other ST5-MRSA-I isolates shared t045 and dt10a. Taken together, these data suggest that B1, the only ST5-MRSA-I isolate from UCTPH, was divergent from the remaining ST5-MRSA-I isolated at MMH, GSH and RCCH. In this context, it was of interest that ST5-MRSA-I isolates from MMH, GSH and RCCH all corresponded to t045 and dt10a, and shared high levels of similarity as determined by PFGE. It is possible that ST5-MRSA-I has only recently been introduced into local hospitals, and that the low degree of genetic diversity observed in this study is the result of the founder effect. Alternatively, it is possible that this study has obtained a snapshot of the local ST5-MRSA-I population after a bottleneck event, resulting in the limited genetic diversity. It is also possible that these results reflect a genetic background that does not readily accommodate genetic change, although this seems less likely given the diversity previously observed within the ST5 genotype (Nübel *et al.* 2008). Regardless of the reasons for the lack of variation in this clone, these data suggest that *dru* typing may not be suitable for the discrimination of ST5-MRSA-I isolates from hospitals in Cape Town at present.

In this study, the predominant clone did not correspond to any pandemic MRSA lineages. Instead, the predominant clone in hospitals in Cape Town corresponded to the infrequently described multidrug-resistant, *pvl*-negative ST612-MRSA-IVd (3-3-1-1-4-88-83). As it is a DLV of ST8 (3-3-1-1-4-4-3) varying at the *tpi* and *yqiL* loci, ST612 was assigned to CC8 (Figure 5.7). The ST612-MRSA-IVd clone collectively accounted for 44 % of MRSA, including all isolates from PFGE clusters D and E, as well as the sporadic isolates S1, S4, S5 and S6. Three and four isolates from clusters D and E, respectively, were classified as ca-MRSA (Appendix A), which hints that this clone may be present in both hospitals and communities in Cape Town. These data also indicate that there is little difference between ca- and ha-ST612-MRSA-IVd, suggesting that there has been transmission of MRSA between hospitals and communities in Cape Town. There may be a similar prevalence of this clone at Tygerberg Hospital as a recent study characterising *S. aureus* isolates from that institution assigned 43.75 % of MRSA to a single *spa*-CC, including isolates

corresponding to *spa* types detected among ST612-MRSA-IVd in the current study (Salaam-Dreyer, 2010).

Although apparently most prevalent in the Western Cape, ST612-MRSA-IV in fact represents the predominant South African clone, accounting for 26.13 % of isolates included in a nationwide study on the epidemiology of MRSA from public and private diagnostic laboratories (Moodley *et al.*, 2010). In that study, two major ST612-MRSA-IV PFGE clusters were identified, and accounted for 19.68 % and 6.45 % of isolates, respectively. Isolates belonging to the larger PFGE cluster were obtained from all nine provinces; MRSA from the smaller PFGE cluster were obtained from all provinces, except for the North West, Gauteng and Limpopo provinces. A low prevalence of SCC*mec* type IV (4 %) has been reported among MRSA from the Steve Biko Academic Hospital situated in Pretoria, Gauteng (Makgotlho *et al.*, 2009), suggesting that ST612-MRSA-IVd may be present at a low frequency at least in that institution; however, these isolates may have corresponded to a clonal type other than ST612-MRSA-IVd as they were *pvl*-positive, whereas all ST612-MRSA-IV isolates described to date have been *pvl*-negative. Two regional studies, both carried out in KwaZulu-Natal (Essa *et al.*, 2009; Shittu *et al.*, 2009), have reported results that conflict with those described by Moodley *et al.* (2010). ST612-MRSA-IV was not identified in these studies; instead, Essa *et al.* (2009) reported that 66.67 % of isolates corresponded to ST8-MRSA-IV, while Shittu *et al.* (2009) indicated that two DLVs of ST8, ST1173-MRSA-IV (3-3-1-1-4-131-3) and ST1338-MRSA-IV (3-3-1-1-4-131-83), collectively accounted for 62.3 % of the MRSA.

ST612-MRSA-IVd isolates from hospitals in Cape Town exhibited a marked degree of genetic variation as indicated by PFGE, and by the detection of four *spa* types and seven *dru* types among the fourteen representative isolates. This degree of genetic variation may not be unique to ST612-MRSA-IV isolates included in the current study. Five *spa* types were present among the fourteen putative ST612-MRSA-IV isolates from Tygerberg Hospital (Salaam-Dreyer, 2010), while eleven *spa* types were detected among the two major and twelve minor PFGE clusters corresponding to ST612-MRSA-IV isolates obtained from public and private laboratories across South Africa (Moodley *et al.*, 2010). It is possible that ST612-MRSA-IV represents an ancient clone that has undergone clonal expansion in South Africa, thus gradually

accumulating the genetic variation observed among local isolates. Alternatively, it is possible that the genetic background of ST612-MRSA-IV is readily able to accommodate mutations and the acquisition of mobile genetic elements, resulting in the diversity observed in recent studies. These explanations are not mutually exclusive, and may both have played a role in generating the level of genetic diversity observed in ST612-MRSA-IV isolates from South Africa. Given the variation observed at the *spa* and *dru* loci of ST612-MRSA-IV isolates, it is possible that *spa* typing and *dru* typing, in conjunction with PFGE, will prove particularly useful for investigating potential outbreaks and intra- and inter-hospital transmission in South Africa.

While ST612-MRSA-IV appears to be endemic to South Africa, it does not seem to be prevalent elsewhere. To the best of the author's knowledge, this clone has only been reported previously in Australia and the UK, and then rarely. In Australia, ST612-MRSA-IV, dubbed WA MRSA-20, has only been isolated once each in 2004 and 2008 (Coombs *et al.*, 2005; Nimmo and Coombs, 2008; Coombs *et al.*, 2009). Both isolates were obtained from healthcare workers; the 2004 isolate was cultured from a nasal swab and classified as ca-MRSA, while the 2008 isolate was cultured from a clinical specimen (Coombs *et al.*, 2005; Coombs *et al.*, 2009). To date, ST612-MRSA-IV isolates reported in the UK appear to be restricted to equine-associated veterinary workers (Williams, 2008; Clegg, 2010). That the dominant clone present in South Africa should correspond to an unusual genotype apparently largely restricted to the region was not completely surprising. Studies carried out in China (Fan *et al.*, 2009), Mali (Ruimy *et al.*, 2008), Cameroon, Madagascar, Morocco, Senegal and Niger (Breurec *et al.*, 2010) also reported that the *S. aureus* populations in these countries included unusual genotypes largely endemic to the region, as well as pandemic clones frequently described worldwide. Additionally, the extensive epidemiological studies of Nübel *et al.* (2008 and 2010) and Grundmann *et al.* (2010) have clearly indicated that certain MRSA clones, or variants thereof, are endemic to particular geographical regions.

It is interesting to speculate on the possible evolutionary relationship between ST612-MRSA-IV isolates from South Africa, Australia and the UK. Considering that ST612-MRSA-IV appears to represent a rare lineage, combined with the fact that it is unlikely that unrelated isolates will share the same ST (Spratt, 1999), it is quite

possible that the isolates from these three countries are related. In further support of this hypothesis, ST612-MRSA-IV isolates from Australia and the UK had antimicrobial resistance profiles (resistant to rifampicin, co-trimoxazole and gentamicin) similar to the South African isolates (Frances O'Brien and Julie Pearson, personal communication; Williams, 2008; Clegg, 2010). Interestingly, one of the Australian ST612-MRSA-IV isolates also corresponded with one of the most common South African *spa* types, t064, while the other was consistent with the closely related t7571. Unfortunately, the information available on the UK ST612-MRSA-IV isolates did not include *spa* typing data. It is likely that the frequent exchange of individuals, perhaps particularly healthcare workers, between South Africa, Australia and the UK has resulted in the observed distribution of ST612-MRSA-IV. Given that ST612-MRSA-IV appears to be most prevalent in South Africa, it is tempting to suggest that the clone emerged locally and was exported to Australia and the UK. However, given the prevalence of ST612-MRSA-IV and related putative ancestral clones among veterinary workers in the UK (Moodley *et al.*, 2006; Williams, 2008; Clegg, 2010), it is possible that the clone was imported from that country into South Africa, and subsequently flourished in its new environment

There are several possible routes of descent for ST612-MRSA-IV, regardless of in which country these processes occurred. An ST8 ancestor may have acquired mutations in *tpi* and then *yqiL*, or *yqiL* and then *tpi*, going through one of two possible intermediary SLVs (ST828 or ST995) to become ST612, with SCC*mec* type IV acquired at any stage during this process (Figure 5.14).

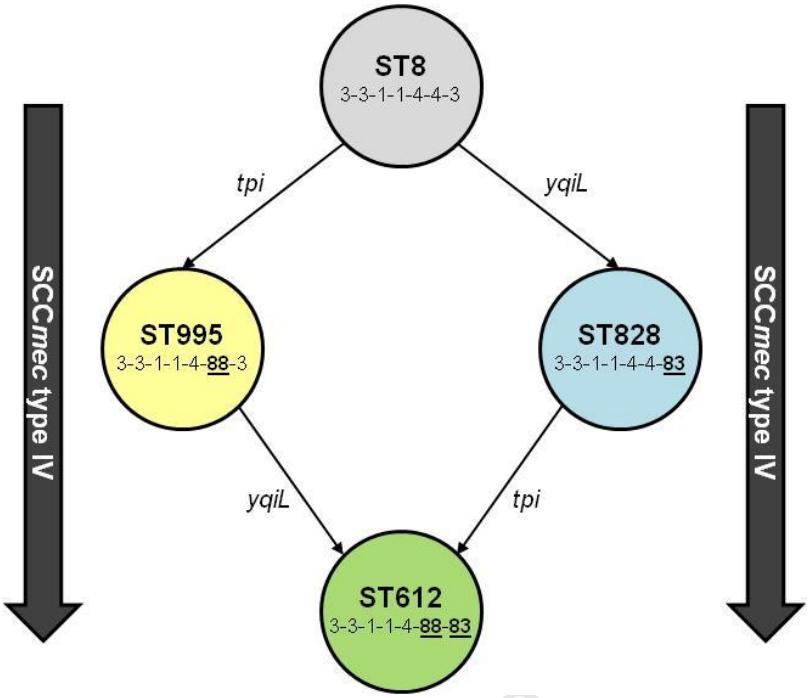


Figure 5.14 Schematic diagram illustrating possible evolutionary histories for ST612-MRSA-IV. Different genotypes are represented by circles with STs and allelic profiles indicated in their centres. Mutations in housekeeping genes are indicated adjacent to the stepwise progressions between ancestral and descendant STs, and in also in the allelic profile of the descendant genotype. SCC*mec* type IV may have been acquired at any stage during the evolution of ST612, as indicated by the grey arrows.

The eBURST analysis of the *S. aureus* MLST database suggested ST828 as the intermediary for ST612 (Figure 5.7); however, it is important to acknowledge that this is simply one possible evolutionary scenario, and it is also possible that ST995 was the intermediary for ST612 (Figure 5.14). ST828 and ST995 represent single entries in the *S. aureus* database, corresponding to an MSSA isolate obtained from the Netherlands, and an ST995-MRSA-IV strain isolated in Norway, respectively (www.mlst.net), where to the best of the author's knowledge, ST612-MRSA-IV has not been described. To date, no MSSA or MRSA intermediaries of ST612-MRSA-IV have been described in South Africa, the UK or Australia. Interestingly, putative ST612-MSSA isolates corresponding to *spa* types typical of ST612-MRSA-IV have been described at Tygerberg Hospital (Salaam-Dreyer, 2010). This may suggest the local emergence of ST612-MRSA-IV upon acquisition of SCC*mec* type IV. Alternatively, these isolates may in fact represent ST612-MRSA from which the

SCC*mec* cassette has been excised, as appears to be common in the case of SCC*mec* type IV (Jansen *et al.*, 2006).

In the context of the emergence of the ST612 genotype, the detection of ST8-MRSA-IV as the predominant clone in a recent study carried out in the KwaZulu-Natal province (Essa *et al.*, 2009) makes it possible to suggest that ST612-MRSA-IV arose locally from this lineage. Further, the identification of ST1173-MRSA-IV, t064, and ST1338-MRSA-IV, t064, two dominant DLVs of ST8, in KwaZulu-Natal hints at the evolution of the ST8-MRSA-IV lineage within South Africa (Shittu *et al.*, 2009). Larger future studies on the epidemiology of MRSA and MSSA isolates from the South Africa may well provide additional clues as to the evolution of ST612-MRSA-IV. Ultimately, it will be necessary to analyse larger portions of the genomes of ST612-MRSA-IV isolates obtained from different time periods and geographical regions, as well as those of any putative ancestral or descendant genotypes, in order to resolve the evolutionary history of this clone.

Regardless of its origins and evolutionary history, ST612-MRSA-IV appears to have been most successful in South Africa. Reasons as to why the success of this clone has been limited to South Africa are likely to be complex and multifactorial. The widespread prevalence of ST612-MRSA-IV in South Africa may be directly related to the transmissibility of this clone, or to factors related to its host population. Alternatively, it is possible that local infection control practices, either in terms of current policies or adherence to the recommended guidelines, are inadequate for controlling ST612-MRSA-IV. Additionally, the possibility that the community is a reservoir of this clone cannot be dismissed until studies examining the molecular epidemiology of both ca-MRSA and carriage isolates have been performed. It is also possible that certain aspects of the biology of ST612-MRSA-IV have contributed to its local success. For example, the presence of the smaller SCC*mec* type IV may have conferred an advantage on this clone as the other major clones present in South Africa are associated with the larger SCC*mec* type I – III elements, which are thought to confer a greater fitness cost (Ma *et al.*, 2002; Okuma *et al.*, 2002; Robinson and Enright, 2003).

Finally, it is interesting to consider the contribution of antimicrobial usage to the success of ST612-MRSA-IV in South Africa. ST612-MRSA-IVd isolates from hospitals in Cape Town were largely multidrug-resistant, and the combined reports of Moodley *et al.* (2010) and Marais *et al.* (2009) suggest that ST612-MRSA-IV isolates from other regions of South Africa may be similarly resistant. ST612-MRSA-IV isolates from hospitals in Cape Town were universally resistant to rifampicin, and all but two were resistant to co-trimoxazole; however, resistance to these antibiotics was rare among other clones detected in hospitals in Cape Town. As described in Chapter 2 [2.4], previous studies have shown that the use of rifampicin and co-trimoxazole selects for *S. aureus* strains resistant to these antibiotics (Martin *et al.*, 1999; Sekiguchi *et al.*, 2006). If the genetic background of ST612-MRSA-IV is in fact plastic, it is possible that the frequent use of these antimicrobial agents in South Africa may have driven the *de novo* acquisition of mutations and genetic elements conferring resistance on local isolates as observed in Japan and Spain (Sekiguchi *et al.*, 2006; Mick *et al.*, 2010). Alternatively, it is possible that the frequent use of rifampicin and co-trimoxazole in South Africa has driven the clonal expansion of a resistant ST612-MRSA-IV ancestral strain. These possibilities will be investigated further in Chapter 6.

CHAPTER 6

The Detection of an Uncommon Double Mutation Conferring High-level Rifampicin-resistance and Additional Conserved Synonymous Single Nucleotide Polymorphisms Suggests Clonal Expansion of ST612-MRSA-IV in Cape Town Hospitals

6.1 Introduction

In South Africa, rifampicin is used in combination therapies for the treatment of *S. aureus* infections and, perhaps more importantly, is one of the first line drugs used for the treatment of *M. tuberculosis* infections (South African Department of Health, 2004). Given the frequent use of rifampicin to treat the latter infections, it is likely that this selective pressure may have effected the emergence and high prevalence rate of rifampicin-resistant MRSA in South Africa. In recent studies carried out on MRSA from public hospitals in KwaZulu-Natal and Gauteng, 73.80 % and 100 % of isolates, respectively were rifampicin-resistant (Shittu and Lin, 2006; Groome *et al.*, 2009). A recent nationwide antimicrobial susceptibility surveillance survey showed that 52.82 % of MRSA obtained from South African public hospitals were rifampicin-resistant (Marais *et al.*, 2009). The proportion of rifampicin-resistant MRSA detected in this study was similar at 45 %. Resistance to rifampicin was universal among the forty-four isolates corresponding to the dominant local clone, ST612-MRSA-IV, accounting for all bar one of the rifampicin-resistant isolates identified in this study. The only other rifampicin-resistant isolate was C31, a representative ST5-MRSA-I isolate from PFGE cluster C. Worryingly, all MRSA isolates selected for rifampicin E-tests had high levels of resistance (MICs ≥ 256 µg/ml).

Rifampicin is a bactericidal antimicrobial agent that inhibits transcription by binding to the β-subunit of the bacterial DNA-dependent RNA polymerase (Chambers, 1997; Greenwood *et al.*, 2007). The β-subunit of RNA polymerase is encoded by *rpoB*, and mutations within conserved regions of the gene have been shown to confer resistance to rifampicin in *S. aureus*, *M. tuberculosis*, *Escherichia coli*, *Neisseria meningitidis* and *Streptococcus pneumonia* (Aubry-Damon *et al.*, 1998). Modification

of the β-subunit decreases the affinity of RNA polymerase for rifampicin, thus conferring resistance to this antibiotic. The majority of mutations that confer rifampicin resistance in *S. aureus* have been mapped to a conserved region of the *rpoB* gene known as the rifampicin resistance-determining region (RRDR), as shown in Figure 6.1 (O'Neill *et al.*, 2006). The RRDR spans amino acids 462 to 550 (*S. aureus* co-ordinates) and includes two conserved regions shown to be rifampicin resistance hotspots in the corresponding regions in *E. coli* and *M. tuberculosis* (Aboshkiwa *et al.*, 1995; Aubry-Damon *et al.*, 1998; Wichelhaus *et al.*, 1999; O'Neill *et al.*, 2006). These conserved regions are known as clusters I and II, and also include most mutations known to confer rifampicin resistance in *S. aureus* (Figure 6.1) (Aubry-Damon *et al.*, 1998; Wichelhaus *et al.*, 1999).

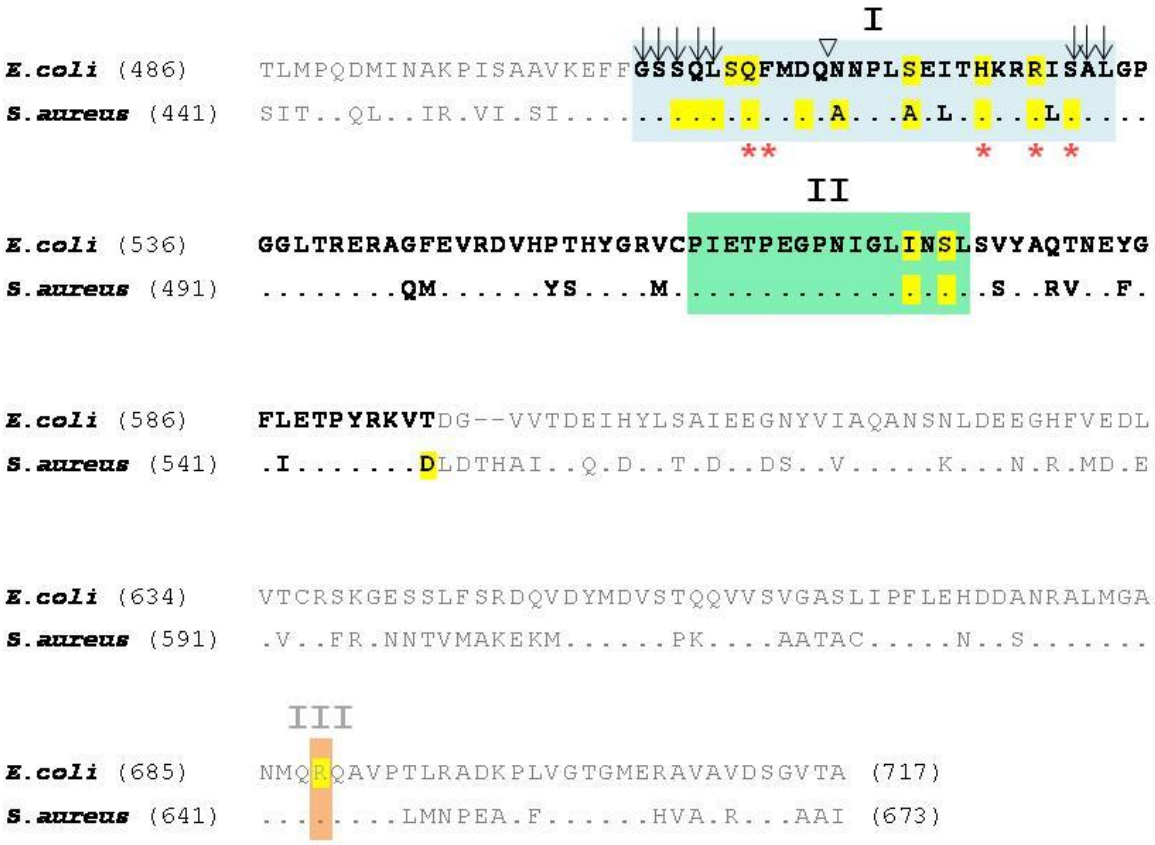


Figure 6.1. Alignment of *E. coli* and *S. aureus* RpoB amino acid sequences. Mutations conferring rifampicin resistance in *E.coli* commonly occur in clusters I, II and III, which are highlighted in blue, green and orange, respectively. In *S. aureus* most mutations conferring resistance to rifampicin occur within the rifampicin-resistance determining region (bold black text). Dots (.) indicate identical amino acids and dashes (–) represent gaps. For each sequence, commonly mutated residues are highlighted in yellow. Deletions are indicated by downward-pointing arrows and insertions by inverted triangles. Residues known to be involved in rifampicin binding in *S. aureus* are marked with a red asterisk. Adapted from Aubry-Damon *et al.* (1998), Wichelhaus *et al.* (1999) and O'Neill *et al.* (2006).

Although there are few studies describing rifampicin resistance genotypes in *S. aureus*, both *in vitro* and *in vivo* data suggest a bias towards certain mutations (Figure 6.1) (O'Neill *et al.*, 2006). Additionally, the level of rifampicin resistance has been shown to be dependent on the location and nature of amino acid substitutions within the β-subunit (Aubry-Damon *et al.*, 1998; Wichelhaus *et al.*, 1999; Wichelhaus *et al.*, 2002). Residues commonly mutated in *S. aureus* include Q_{468}, F_{469}, H_{481}, R_{484}, and S_{486}, all of which have been shown to be involved in rifampicin binding (Figures 6.1 and 6.2) (O'Neill *et al.*, 2006).

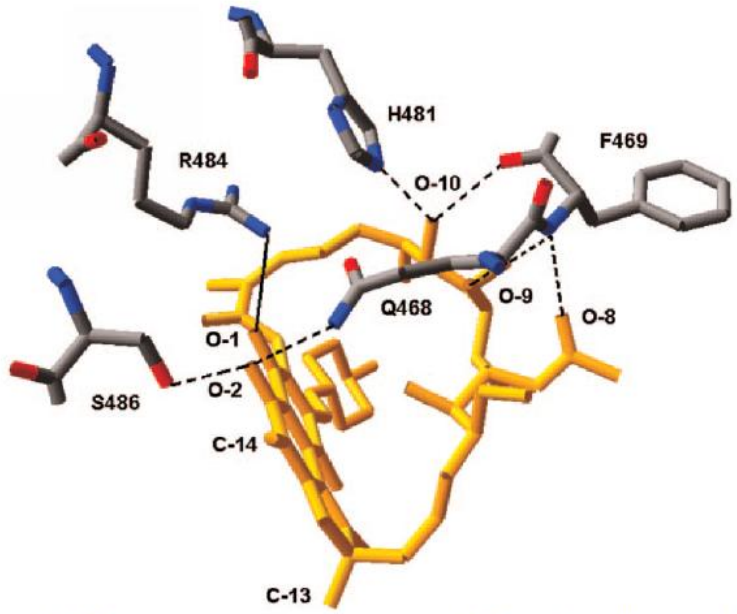


Figure 6.2 Rifampicin binding to the β-subunit of *S. aureus* RNA polymerase. Rifampicin is shown in yellow. The five important β-subunit residues known to make direct H-bonding contact with the rifampicin molecule (Q_{468}, F_{469}, H_{481}, R_{484}, and S_{486}) are marked. Figure taken from O'Neill *et al.* (2006).

The level of resistance conferred by a particular mutation is dependent on the type of amino acid substitution (Wichelhaus *et al.*, 1999), as illustrated by changes at H_{481}, one of the most frequently substituted residues identified to date (O'Neill *et al.*, 2006). At H_{481}, substitution of the histidine residue with asparagine confers low level rifampicin resistance, while substitution with tyrosine confers high level resistance. Although single mutations such as $H_{481}Y$ can confer high-level rifampicin-resistance, clinical strains with high levels of resistance frequently carry two or more mutations (Wichelhaus *et al.*, 1999; O'Neill *et al.*, 2006). A large proportion of double and triple mutants carry the $H_{481}N$ substitution, which alone confers low level resistance but, in

conjunction with additional mutations, is associated with high-level resistance (Wichelhaus *et al.*, 1999; Wichelhaus *et al.*, 2002; O'Neill *et al.*, 2006). These results indicate that one knock-on effect of secondary and tertiary mutations within the *rpoB* gene is increased levels of rifampicin resistance (O'Neill *et al.*, 2006).

Although mutations within *rpoB* confer a selective advantage in the presence of rifampicin, they do come at a physiological price (Reynolds, 2000; Lipsitch, 2001; Wichelhaus *et al.*, 2002). A number of *in vitro* studies in *S. aureus* and *E. coli* have shown that rifampicin-resistant mutants are often less fit than their parent strains (Reynolds, 2000; Wichelhaus *et al.*, 2002). It has been suggested that resistance modifications in RNA polymerase decrease the affinity of the enzyme for DNA and destabilise the transcription complex, which results in lowered levels of transcription (O'Neill *et al.*, 2006). In the absence of rifampicin, mutations in *rpoB* would be disadvantageous and one might expect reversion to the wild-type protein. Reynolds *et al.* (2000) showed that, in *E. coli*, this is not the case as intragenic, or extragenic, compensatory mutations restored levels of transcription and bacterial fitness. Similarly, O'Neill *et al.* (2006) showed that the secondary mutations present in clinical *S. aureus* isolates not only increased levels of rifampicin resistance, but in some cases restored bacterial fitness, or both restored fitness and increased levels of resistance.

As mentioned previously, the available information on rifampicin resistance genotypes is limited to a small number of studies which, to the best of the author's knowledge, have not included rifampicin-resistant MRSA from South Africa. The investigations described in this chapter were prompted by two related questions: what is the mechanism of rifampicin resistance in the local and previously described ST612-MRSA-IV isolates, and is the prevalence of rifampicin-resistance in this clone due to the acquisition of *de novo* mutations in *rpoB* or the dissemination of a rifampicin-resistant ancestor?

6.2 Experimental Protocol

6.2.1 Selection of MRSA for *rpoB* genotyping

Twelve MRSA isolates described in this study were selected for *rpoB* genotyping, including 2 rifampicin susceptible isolates: the sporadic isolate designated S7, and the representative from cluster F known as F9 (Appendix A). Isolate C31 (ST5-MRSA-I), the only rifampicin-resistant isolate outside the local dominant clone, was included as were 9 ST612-MRSA-IV isolates identified in this study: 2 sporadic isolates, S1 and S4, 2 isolates from cluster D (D2 and D4) and 5 isolates from cluster E (E5, E8, E19, E26 and E32), which were selected to include MRSA from across the PFGE cluster (Appendix A). Additionally, the 2 ST612-MRSA-IV isolated from healthcare workers in Australia (Coombs *et al.*, 2005; Nimmo and Coombs, 2008; Coombs *et al.*, 2009), as well as 2 previously described ST612-MRSA-IV isolates from South Africa (Goering *et al.*, 2008b), were also included for *rpoB* genotyping. The Australian ST612-MRSA-IV isolates (04-17532 and 09-15543) were obtained from Dr Frances O'Brien and Ms Julie Pearson (Department of Microbiology and Infectious Diseases, PathWest Laboratory Medicine WA, Royal Perth Hospital, Perth, Australia), while the 2 isolates previously described in South Africa (N83 and N84) were provided by Professor Richard Goering (Department of Medical Microbiology and Immunology, Creighton University Medical Center, Omaha, NE, USA). All isolates selected for *rpoB* genotyping had rifampicin E-test MICs ≥ 256 µg/ml, except isolate S7 and the representative of cluster F, which were rifampicin susceptible with E-test MICs ≤ 0.016 µg/ml.

6.2.2 *rpoB* genotyping

A 702 bp region of *rpoB* spanning amino acid residues 441 to 673 (*S. aureus* co-ordinates), including the RRDR (Figure 6.1), was amplified as described by Aubry-Damon *et al.* (1998). Each PCR was carried out in a final volume of 100 µl with 1X PCR buffer, 1.5 mM $MgCl_2$ (JMR Holdings, London, UK), 40 pmol of the primers F3 and F4 (Synthetic DNA Laboratory, University of Cape Town, Cape Town, South Africa) (Appendix E) and 400 µM of each dNTP (Thermo Scientific, Wilmington, DE, USA). One hundred nanograms of template DNA and 1 U of *Taq* polymerase (JMR Holdings, London, UK) were added to the reaction volume. Amplification was carried out using an Applied Biosystems 2720 Thermocycler (Applied Biosystems, Carlsbad, CA, USA). The PCR cycling conditions consisted of an initial denaturation step of 4

min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 45 s, elongation at 72°C for 45 s, with a final extension step of 3 min at 72°C.

The PCR products were separated on 1.5 % agarose gels and visualised as previously described [3.2.2]. The 702 bp fragment was purified and both strands were sequenced at the Central Analytical Facility at the University of Stellenbosch as described in Chapter 3 [3.2.3.2]. After the sequences were viewed and edited [3.2.3.2], they were aligned to the *rpoB* sequence of RN4220, a rifampicin susceptible *S. aureus* strain (accession number X64172), using the ClustalW algorithm in BioEdit Sequence Alignment Editor (version 7.0.5.2) (Hall, 1999). The aligned sequences were trimmed in order to exclude regions with poor quality sequencing data and the resulting final fragment used for analysis was 669 nucleotides in length. The predicted amino acid sequences of the selected isolates, spanning amino acid residues 442 to 664, were inferred using the translation function in BioEdit Sequence Alignment Editor (version 7.0.5.2) (Hall, 1999).

6.3 Results

6.3.1 Characterisation of mutations in the *rpoB* gene

Three *rpoB* genotypes were detected among the 14 rifampicin-resistant isolates characterised in this study. The *rpoB* genotypes and other available molecular characteristics of all 16 isolates included in this investigation are shown in Table 6.1. A multiple sequence alignment of the DNA and amino acid sequences is shown in Appendix H. No amino acid substitutions were observed in the RpoB protein sequences of rifampicin-susceptible isolates S7 and F9. C31 (ST5-MRSA-I), the only rifampicin-resistant isolate identified outside the dominant local clonal type, carried a single $H_{481}Y$ substitution known to confer high-level rifampicin resistance (Table 6.1; Appendix H). The 9 local ST612-MRSA-IV isolates all carried the same double mutational changes within the RRDR, $H_{481}N$, $I_{527}M$ (Table 6.1; Appendix H), which have previously been associated with high-level rifampicin resistance in *S. aureus*. Both of the ST612-MRSA-IV previously identified in South Africa also carried the $H_{481}N$, $I_{527}M$ double substitution. These mutational changes were also observed in the 2 ST612-MRSA-IV isolates from Australia; however an additional novel amino acid substitution, $K_{579}R$, was observed outside the RRDR in isolate 09-15543 (Table 6.1; Appendix H).

In addition to the mutations associated with amino acid substitutions in RpoB, synonymous single nucleotide polymorphisms (SNPs) were detected in all 16 isolates when the *rpoB* nucleotide sequences were compared to that of RN4220 (Table 6.1; Appendix H). All isolates contained an identical synonymous SNP, thymine instead of guanine, at the third base of the codon for alanine at position 498 (Table 6.1; Appendix H). Otherwise between 1 and 3 additional synonymous SNPs particular to each clonal type were identified in the nucleotide sequences. Of note is the conserved cytosine present instead of thymine at the third base of the codon for arginine at amino acid position 512, which was detected in the 9 local and 4 previously identified ST612-MRSA-IV isolates (Table 6.1; Appendix H).

Table 6.1. Molecular typing data and *rpoB* genotypes of selected methicillin-resistant *S. aureus* isolates from hospitals in Cape Town, and previously described ST612-MRSA-IV from South Africa and Australia

MRSA isolate [a]	Clonal type (*spa* type; *dru* type); clonal complex	PFGE cluster	Rifampicin MIC (µg/ml)	Nucleotide substitution	Amino acid position[b]	Amino acid substitution
S7	ST22-MRSA-IV (t032; dt9j); CC22	Sporadic Isolate	≤0.016	GCG→GCT	498	-
				CAT→CAC	554	-
				AAT→AAC	599	-
F9	ST36-MRSA-II [c] (t021; dt7s); CC30	F	≤0.016	AAC→AAT	474	-
				GCG→GCT	498	-
				GTA→GTG	502	-
				ACA→ACG	518	-
C31	ST5-MRSA-I (t045; dt10a); CC5	C	≥256	CAT→TAT	481	$H_{481}Y$
				GCG→GCT	498	-
				AAT→AAC	630	-
				GGT→GGA	658	-
S1	ST612-MRSA-IV (t1443; dt10aq); CC8	Sporadic Isolate	≥256	CAT→AAT	481	$H_{481}N$
				GCG→GCT	498	-
				CGT→CGC	512	-
				ATT→ATG	527	$I_{527}M$
S4	ST612-MRSA-IV (t1257; dt10i); CC8	Sporadic Isolate	≥256	CAT→AAT	481	$H_{481}N$
				GCG→GCT	498	-
				CGT→CGC	512	-
				ATT→ATG	527	$I_{527}M$

D2	ST612-MRSA-IV [c] (t064; dt10i); CC8	D	≥256	CAT→AAT	481	$H_{481}N$
				GCG→GCT	498	-
				CGT→CGC	512	-
				ATT→ATG	527	$I_{527}M$
D4	ST612-MRSA-IV (t064; dt10i); CC8	D	≥256	CAT→AAT	481	$H_{481}N$
				GCG→GCT	498	-
				CGT→CGC	512	-
				ATT→ATG	527	$I_{527}M$
E5	ST612-MRSA-IV [c] (t1443; dt9ac); CC8	E	≥256	CAT→AAT	481	$H_{481}N$
				GCG→GCT	498	-
				CGT→CGC	512	-
				ATT→ATG	527	$I_{527}M$
E8	ST612-MRSA-IV [c] (t064; dt9ad); CC8	E	≥256	CAT→AAT	481	$H_{481}N$
				GCG→GCT	498	-
				CGT→CGC	512	-
				ATT→ATG	527	$I_{527}M$
E19	ST612-MRSA-IV [c] (t1443; dt10o); CC8	E	≥256	CAT→AAT	481	$H_{481}N$
				GCG→GCT	498	-
				CGT→CGC	512	-
				ATT→ATG	527	$I_{527}M$
E26	ST612-MRSA-IV [c] (t1443; dt10i); CC8	E	≥256	CAT→AAT	481	$H_{481}N$
				GCG→GCT	498	-
				CGT→CGC	512	-
				ATT→ATG	527	$I_{527}M$

E32	ST612-MRSA-IV (t1443; dt8v); CC8	E	≥256	CAT→AAT	481	$H_{481}N$
				GCG→GCT	498	-
				CGT→CGC	512	-
				ATT→ATG	527	$I_{527}M$
N83 [d]	ST612MRSA-IV (t064; dt10i); CC8	ND [e]	≥256	CAT→AAT	481	$H_{481}N$
				GCG→GCT	498	-
				CGT→CGC	512	-
				ATT→ATG	527	$I_{527}M$
N84 [d]	ST612MRSA-IV (t064; dt10i); CC8	ND	≥256	CAT→AAT	481	$H_{481}N$
				GCG→GCT	498	-
				CGT→CGC	512	-
				ATT→ATG	527	$I_{527}M$
04-17532 [d]	ST612-MRSA-IV (t064; dt10i); CC8	ND	≥256	CAT→AAT	481	$H_{481}N$
				GCG→GCT	498	-
				CGT→CGC	512	-
				ATT→ATG	527	$I_{527}M$
09-15543 [d]	ST612MRSA-IV [c] (t7571; dt10i); CC8	ND	≥256	CAT→AAT	481	$H_{481}N$
				GCG→GCT	498	-
				CGT→CGC	512	-
				ATT→ATG	527	$I_{527}M$
				AAA→AGA	579	$K_{579}R$

[a] Isolate designations as introduced in Chapter 2.
[b] *S. aureus* co-ordinates.
[c] Sequence Types (STs) inferred from those assigned to representative isolates from respective PFGE clusters.
[d] Previously described ST612-MRSA-IV isolates obtained from South Africa (N83 and N84) and Australia (04-17532 and 09-15543).
[e] ND, not determined.

6.4 Discussion

It has long been acknowledged that the use of antimicrobial agents in the clinical setting exerts selective pressure on bacteria resulting in the emergence of drug-resistant organisms (Lowy, 2003; Levy and Marshall, 2004; Hawkey, 2008). The proportion of rifampicin-resistant isolates detected in this study, particularly within the dominant clone, ST612-MRSA-IV, prompted the characterisation of the *rpoB* gene in a selection of MRSA isolates. Analysis of the RRDR of one ST5-MRSA-I isolate (C31) and thirteen representatives of ST612-MRSA-IV that all expressed high-level rifampicin-resistance identified three *rpoB* genotypes; no amino acid substitutions were detected in the rifampicin-susceptible isolates S7 and F9.

C31, ST5-MRSA-I, was the only rifampicin-resistant isolate outside of the dominant clonal type and carried a single mutational change within *rpoB*: $H_{481}Y$. This substitution, associated with high-level resistance, is one of the most common rifampicin resistance genotypes and has previously been reported in several laboratory mutants and clinical isolates (Aubry-Damon *et al.*, 1998; Wichelhaus *et al.*, 1999; Schmitz *et al.*, 2000; Wichelhaus *et al.*, 2002; O'Neill *et al.*, 2006). Molecular modelling has provided valuable insights as to how the $H_{481}Y$ mutation is refractory to rifampicin-binding. The imidazole side chain in histidine typically forms a hydrogen (H) bond with rifampicin (Figure 6.2) but, because the phenolic side chain of tyrosine is larger than the imidazole ring of histidine, the conformation of the modified RNA polymerase is altered, thereby eliminating the original H-bond between rifampicin and its target. Additionally, the altered conformation allows for a slight movement of rifampicin within the binding cavity, which decreases drug-enzyme hydrophobic interactions. It is these changes that are thought to be responsible for the significant decrease in the binding energy of RNA polymerase for rifampicin and the elevated MICs associated with the $H_{481}Y$ genotype (O'Neill *et al.*, 2006).

Mick *et al.* (2010), detected four markedly different rifampicin resistance genotypes among thirty-two ST228-MRSA-IV isolates, expressing varied levels of resistance, which were collected from a single hospital over three years. This contrasted with the results obtained in the current study where only two *rpoB* genotypes were identified among the nine local and four previously described ST612-MRSA-IV isolates. One genotype, $H_{481}N$, $I_{527}M$, accounted for twelve of the thirteen

ST612-MRSA-IV isolates, including two previously identified South African isolates, and one of the Australian isolates. This genotype is relatively uncommon and, to the best of the author's knowledge, has only been reported previously in two clinical rifampicin-resistant MRSA isolates from Italy (Wichelhaus *et al.*, 1999; O'Neill *et al.*, 2006). The third *rpoB* genotype was present in the remaining Australian ST612-MRSA-IV isolate and included a triple amino acid substitution: $H_{481}N$, $I_{527}M$, $K_{579}R$. To the best of the author's knowledge, $K_{579}R$, which occurs outside the RRDR, has not been reported previously, hence $H_{481}N$, $I_{527}M$, $K_{579}R$ is a novel genotype.

Studies have shown that substituting asparagine for histidine at position 481 weakens the H-bond with rifampicin, thereby reducing the affinity of RNA polymerase for the drug (O'Neill *et al.*, 2006); however, the effects of the $I_{527}M$ substitution have not been characterised. As the single mutational change $H_{481}N$ typically confers low-level rifampicin resistance, it can be assumed that high-level resistance develops in a stepwise fashion due to the acquisition of additional mutations within *rpoB* (Wichelhaus *et al.*, 1999). That $H_{481}N$, $I_{527}M$ isolates described to date have expressed high-level rifampicin resistance (Wichelhaus *et al.*, 1999), supports the assumption that the secondary $I_{527}M$ substitution augments the level of resistance conferred by the primary substitution. In addition to these substitutions, the most recent of the Australian ST612-MRSA-IV isolates, 09-15543, isolated in 2009, contains a third novel $K_{579}R$ substitution, which likely represents the latest substitution in this clone. The impact of the $K_{579}R$ substitution on rifampicin-resistance is yet to be determined.

A number of synonymous SNPs were detected in the sixteen isolates when using the nucleotide sequence of RN4220 as a reference (Table 6.1; Appendix H). One SNP (GCG→GCT) in the codon for alanine at position 498 was common to all sixteen isolates, which belonged to a number of *S. aureus* lineages (Table 6.1). The SNP (GCG→GCT) has also been reported in *S. aureus* strains ATCCBAA44 and PER88 (ST247-MRSA-I control strains) and ST228-MRSA-I isolates from Spain (Mick *et al.*, 2010). Codon usage tables derived from the genome sequences of six *S. aureus* strains (NCTC8325 (ST8-MSSA), COL (ST250-MRSA-I), Newman (ST8-MRSA-II), USA300 (ST8-MRSA-IV), N315 (ST5-MRSA-II) and Mu50 (ST5-VISA-II)), indicated that the codon GCT is twice as prevalent as GCG (http://www.kazusa.or.jp/codon/). Given these observations, it seems more likely that the SNP (GCT→GCG) arose

once in RN4220, rather than on separate occasions in diverse *S. aureus* lineages. This can, however, only be confirmed when more *rpoB* sequences of *S. aureus* isolates from a variety of genetic backgrounds become available.

The only other conserved synonymous SNP (CGT→CGC in the codon for arginine at amino acid position 512) was observed in all ST612-MRSA-IV isolates (Table 6.1; Appendix H). This mutation was noteworthy for two reasons: firstly, AT-rich organisms such as *S. aureus* more commonly contain thymine or adenine at the third base of codons rather than cytosine or guanine (Ermolaeva, 2001; http://www.kazusa.or.jp/codon/); secondly, codon usage tables indicated that the codon CGT is more common than CGC in *S. aureus* (http://www.kazusa.or.jp/codon/). Thus it is possible to suggest that the SNP (CGT→CGC) did not arise on multiple occasions, but was instead inherited from a common ancestor, and has been conserved within ST612-MRSA-IV. Further, this supports a hypothesis of clonal expansion of a rifampicin-resistant ST612-MRSA-IV ancestor in hospitals in Cape Town. Significantly, the four previously described ST612-MRSA-IV isolates carried the same synonymous SNPs observed in the local isolates, suggesting that ST612-MRSA-IV described in this study may share a common ancestor with the isolates previously described in South Africa and Australia.

Although the reasons for the dominance of ST612-MRSA-IV in hospitals in Cape Town are likely to be complex, it is probable that the frequent use of rifampicin in South Africa has contributed to the emergence and local expansion of this clone. Albeit that resistance mutations frequently come at an initial fitness cost to the organism (Lipsitch, 2001), it has been shown that rifampicin-resistant *E. coli* do not revert to a wild-type susceptible RNA polymerase in the absence of rifampicin (Reynolds, 2000). Rather, they persist because of their capacity to develop compensatory mutations, which restore bacterial fitness (Reynolds, 2000). Similarly, *S. aureus* isolates have also been shown to acquire compensatory mutations that negate the fitness costs caused by the primary resistance mutation (O'Neill *et al.*, 2006). Disturbingly, if the ST612-MRSA-IV isolates from local hospitals have accumulated intragenic or extragenic compensatory mutations, reduced use of rifampicin may have little to no effect on the prevalence of this multidrug-resistant clone (Wichelhaus *et al.*, 2002).

CHAPTER 7

Concluding Remarks

At the onset of this study, there was a paucity of comprehensive molecular epidemiological data available on MRSA from South Africa; however, a number of reports describing aspects of the molecular epidemiology of MRSA from South Africa have been published in the past eighteen months (Essa *et al.*, 2009; Makgotlho *et al.*, 2009; Shittu *et al.*, 2009; Moodley *et al.*, 2010), which is evidence of an increasing global appreciation of the importance of epidemiological investigations. To the best of the author's knowledge, the current study represents the first comprehensive molecular characterisation of MRSA from hospitals in Cape Town. Additionally, along with the studies reported by Shittu *et al.* (2009) and Moodley *et al.* (2010), this represents one of the first comprehensive molecular characterisations of MRSA from South Africa, including techniques for the investigation of aspects of both local and global epidemiology. The results of this study have essentially provided a snapshot of the molecular epidemiology of MRSA present in five hospitals in Cape Town during 2007 and 2008.

PFGE proved a useful starting point in the molecular characterisation of the hundred isolates, providing valuable insights into the epidemiology of MRSA from hospitals in Cape Town. A comprehensive molecular characterisation was carried out including MRSA isolates representative of the PFGE clusters, as well as the sporadic isolates, using a combination of SCC*mec* typing, MLST and *spa* typing, as is the international convention. Using this data, it was possible to determine the identities of the MRSA clones present in hospitals in Cape Town, and investigate the epidemiology of local isolates within the broader South African and global contexts.

Several frequently described pandemic clones were identified among the hundred isolates, some of which have been previously reported in South Africa, albeit at different frequencies (Essa *et al.*, 2009; Shittu *et al.*, 2009; Moodley *et al.*, 2010). Two well-known pandemic HA-MRSA clones, ST239-MRSA-III and ST36-MRSA-II, were identified in this study but, in contrast to reports from other regions of South Africa, these clones both corresponded to minor PFGE clusters. Based on recent

studies, it seems likely that ST239-MRSA-III and ST36-MRSA-II were imported into South Africa, and have subsequently disseminated throughout the country due to emigration, tourism and local travel (Enright *et al.*, 2002; Robinson and Enright, 2003; Smyth *et al.*, 2009; Harris *et al.*, 2010).

The second-most prevalent clone identified in this study, ST5-MRSA-I, also corresponded to a pandemic HA-MRSA lineage. As suggested for ST239-MRSA-III and ST36-MRSA-II, ST5-MRSA-I may have been imported into South Africa; however, in the light of recent studies regarding the evolution of ST5-MRSA (Nübel *et al.*, 2008), it is also interesting to consider the possibility that this clone emerged locally. ST5-MRSA-I was markedly more prevalent in hospitals in Cape Town than in other regions of South Africa (Essa *et al.*, 2009; Shittu *et al.*, 2009; Moodley *et al.*, 2010), and also appears to be present in local communities. It remains to be determined whether the nationwide differences in the prevalence of ST5-MRSA-I, ST239-MRSA-III and ST36-MRSA-II might be due to the uneven dissemination of these clones, or to regional changes in clonal composition, or to the fact that these clones are equipped to thrive in some regions, but not others. The high prevalence of ST5-MRSA-I detected in the current study may also be linked to the recent dissemination of this clone in the maternity and neonatal services at GSH and MMH, which is currently being investigated further.

Interestingly, all but one of the local ST5-MRSA-I isolates carried the *ccrC* gene, which is uncommon in strains containing SCC*mec* type I. Similarly, isolate S2, corresponding to ST72-MRSA-V, a lineage not previously reported in South Africa, was also atypical in terms of its SCC*mec* content, as ST72-MRSA isolates are typically associated with SCC*mec* types II and IV (McDougal *et al.*, 2003; Kim *et al.*, 2007; Park *et al.*, 2007b; Peck *et al.*, 2009; Bae *et al.*, 2010; Lim *et al.*, 2010; Tavares *et al.*, 2010). Additionally, isolate S2 appeared to contain at least two alleles of *ccrC*, and may also contain divergent J regions. It is possible that the ST5-MRSA-I and ST72-MRSA-V isolates contain composite SCC*mec* elements, which include multiple *ccr* complexes (Ito *et al.*, 2009). Mosaic SCC*mec* elements are commonly described among CNS species, and it is possible that the unusual SCC*mec* elements that appear to be present in local MRSA originated in the CNS population present in hospitals in Cape Town (Garza-González *et al.*, 2010). Additional studies are required to further characterise the SCC*mec* content of the ST5-MRSA-I and ST72-

MRSA-V isolates. If these isolates do contain novel mosaic SCC*mec* elements, studies characterising the origins of these elements may improve our understanding of the local evolution of MRSA and the genetic exchanges taking place between *S. aureus* and CNS.

While a number of commonly described pandemic clones were identified in this study, the dominant clone present in hospitals in Cape Town was the infrequently described ST612-MRSA-IVd. ST612-MRSA-IV has recently been shown to be endemic in South Africa (Moodley *et al.*, 2010), but does not appear to be common elsewhere. To the best of the author's knowledge, the clone has only been previously reported on two occasions in Australia (Coombs *et al.*, 2005; Coombs *et al.*, 2009), and also rarely in horses and their veterinary workers in the UK (Williams, 2008; Clegg, 2010). This multidrug-resistant clone was detected in all of the included Cape Town hospitals, and also appears to be present in local communities.

It is interesting to consider the emergence and evolution of ST612-MRSA-IV. It is not yet clear whether ST612-MRSA-IV was imported into South Africa or whether it emerged locally in either the hospital or community setting. Given the restricted geographical distribution of ST612-MRSA-IV, it is quite possible that this clone emerged locally, and has since undergone clonal expansion to become the dominant clone in South Africa, subsequently being disseminated to Australia and the UK. The detection of an uncommon double mutation that confers high-level rifampicin resistance and a unique synonymous SNP in local ST612-MRSA-IV supports the suggestion of clonal expansion in hospitals in Cape Town. Furthermore, the fact that ST612-MRSA-IV isolates described previously in South Africa and Australia shared the same uncommon double mutation and unique SNP suggests that these MRSA may share a common ancestor with those present in hospitals in Cape Town. Further studies investigating larger portions of the ST612-MRSA-IV genome are required to clarify the emergence and evolution of this clone.

The genetic diversity detected among ST612-MRSA-IV isolates both in the current study and in that of Moodley *et al.* (2010) was also of interest. In both studies, a high degree of variation was observed in the PFGE macrorestriction profiles of

ST612-MRSA-IV isolates with these strains assigned to multiple clusters. Additionally, several *spa* types were detected among ST612-MRSA-IV isolates in both studies. In the present study, *dru* typing, a new single-locus sequence-based typing technique was also used to characterise the MRSA. Although *dru* typing was unable to differentiate the highly similar ST5-MRSA-I isolates, the technique detected a marked degree of genetic variation among the ST612-MRSA-IV isolates. Collectively, these data may suggest that ST612-MRSA-IV represents an ancient South African clone that has accumulated genetic variation over time, or, alternatively, it is possible that the genetic background of this clone is highly plastic and readily, and rapidly, accommodates genetic changes. These explanations are not mutually exclusive and both factors may have contributed to the level of genetic diversity observed among ST612-MRSA-IV isolates.

The six PFGE clusters corresponding to the major four clones included isolates from between two and five hospitals in Cape Town, which suggested the transmission of MRSA within and between local healthcare institutions. It was noted that three of these clones (ST239-MRSA-III, ST36-MRSA-III, and particularly ST612-MRSA-IV) included large proportions of multidrug-resistant isolates that were obtained from multiple hospitals. The transmission of multidrug-resistant MRSA within and between local hospitals is worrying given the therapeutic challenges posed by these strains. These findings have highlighted the need for further investigations into the underlying factors contributing to the dissemination of MRSA in hospitals in Cape Town, and have provided an indication of where such studies should be directed.

In addition to providing an understanding of the local epidemiology of MRSA from hospitals in Cape Town, the current study has also raised several questions that may form the basis of future community-based studies on MRSA. Of particular interest is the possible role that widespread use of rifampicin and co-trimoxazole in local communities plays in shaping the MRSA population structure, and future studies will be carried out to investigate this issue. Additionally, future studies will be required to determine whether or not there are in fact differences between adult and paediatric community-acquired MRSA as hinted by the current study. Current molecular markers of CA-MRSA have been determined on the basis of studies carried out largely in developed nations; therefore, future studies describing the molecular

epidemiology of CA-MRSA from South Africa will also prove invaluable in determining molecular markers appropriate for the local identification of these clones.

In the future, it will also be important to carry out studies similar to the one described here at regular intervals in order to monitor changes in the epidemiology of MRSA from hospitals in Cape Town. These studies will generate the data necessary for the evaluation and modification of existing infection control policies and antibiotic prescription guidelines, and will inform future studies, which will ultimately improve the control of MRSA in local hospitals.

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Table A1 Laboratory and experimental data obtained for 100 methicillin-resistant *S. aureus* isolates from hospitals in Cape Town[a]

MRSA isolate	Laboratory data obtained from the NHLS															Experimental data obtained during this study					
	Antibiogram											Date of isolation	Specimen type	Hospital	Ward	PFGE cluster	SCC*mec* type	ST	*spa* type	*dru* type	*rpoB* Amino Acid Substitutions
	P	Clo	E	Cli	R	Sxt	Ci	G	F	V	L										
A1	R	R	R	R	S	R	R	R	S	S	-	10.02.2007	Blood	GSH	ICU	A	III	239	t037	dt14e[b]	-
A2	R	R	R	R	S	R	R	R	S	S	S	01.10.2007	Pus swab	GSH	OPC	A	III	-	-	-	-
A3	R	R	R	R	S	R	R	R	S	S	S	17.10.2008	RTS	RCCH	ICU	A	III	-	-	-	-
A4	R	R	R	R	S	R	R	R	S	S	S	13.02.2007	RTS	RCCH	OPC	A	III	-	-	-	-
S1	R	R	S	S	R	R	R	R	S	S	S	23.04.2008	CVCT	GSH	MED	Sporadic Isolate	IVd	612	t1443	dt10aq[b]	$H_{481}N$; $I_{527}M$
S2	R	R	R	R	S	S	S	S	S	S	-	10.07.2007	Pus swab	GSH	OPC	Sporadic Isolate	V	72	t3092	dt11a	-
S3	R	R	R	R	S	S	R	R	S	S	-	12.07.2007	Blood	RCCH	ICU	Sporadic Isolate	II	36	t021	dt4c[b]	-
S4	R	R	R	R	R	R	R	R	S	S	S	23.07.2008	Pus swab	RCCH	MED	Sporadic Isolate	IVd	612	t1257	dt10i	$H_{481}N$; $I_{527}M$
B1	R	R	R	R	S	S	R	S	S	S	S	16.04.2008	RTS	UCTPH	SUR	B	I	5	t7185[b]	dt10ar[b]	-
B2	R	R	R	R	S	S	S	R	S	S	S	26.08.2008	Pus swab	GSH	SUR	B	I	5	t045	dt10a	-
C1	R	R	R	R	S	R	S	S	S	S	-	17.06.2007	Urine	MMH	GYN	C	I	-	-	-	-
C2	R	R	S	S	S	S	S	S	S	S	-	23.06.2007	RTS	GSH	GYN	C	I	-	-	-	-
C3	R	R	R	R	S	S	S	S	S	S	-	26.06.2007	Urine	MMH	GYN	C	I	-	-	-	-
C4	R	R	R	R	S	S	S	R	S	S	S	07.10.2007	Urine	MMH	ICU	C	I	-	-	-	-
C5	R	R	R	R	S	S	S	S	S	S	S	14.12.2007	Pus	MMH	GYN	C	I	5	t045	dt10a	-
C6	R	R	R	R	S	S	S	S	S	S	S	13.04.2008	Pus swab	MMH	GYN	C	I	-	-	-	-
C7	R	R	R	R	S	S	S	S	S	S	-	26.04.2008	Pus swab	MMH	GYN	C	I	-	-	-	-
C8	R	R	R	R	S	S	S	R	S	S	S	16.07.2008	Pus swab	MMH	ICU	C	I	-	-	-	-
C9	R	R	R	R	S	S	S	S	S	S	S	05.10.2007	Urine	MMH	GYN	C	I	-	-	-	-
C10	R	R	R	R	S	S	S	S	S	S	-	22.04.2008	Pus swab	MMH	GYN	C	I	-	-	-	-
C11	R	R	R	R	S	S	S	R	S	S	S	11.06.2008	Pus swab	GSH	GYN	C	I	-	-	-	-
C12[c]	R	R	R	R	S	S	S	R	S	S	-	15.02.2007	Pus swab	RCCH	NP	C	I	-	-	-	-

Table A1 cont.

MRSA isolate	P	Clo	E	Cli	R	Sxt	Ci	G	F	V	L	Date of isolation	Specimen type	Hospital	Ward	PFGE cluster	SCC*mec* type	ST	*spa* type	*dru* type	*rpoB* Amino Acid Substitutions
C13	R	R	R	R	S	S	S	R	S	S	-	27.02.07	Pus swab	GSH	GYN	C	I	-	-	-	-
C14	R	R	R	R	S	S	S	R	S	S	-	28.02.07	Pus swab	GSH	ICU	C	I	-	-	-	-
C15	R	R	S	S	S	S	S	S	S	S	-	12.06.07	Urine	MMH	GYN	C	I	-	-	-	-
C16[c]	R	R	S	S	S	S	S	S	S	S	-	12.06.07	Urine	MMH	GYN	C	I	5	t045	dt10a	-
C17[c]	R	R	R	R	S	S	S	S	S	S	-	18.06.07	Urine	RCCH	OPC	C	I	-	-	-	-
C18	R	R	R	R	S	S	S	S	S	S	-	07.09.07	Urine	MMH	GYN	C	I	-	-	-	-
C19	R	R	R	R	S	S	S	S	S	S	-	12.12.07	Blood	GSH	ICU	C	I	-	-	-	-
C20	R	R	R	R	S	S	S	S	S	S	-	12.12.07	Blood	GSH	ICU	C	I	-	-	-	-
C21	R	R	R	R	S	S	S	S	S	S	S	22.01.08	Pus swab	GSH	SUR	C	I	-	-	-	-
C22	R	R	R	R	S	S	S	S	S	S	-	16.04.08	RTS	MMH	ICU	C	I	-	-	-	-
C23	R	R	R	R	S	S	S	R	S	S	S	04.06.08	CVCT	RCCH	SUR	C	I	-	-	-	-
C24	R	R	R	R	S	S	S	R	S	S	S	26.06.08	Pus swab	GSH	SUR	C	I	-	-	-	-
C25	R	R	R	R	S	S	S	S	S	S	-	24.07.08	Pus swab	GSH	SUR	C	I	-	-	-	-
C26	R	R	R	R	S	S	S	S	S	S	S	28.07.08	Pus swab	GSH	SUR	C	I	-	-	-	-
C27	R	R	R	R	S	S	S	R	S	S	-	26.02.07	Pus swab	MMH	ICU	C	I	-	-	-	-
C28	R	R	R	R	S	S	S	S	S	S	S	30.05.08	Pus swab	MMH	GYN	C	I	-	-	-	-
C29	R	R	R	R	S	S	S	S	S	S	S	11.06.08	Pus swab	GSH	EMERG	C	I	-	-	-	-
C30	R	R	R	R	S	S	S	R	S	S	S	17.06.08	Pus swab	MMH	OPC	C	I	-	-	-	-
C31	R	R	R	R	R	S	S	S	S	S	S	20.06.08	Pus swab	MMH	GYN	C	I	5	t045	dt10a	$H_{481}Y$
C32	R	R	R	R	S	S	S	S	S	S	S	27.09.2008	CVCT	RCCH	SUR	C	I	-	-	-	-
C33	R	R	R	R	S	S	S	S	S	S	S	28.09.2008	Pus swab	GSH	OPC	C	I	-	-	-	-
C34	R	R	R	R	S	S	S	S	S	S	S	28.09.2008	Pus swab	MMH	ICU	C	I	-	t045	dt10a	-
C35	R	R	R	R	S	S	S	S	S	S	S	14.12.08	Pus swab	GSH	GYN	C	I	-	-	-	-
D1	R	R	S	S	R	R	R	R	S	S	S	16.04.08	Pus swab	GSH	SUR	D	IVd	-	-	-	-

Table A1 cont.

MRSA isolate	P	Clo	E	Cli	R	Sxt	Ci	G	F	V	L	Date of isolation	Specimen type	Hospital	Ward	PFGE cluster	SCC*mec* type	ST	*spa* type	*dru* type	*rpoB* Amino Acid Substitutions
D2	R	R	R	R	R	R	S	R	S	S	S	24.08.2008	Pus swab	RCCH	SUR	D	IVd	-	t064	dt10i	$H_{481}N$; $I_{527}M$
D3[c]	R	R	R	R	R	R	S	S	S	S	S	06.11.2008	Pus swab	GSH	MED	D	IVd	-	-	-	-
D4[c]	R	R	R	R	R	R	S	R	S	S	S	10.10.2008	RTS	GSH	EMERG	D	IVd	612	t064	dt10i	$H_{481}N$; $I_{527}M$
D5	R	R	R	R	R	R	S	R	S	S	-	12.06.2007	Pus swab	RCCH	MED	D	IVd	-	-	-	-
D6	R	R	R	R	R	R	S	S	S	S	S	11.06.2008	Pus swab	VH	MED	D	IVd	-	t064	dt10i	-
D7[c]	R	R	S	S	R	R	S	R	S	S	-	28.02.2007	Pus swab	GSH	MED	D	IVd	-	-	-	-
S5	R	R	S	S	R	R	S	R	S	S	S	12.02.2008	Pus swab	RCCH	NP	Sporadic Isolate	IVd	612	t064	dt10i	-
E1[c]	R	R	S	S	R	R	R	R	S	S	-	10.06.2007	Pus swab	RCCH	ICU	E	IVd	-	-	-	-
E2	R	R	R	R	R	R	R	S	S	S	-	28.07.2007	Blood	GSH	ICU	E	IVd	-	-	-	-
E3	R	R	S	S	R	R	R	R	S	S	S	23.04.2008	Pus swab	RCCH	SUR	E	IVd	612	t1443	dt10i	-
E4	R	R	R	R	R	R	R	R	S	S	-	28.01.2007	RTS	GSH	ICU	E	IVd	612	t2196	dt10ap[b]	-
E5	R	R	S	S	R	R	R	R	S	S	S	23.07.2008	Pus swab	GSH	SUR	E	IVd	-	t1443	dt9ac[b]	$H_{481}N$; $I_{527}M$
E6[c]	R	R	R	S	R	R	S	R	S	S	S	01.09.2008	Pus	RCCH	ICU	E	IVd	-	-	-	-
E7	R	R	R	R	R	R	S	R	S	S	-	12.06.2007	RTS	VH	OPC	E	IVd	-	-	-	-
E8	R	R	R	R	R	R	R	R	S	S	S	11.02.2008	RTS	UCTPH	SUR	E	IVd	-	t064	dt9ad[b]	$H_{481}N$; $I_{527}M$
E9	R	R	S	S	R	R	S	R	S	S	-	11.06.2007	Pus swab	GSH	OPC	E	IVd	-	-	-	-
E10	R	R	S	S	R	R	S	R	S	S	-	29.01.2007	RTS	GHS	OPC	E	IVd	-	-	-	-
E11	R	R	R	R	R	R	R	R	S	S	S	12.11.2008	Pus swab	GSH	MED	E	IVd	-	-	-	-
E12	R	R	S	S	R	R	R	R	S	S	-	18.07.2007	Pus swab	GSH	OPC	E	IVd	-	-	-	-
E13	R	R	S	S	R	R	R	R	S	S	S	23.05.2008	Pus swab	GSH	SUR	E	IVd	-	-	-	-
E14	R	R	R	R	R	R	R	R	S	S	-	26.02.2007	Pus swab	VH	MED	E	IVd	-	-	-	-
E15	R	R	S	S	R	R	R	R	S	S	-	11.07.2007	Pus swab	GSH	MED	E	IVd	-	-	-	-
E16	R	R	S	S	R	R	R	R	S	S	S	31.12.2007	CVCT	GSH	ICU	E	IVd	-	-	-	-

Table A1 cont.

MRSA isolate	P	Clo	E	Cli	R	Sxt	Ci	G	F	V	L	Date of isolation	Specimen type	Hospital	Ward	PFGE cluster	SCC*mec* type	ST	*spa* type	*dru* type	*rpoB* Amino Acid Substitutions
E17	R	R	S	S	R	R	R	R	S	S	S	23.07.2008	Pus swab	GSH	SUR	E	IVd	-	-	-	-
E18	R	R	S	S	R	R	R	R	S	S	S	16.04.2008	Pus swab	GSH	SUR	E	IVd	-	-	-	-
E19	R	R	S	S	R	R	R	R	S	S	-	28.02.2008	Pus	RCCH	MED	E	IVd	-	t1443	dt10o	$H_{481}N$; $I_{527}M$
E20	R	R	S	S	R	S	R	S	S	S	-	11.07.2007	Pus swab	GSH	OPC	E	IVd	-	-	-	-
E21	R	R	S	S	R	R	R	R	S	S	S	25.06.2008	Pus swab	GSH	SUR	E	IVd	-	-	-	-
E22[c]	R	R	S	S	R	R	R	R	S	S	-	28.02.2007	Urine	RCCH	EMERG	E	IVd	-	-	-	-
E23	R	R	R	R	R	R	R	R	S	S	-	06.07.2007	Pus swab	RCCH	NP	E	IVd	-	-	-	-
E24	R	R	S	S	R	R	R	R	S	S	S	18.07.2007	Pus	GSH	OPC	E	IVd	-	-	-	-
E25[c]	R	R	R	R	R	R	R	R	S	S	S	08.04.2008	Pus swab	RCCH	SUR	E	IVd	-	-	-	-
E26	R	R	S	S	R	R	R	R	S	S	-	29.02.2008	RTS	VH	MED	E	IVd	-	t1443	dt10i	$H_{481}N$; $I_{527}M$
E27	R	R	S	S	R	R	R	R	S	S	S	11.03.2008	Pus swab	GSH	ICU	E	IVd	-	-	-	-
E28	R	R	R	R	R	R	R	R	S	S	S	04.06.2008	Pus swab	MMH	ICU	E	IVd	-	-	-	-
E29	R	R	S	S	R	R	R	R	S	S	-	12.06.2007	Pus swab	GSH	MED	E	IVd	-	-	-	-
E30	R	R	R	S	R	R	R	R	S	S	S	29.09.2007	Pus swab	GSH	MED	E	IVd	-	-	-	-
E31	R	R	R	R	R	R	R	R	S	S	S	29.09.2008	Pus swab	RCCH	OPC	E	IVd	-	-	-	-
E32	R	R	S	S	R	R	R	R	I	S	S	03.11.2008	CVCT	GSH	MED	E	IVd	612	t1443	dt8v[b]	$H_{481}N$; $I_{527}M$
E33	R	R	R	R	R	S	S	R	S	S	S	17.06.2008	CVCT	RCCH	SUR	E	IVd	-	-	-	-
S6	R	R	S	S	R	R	S	R	S	S	-	23.09.2007	Blood	GSH	MED	Sporadic Isolate	IVd	612	t064	dt10i	-
S7	R	R	R	R	S	S	R	S	I	S	S	12.11.2008	Pus swab	GSH	MED	Sporadic Isolate	IVh	22	t032	dt9j	None
F1	R	R	R	R	S	S	R	S	S	S	S	09.10.2008	RTS	GSH	ICU	F	II	-	-	-	-
F2	R	R	R	R	S	S	R	S	S	S	S	16.10.2008	RTS	UCTPH	OPC	F	II	-	-	-	-
F3	R	R	R	R	S	S	R	R	S	S	S	09.06.2008	Pus swab	GSH	EMERG	F	II	-	-	-	-
F4	R	R	R	R	S	S	R	S	S	S	S	22.06.2008	Pus swab	UCTPH	ICU	F	II	-	-	-	-

Table A1 cont.

MRSA isolate	P	Clo	E	Cli	R	Sxt	Ci	G	F	V	L	Date of isolation	Specimen type	Hospital	Ward	PFGE cluster	SCC*mec* type	ST	*spa* type	*dru* type	*rpoB* Amino Acid Substitutions
F5	R	R	R	R	S	S	R	R	S	S	S	28.06.2008	Pus swab	GSH	ICU	F	II	-	-	-	-
F6	R	R	R	R	S	S	R	S	S	S	S	15.07.2008	Pus swab	GSH	SUR	F	II	-	-	-	-
F7	R	R	R	R	S	S	R	S	S	S	S	31.01.2008	Pus swab	GSH	SUR	F	II	-	-	-	-
F8	R	R	R	R	S	S	R	S	S	S	-	24.09.2007	Blood	UCTPH	ICU	F	II	36	t012	dt9a	-
F9	R	R	R	R	S	S	R	S	S	S	S	12.12.2007	CVCT	GSH	ICU	F	II	-	t021	dt7s[b]	None
F10	R	R	R	R	S	S	S	R	S	S	-	27.02.2008	Pus swab	GSH	SUR	F	II	-	-	-	-
F11	R	R	R	R	S	S	R	S	S	S	S	31.12.2007	Pus swab	GSH	SUR	F	II	-	-	-	-
S8	R	R	S	S	S	S	S	S	S	S	S	23.06.2008	Pus swab	RCCH	OPC	Sporadic Isolate	IVb	650	t002	dt10t	-

[a] NHLS, National Health Laboratory Service; MRSA, methicillin-resistant *S. aureus*; P, penicillin; Clo, cloxacillin; E, erythromycin; Cli, clindamycin; R, rifampicin; Sxt, co-trimoxazole; Ci, ciprofloxacin; G, gentamicin; F, fusidic acid; V, vancomycin; L, linezolid; PFGE, pulsed-field gel electrophoresis; SCC*mec*, staphylococcal cassette chromosome *mec*; ST, Sequence Type (multilocus sequence typing); RTS, respiratory tract specimen; CVCT, central venous catheter tips; GSH, Groote Schuur Hospital; RCCH, Red Cross War Memorial Children's Hospital; UCTPH, University of Cape Town Private Hospital; MMH, Mowbray Maternity Hospital; VH, Victoria Hospital; ICU, intensive care unit; OPC, outpatient clinic; MED, medical ward; SUR, surgical ward; GYN, gynaecology and obstetrics; EMERG, emergency services; NP, data not provided; (-), test or experiment not carried out.

[b] Novel *spa* or *dru* type.

[c] Putative community-acquired methicillin-resistant *S. aureus*.

APPENDIX B

Buffers and Solutions

All buffers and solutions made up to the appropriate final volume using distilled water.

ESP Buffer

10 mM Tris-Cl

1.0 mM EDTA

1% SDS

1 mg/ml Proteinase K

pH 8.0

Add 0.788g Tris-Cl and 0.186g EDTA to approximately 480 ml of distilled water, then adjust to pH 8.0 and make up to a final volume of 500 ml. Dissolve 1g SDS and 100mg proteinase K (Roche Diagnostics GmbH, Mannheim, Germany) in 100 ml of this buffer. Prepare 20 ml aliquots and store at -20º C for up to 6 months.

EC Buffer

6 mM Tris-Cl

1.0 M NaCl

0.1 M EDTA

0.5% Brij 58

0.5% Sarkosyl (pH 7.5)

Autoclave and store at room temperature up to 6 months.

TAE Buffer (50X)

242 g Tris base

100 ml EDTA (0.5M, pH 8.0)

57.1 ml Glacial Acetic Acid

Make up to final volume of 1L using distilled water.

Store at room temperature.

TBE Buffer (10X)

54 g Tris base

27.5 g Boric acid

4.65 g EDTA

pH 8.0

Make up to final volume of 500 ml using distilled water.

Autoclave and store at room temperature.

TE Buffer

10 mM Tris HCl

1 mM EDTA

pH 8.0

Autoclave and store at room temperature for up to 6 months

TEN Buffer

0.1M Tris Cl

0.15M NaCl

0.1M EDTA

Autoclave and store at room temperature for up to 6 months

APPENDIX C

Molecular Weight Markers

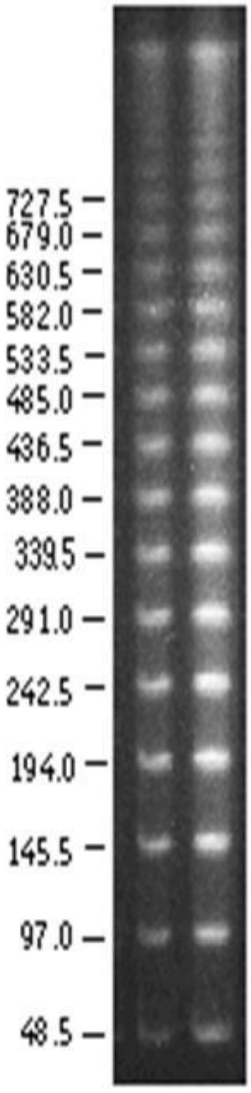


Figure C1 Lambda Ladder PFG Marker N0340S. Manufactured by New England BioLabs Inc (Ipswich, MA, USA).
(http://www.neb.com/nebecomm/products/productN0340.asp)

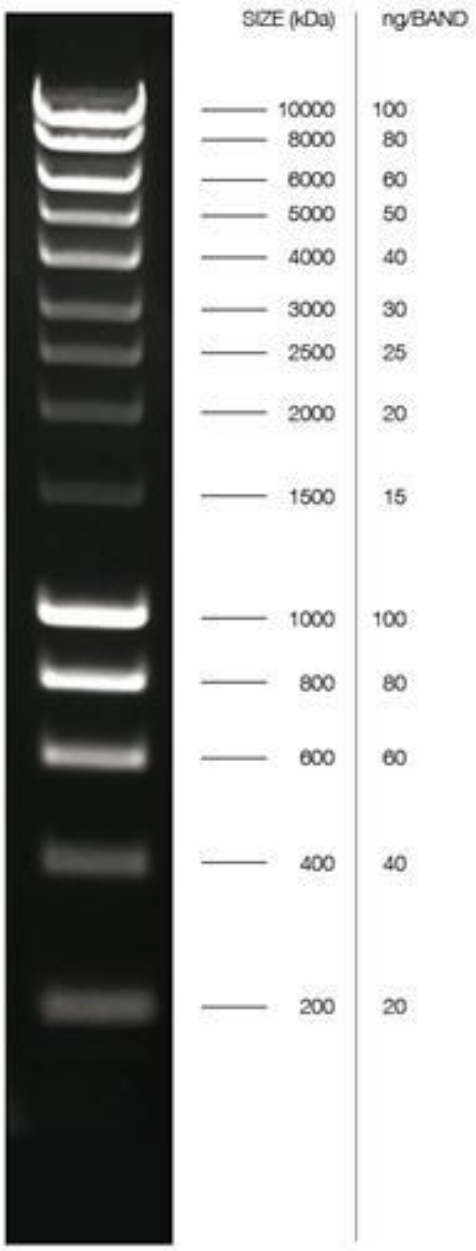


Figure C2 Hyperladder I Molecular Weight Marker. Manufactured by Bioline (Bioline, London, UK).
(http://www.bioline.com/ProductImageBank/MolecularWeightMarkers/HyperLadderI_400.jpg)

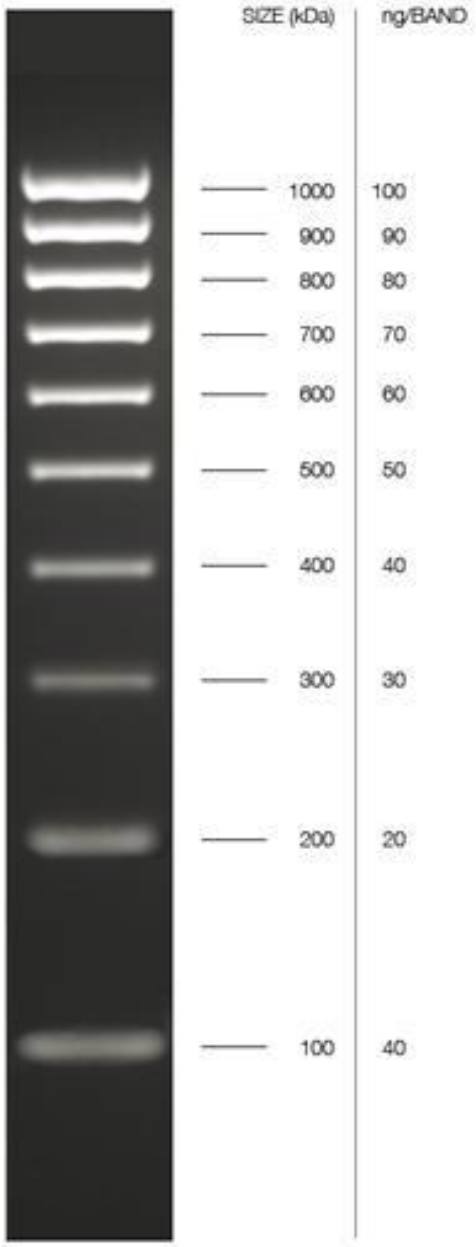


Figure C3 Hyperladder IV Molecular Weight Marker. Manufactured by Bioline (Bioline, London, UK). (http://www.bioline.com/ProductImageBank/MolecularWeightMarkers/HyperLadderIV_400.jpg)

APPENDIX D

Macrorestriction Profile of NCTC8325

Figure D1 *Sma*I macrorestriction profile for *S. aureus* pulsed-field gel electrophoresis control strain NCTC8325. Molecular weights are only indicated for fragments used during gel normalisation. Adapted from Reed *et al*. (2007)

Table E1 Primer sequences

Method	Primer name	Primer sequence (5' – 3')	Relevant control strain	Reference
Multiplex PCR assay for the detection of SCC*mec* types I – VI [3.2.3.1]	CIF2 F2	TTCGAGTTGCTGATGAAGAAGG	COL	Milheiriço *et al.* (2007a)
	CIF2 R2	ATTTACCACAAGGACTACCAGC		
	ccrC F2	GTACTCGTTACAATGTTTGG	WIS	
	ccrC R2	ATAATGGCTTCATGCTTACC		
	RIF5 F10	TTCTTAAGTACACGCTGAATCG	ANS46	
	RIF5 R13	GTCACAGTAATTCCATCAATGC		
	SCCmec V J1 F	TTCTCCATTCTTGTTCATCC	WIS	
	SCCmec V J1 R	AGAGACTACTGACTTAAGTGG		
	dcs F2	CATCCTATGATAGCTTGGTC	COL, BK2464, MW2, and HDE288	
	dcs R1	CTAAATCATAGCCATGACCG		
	ccrB2 F2	AGTTTCTCAGAATTCGAACG	BK2464 and MW2	
	ccrB2 R2	CCGATATAGAAWGGGTTAGC		
	kdp F1	AATCATCTGCCATTGGTGATGC	BK2464	
	kdp R1	CGAATGAAGTGAAAGAAAGTGG		
	SCCmec III J1 F	CATTTGTGAAACACAGTACG	ANS46	
	SCCmec III J1 R	GTTATTGAGACTCCTAAAGC		

Multiplex PCR assay for the detection of SCC*mec* types I – VI [3.2.3.1]	mecI P2	ATCAAGACTTGCATTCAGGC	BK2464 and ANS46	Milheiriço *et al.* (2007a)
	mecI P3	GCGGTTTCAATTCACTTGTC		
	mecA P4	TCCAGATTACAACTTCACCAGG	All MRSA (internal positive control)	
	mecA P7	CCACTTCATATCTTGTAACG		
PCR assays for the detection of *ccr* complexes 1 and 5 [3.2.3.3]	α1	AACCTATATCATCAATCAGTACGT	COL	Kondo *et al.*, (2007)
	βc	ATTGCCTTGATAATAGCCITCT		
	γR	CCTTTATAGACTGGATTATTCAAAATAT	WIS	
	γF	CGTCTATTACAAGATGTTAAGGATAAT		
PCR assays for the detection of class A, B, and C2 mec complexes [3.2.3.3]	mI6	CATAACTTCCCATTCTGCAGATG	ANS46 (A)	Kondo *et al.*, (2007)
	IS7	ATGCTTAATGATAGCATCCGAATG	COL (B)	
	IS2(iS-2)	TGAGGTTATTCAGATATTTCGATGT	WIS (C2)	
	mA7	ATATACCAAACCCGACAACTACA	A/B/C2	

Multiplex PCR assay for the detection of SCC*mec* type IV subtypes IVa – Ivh [3.3.4]	*ccrB*2 F	CGAACGTAATAACATTGTCG	All SCC*mec* type IV isolates (internal positive control)	Milheiriço *et al.* (2007b)
	*ccrB*2 R	TTGGCWATTTTACGATAGCC		
	J IVa F	ATAAGAGATCGAACAGAAGC	JCSC4744	
	J IVa R	TGAAGAAATCATGCCTATCG		
	J IVb F	TTGCTCATTTCAGTCTTACC	JCSC2172	
	J IVb R	TTACTTCAGCTGCATTAAGC		
	J IVc F	CCATTGCAAATTTCTCTTCC	DEN2949; AR43/3330.1	
	J IVc R	ATAGATTCTACTGCAAGTCC		
	J IVd F	TCTCGACTGTTTGCAATAGG	BK2529	
	J IVd R	CAATCATCTAGTTGGATACG		
	J IVg F	TGATAGTCAAAGTATGGTGG	M03-68	
	J IVg R	GAATAATGCAAAGTGGAACG		
	J IVh F	TTCCTCGTTTTTTCTGAACG	HAR22	
	J IVh R	CAAACACTGATATTGTGTCG		

PCR assay for the detection of *pvl* [4.2.2]	*luk-PV-1*	ATCATTAGGTAAAATGTCTGGACATGATCCA	ATCC49775	Lina *et al.* (1999)
	luk-PV-2	GCATCAASTGTATTGGATAGCAAAAGC		
Multilocus sequence typing [5.2.3]	*arcC*-Up	TTGATTCACCAGCGCGTATTGTC	ATCC43300	Enright *et al.* (2000)
	arcC-Dn	TTGATTCACCAGCGCGTATTGTC		
	aroE-Up	ATCGGAAATCCTATTTCACATTC		
	aroE-Dn	GGTGTTGTATTAATAACGATATC		
	glpF-Up	CTAGGAACTGCAATCTTAATCC		
	glpF-Dn	TGGTAAAATCGCATGTCCAATTC		
	gmk-Up	ATCGTTTTATCGGGACCATC		
	gmk-Dn	TCATTAACTACAACGTAATCGTA		
	pta-Up	GTTAAAATCGTATTACCTGAAGG		
	pta-Dn	GACCCTTTTGTTGAAAAGCTTAA		
	tpi-Up	TCGTTCATTCTGAACGTCGTGAA		
	tpi-Dn	TTTGCACCTTCTAACAATTGTAC		
	yqiL-Up	CAGCATACAGGACACCTATTGGC		
	yqiL-Dn	CGTTGAGGAATCGATACTGGAAC		

spa typing [5.2.2]	spa-1113f	TAAAGACGATCCTTCGGTGAGC	COL	Harmsen *et al.* (2003); Ridom GmbH protocol (version 2.1.1)
	spa-1514r	CAGCAGTAGTGCCGTTTGCTT		
dru typing [5.2.4]	*dru-1*	GTTAGCATATTACCTCTCCTTGC	COL	Goering *et al.* (2008a)
	dru-2	GCCGATTGTGCTTGATGAG		
rpoB genotyping [6.2.2]	F3	AGTCTATCACACCTCAACAA	COL	Aubry-Damon *et al.* (1998)
	F4	TAATAGCCGCACCAGAATCA		

APPENDIX F

BLAST Results

```
>dbj|AB121219.1| Staphylococcus aureus DNA, type-V staphylococcal cassette chromosome
mec: strain  JCSC3624(WIS)
Length=28612

 Score =  747 bits (404),  Expect = 0.0
 Identities = 434/449 (96%), Gaps = 0/449 (0%)
 Strand=Plus/Plus

Query  1      GTACTCGTTACAATGTTTGGGTTAATAGGATCAATCGAACGTTCAACACTGATCAGTAAT  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16495  GTACTCGTTACAATGTTTGGGTTAATAGGATCAATCGAACGTTCAACACTGATCAGTAAT  16554

Query  61     GTCAAGATGTCGATGAATGCTAAGGCACGGAGCGGAGAGGCAATCACCGGTCGTGTTTTA  120
              |||||||||||||||||||||||||| || || ||||||||||||||||||||||||||||
Sbjct  16555  GTCAAGATGTCGATGAATGCTAAGGCGCGTAGTGGAGAGGCAATCACCGGTCGTGTTTTA  16614

Query  121    GGCTACAAATTATCACTTAATCCATTGACACAGAAAAATGATTTAGTTATTGATGAAAAT  180
              |||||||||||||||||||||||||||||||||||||||||||||| ||||| |||||||||
Sbjct  16615  GGCTACAAATTATCACTTAATCCATTGACACAGAAAAATGATTTGGTTATCGATGAAAAT  16674

Query  181    GAAGCTCATATTGTACGGGAAATCTTTGATTTATATTTGAATCACAATAAAGGACTTAAA  240
              |||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16675  GAAGCTAATATTGTACGGGAAATCTTTGATTTATATTTGAATCACAATAAAGGACTTAAA  16734

Query  241    GCAATCACGACAATTCTAAATCAAAAAGGATATCGCACCATTAATCAAAAACCATTTTCA  300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16735  GCAATCACGACAATTCTAAATCAAAAAGGATATCGCACCATTAATCAAAAACCATTTTCA  16794

Query  301    GTGTTCGGCGTGAAATATATTTTGAATAATCCAGTCTATAAAGGTTTTGTTAGATTTAAT  360
              ||||| |||||||||||||||||||||||||||||||||||| | ||| ||||| |||
Sbjct  16795  GTGTTTGGCGTGAAATATATTTTGAATAATCCAGTCTATAAAGGGTATGTCAGATTCAAT  16854

Query  361    AACCATCAAAACTGGGCAGTTCAGCGAAGAGGTGGTAAAAGTGATGAAAATGATGTGATA  420
              |||||||||| ||||| |||||||||||| ||||||||||||||| ||||||||||||||
Sbjct  16855  AACCATCAAAATTGGGCTGTTCAGCGAAGAAGTGGTAAAAGTGATAAAAATGATGTGATA  16914

Query  421    TTGGTCAAAGGTAAGCATGAAGCCATTAT  449
              |||||||||||||||||||||||||||||
Sbjct  16915  TTGGTCAAAGGTAAGCATGAAGCCATTAT  16943
```

Figure F1 BLAST result for the 449 bp product amplified from C17 with primer pair ccrC F2/ccrC R2. Print-screen of the portion of the BLAST results indicating that the 449 bp product amplified from isolate C17 was 96% identical to the *ccrC* gene present in SCC*mec* type V control strain WIS.

>gb|AY894415.1| Staphylococcus aureus mec complex C2 region of SCCmecVa, partial sequence; and hypothetical proteins genes, complete cds
Length=7907

Score = 1266 bits (685), Expect = 0.0
Identities = 715/728 (99%), Gaps = 7/728 (0%)
Strand=Plus/Plus

```
Query  1     AATTTTTATCTTTTTCATCAATATCCTCCTTATATAAAGACTACATTTGTAATATATTAC  60
             |||||||||||||||||||||||||||||||||| |||||||||||||||||||| |||
Sbjct  5088  AATTTTTATCTTTTTCATCAATATCCTCCTTATAT-AAGACTACATTTGTAATATACTAC  5146

Query  61    AAATGTAGTATTTATGTCAAAAATAATGTTATAATTTTTGTGATATGGAGGTGTAGAAGG  120
             |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  5147  AAATGTAGTATTTATGTC-AAAATAATGTTATAATTTTTGTGATATGGAGGTGTAGAAGG  5205

Query  121   TGTTATCATCTTTTTTAATGTTAAGTATAATCAGTTCATTGCTCACGATATGTGTAAttt  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5206  TGTTATCATCTTTTTTAATGTTAAGTATAATCAGTTCATTGCTCACGATATGTGTAATTT  5265

Query  181   ttttAGTGAGAATGCTCTATATAAAATATACGGTTCTGTTGCAAAGTTGAATTTATAGTA  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5266  TTTTAGTGAGAATGCTCTATATAAAATATACGGTTCTGTTGCAAAGTTGAATTTATAGTA  5325

Query  241   TAATTATAACCAAAAGGAGTCTTCTGTATGAACTATTTCAGATATAAACAATTTAACAAG  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5326  TAATTATAACCAAAAGGAGTCTTCTGTATGAACTATTTCAGATATAAACAATTTAACAAG  5385

Query  301   GATGTTATCACTGTAGCCGTTGGCTACTATCTAAGATATGCATTGAGTTATCGTGATATG  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5386  GATGTTATCACTGTAGCCGTTGGCTACTATCTAAGATATGCATTGAGTTATCGTGATATG  5445

Query  361   TCTGAAATATTAAGGGAACGTGGTGTAAACGTTCATCATTTAACGGTCTAGCGTTGAGTT  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5446  TCTGAAATATTAAGGGAACGTGGTGTAAACGTTCATCATTTAACGGTCTAGCGTTGAGTT  5505
```

Figure F2 BLAST result for the PCR product amplified from isolate S2 with the primer pair mA7/IS2 (iS-2) specific for the class C2 *mec* complex. Print-screen of a portion of the BLAST results indicating that the PCR product amplified from isolate S2 with was 99% identical to the class C2 *mec* complex present in the SCC*mec* type V element.

APPENDIX G

Provincial Map of South Africa

Figure G1 Provincial Map of the Republic of South Africa indicating the location of the city of Cape Town. Adapted from http://www.iss.co.za/pubs/monographs/no73/map.html

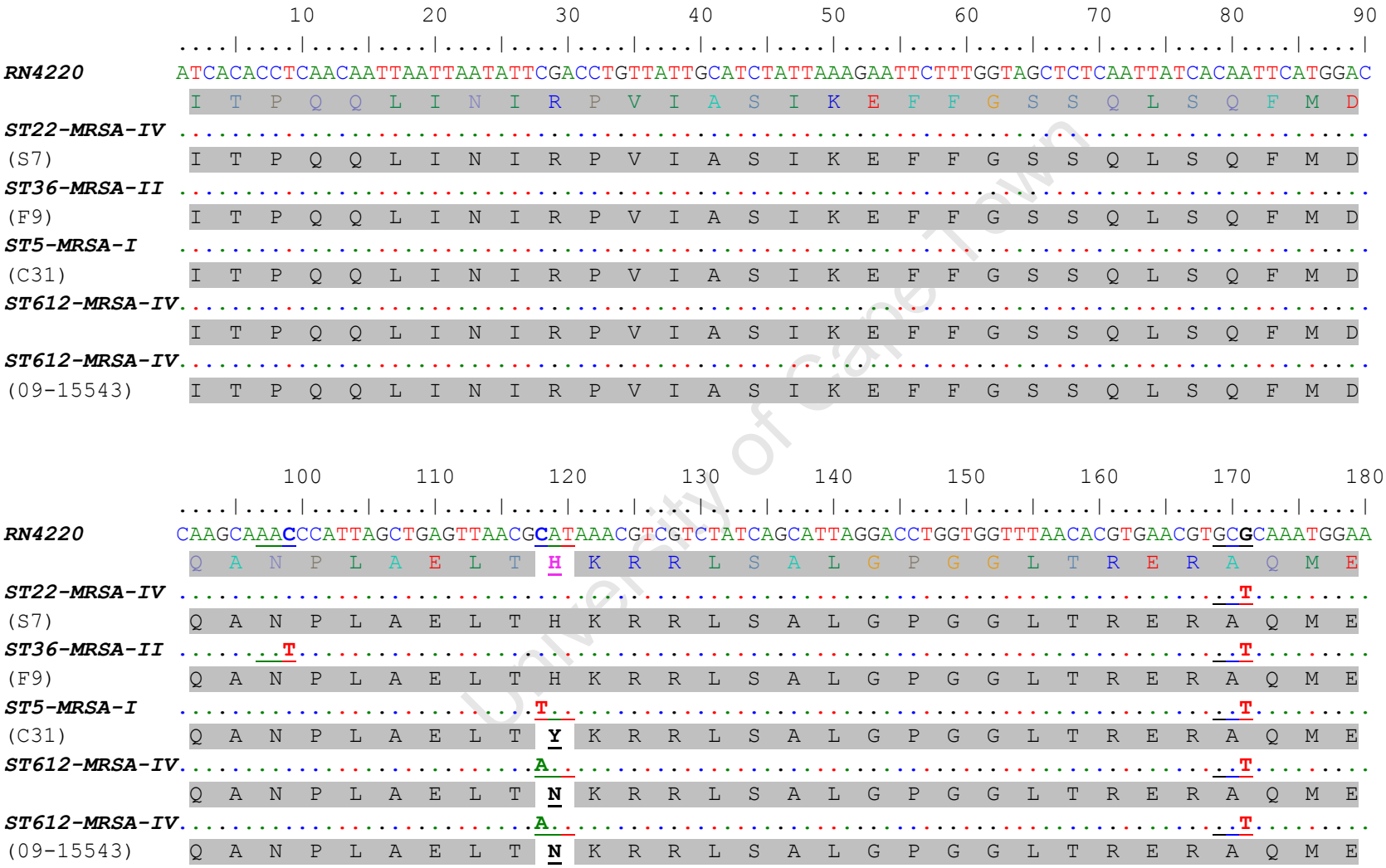
APPENDIX H

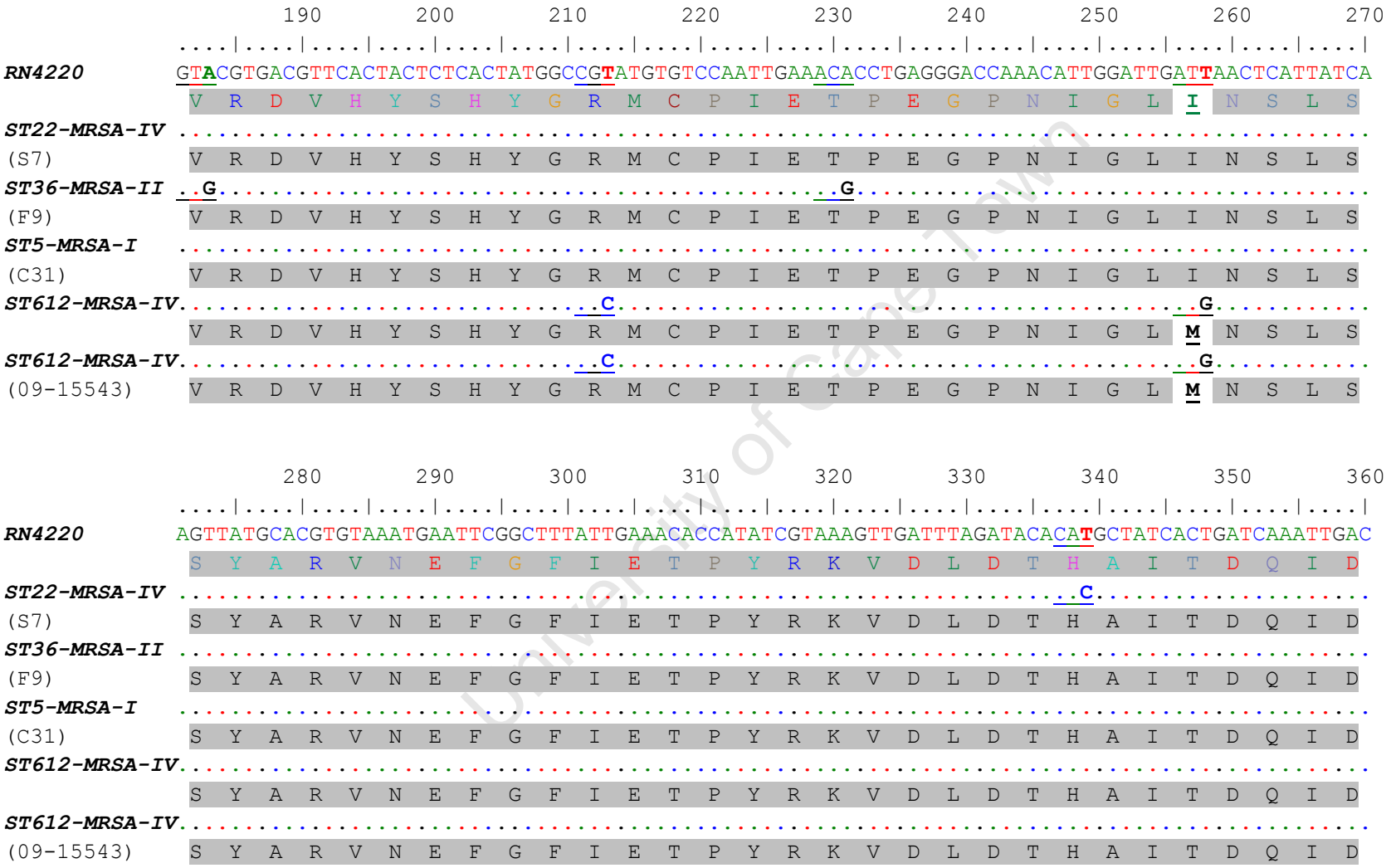
Alignments

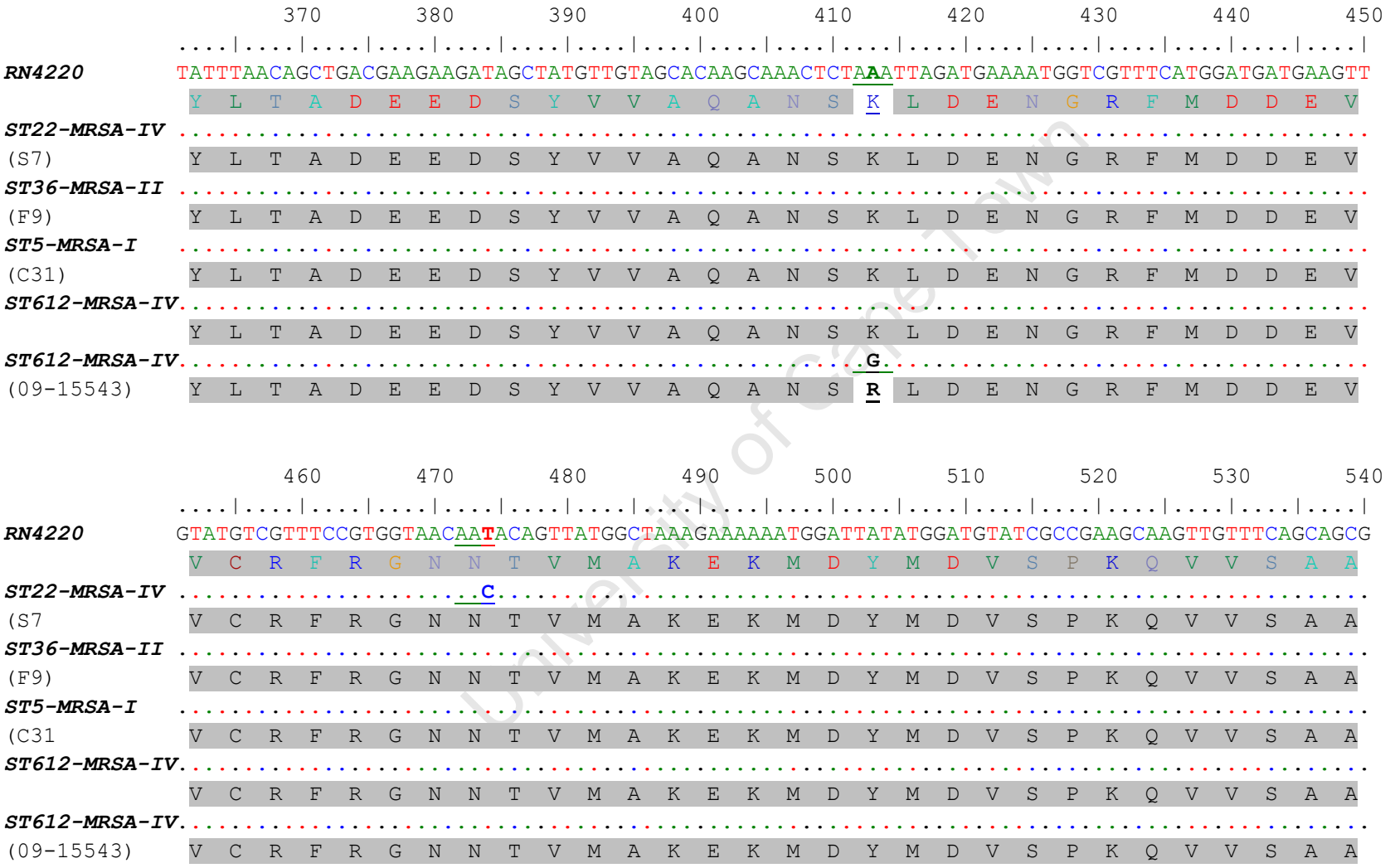
r26 GAGGAAGACAACAAAAA**A**CCTGGT

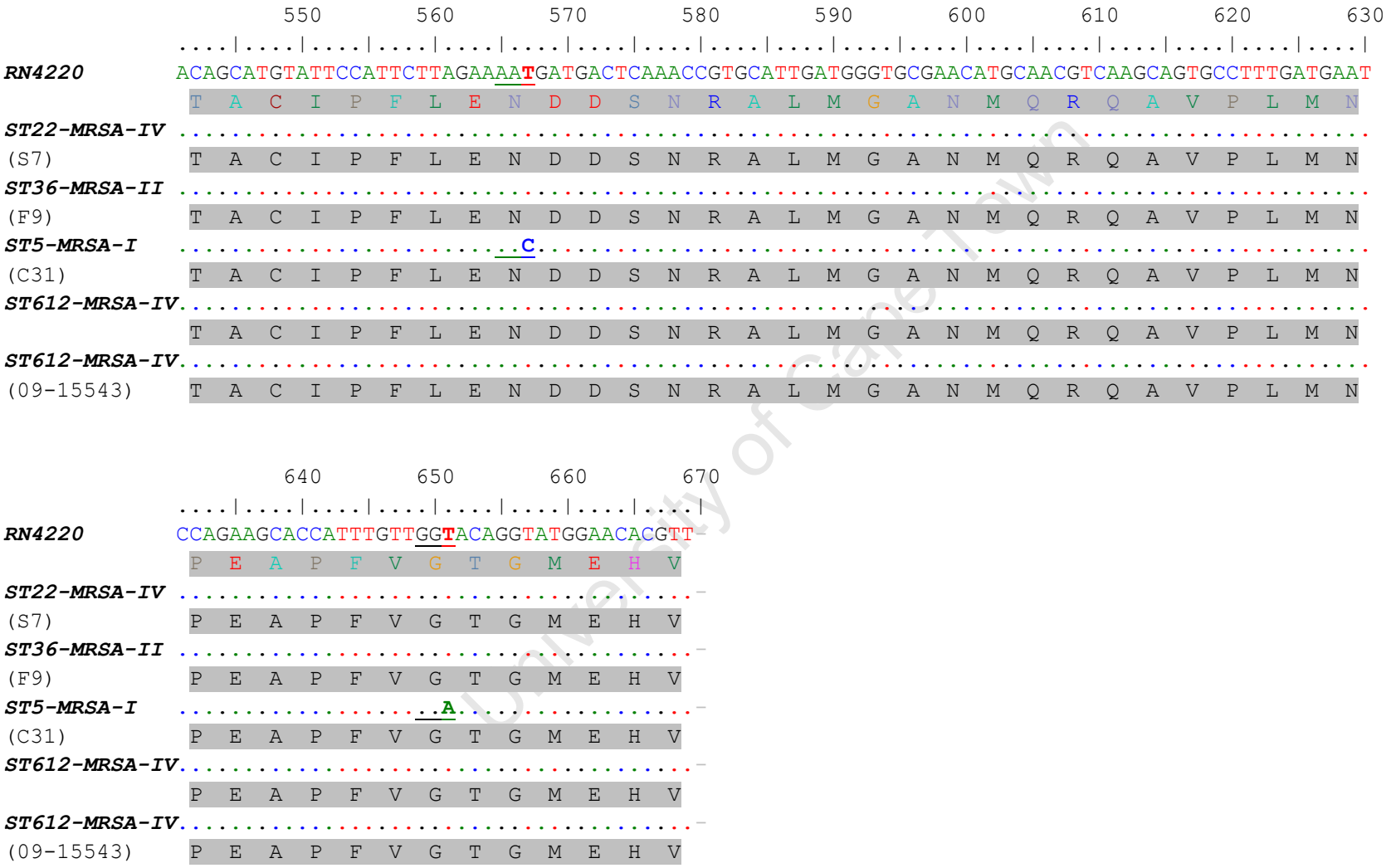
r135 GAGGAAGACAACAAAAA**G**CCTGGT

Figure H1 Ridom StaphType alignment of *spa* repeats r26 and r135 indicating a single nucleotide difference









Multiple alignment of nucleotide and predicted amino acid sequences of *rpoB* amplified from rifampicin-susceptible and -resistant methicillin-resistant *Staphylococcus aureus* isolates. The nucleotide and predicted amino acid sequences of isolates S7 (ST22-MRSA-IV), F9 (ST36-MRSA-II), C31 (ST5-MRSA-I) and 09-15543 (Australian ST612-MRSA-IV), as well as the sequence representative of all other ST612-MRSA-IV isolates selected for *rpoB* genotyping, were aligned to rifampicin-susceptible control strain RN4220 (accession number X64172) using the Clustal W algorithm in BioEdit Sequence Alignment Editor (version 7.0.5.2) (Hall, 1999). For each isolate, the nucleotide sequence is shown above the predicted amino acid sequence. The amino acid sequence of each isolate is presented in full as single letter code. Grey shading indicates identical amino acid sequences and mutations are indicated by breaks in the shading. The nucleotide sequence of RN4220 only is shown in full; dots (.) indicate identical residues in the sequences of the study isolates with discrepant nucleotides shown in full. Mutational changes are also indicated at the nucleotide level. Altered codons are underlined with the relevant nucleotide residue shown in full.