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MOLECULAR CHARACTERISATION OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) FROM SOUTH AFRICA

WILHELM FREDERICK OOSTHUYSEN

Degree of Master of Science in Medicine by Research

A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine by research.

Johannesburg, 2007

DECLARATION

I, Wilhelm F. Oosthuysen, declare that this dissertation is my own work. It is being submitted for the degree Master of Science in Medicine (by research) at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other university.

_____

Wilhelm F. Oosthuysen

B.Sc., B.Sc. (Hons)

9th day of November 2007

DEDICATION

To my family and my only sister.

For your never-ending love and support.

I love you.

PUBLICATIONS, PRESENTATIONS AND AWARDS

PUBLICATIONS

The following manuscript will be submitted for publication and arose from work done for this dissertation.

Oosthuysen W.F., Marais E., Aithma N., Dusé A.G. and the South African MRSA Surveillance Group. *Molecular characterisation of methicillin-resistant Staphylococcus aureus from South Africa*. To be submitted to the Journal of Clinical Microbiology (JCM).

PRESENTATIONS

The following presentations arose from work done for this dissertation.

POSTER PRESENTATIONS

Oosthuysen W.F., Marais E., Dusé A.G. and the South African MRSA Surveillance Group. **"Molecular characterisation of methicillin-resistant *S. aureus* from South Africa"** presented at the joint 17th European Congress on Clinical Microbiology and Infectious Diseases (ECCMID) and the 25th International Congress on Chemotherapy (ICC), 31st March - 3rd April 2007, Munich, Germany (Poster #1296).

ORAL PRESENTATIONS

Oosthuysen W.F. **"Molecular characterisation of methicillin-resistant *Staphylococcus aureus* in South Africa"** presented at the Molecular and Cell Biology Group (MCBG) Symposium, 05 October 2006, Johannesburg Hospital Auditorium, Johannesburg, South Africa.

Oosthuysen W.F. **"Molecular characterisation of methicillin-resistant *Staphylococcus aureus* in South Africa"** presented at the Faculty of Health Sciences Research Day of the University of the Witwatersrand, Johannesburg, 23 August 2006, WITS Medical School, Johannesburg, South Africa.

AWARD

The International Society of Chemotherapy (ISC) Tom Bergan Memorial Award was conferred upon Wilhelm Oosthuysen at the 25th International Congress of Chemotherapy, 3rd April 2007, Munich, for the above-mentioned poster.

ABSTRACT

Few antibiotics are left that are effective against methicillin-resistant *Staphylococcus aureus* (MRSA) and even strains resistant to these agents have been isolated. Previous studies have identified five distinct MRSA clonotypes, which are present globally. No comprehensive national study has previously been undertaken to investigate the MRSA types in South Africa, and this study was aimed at elucidating the genotypic population structure of South African MRSA isolates. *Sma*I digested genomic DNA, separated by pulsed-field gel electrophoresis, was used to characterise 349 *S. aureus* isolates, obtained from various state and private diagnostic laboratories. PFGE results were complemented with those of *spa* typing and staphylococcal cassette chromosome *mec* (SCC*mec*) typing results. Two-hundred-and-five different PFGE patterns were identified, which were grouped into twenty-four clusters. Three were major lineages, containing more than 20% of the isolates with a similarity cut-off of 70%. Only thirty-seven *spa* types were identified (fourteen novel *spa* types), which clustered into six *spa*-Clonal Complexes after BURP analysis. SCC*mec* types I-IV were identified, including variants of each type. Data suggest that the Archaic clone (RSA05), oldest of the epidemic clones, represents one of the major clones in South Africa. Strains that were part of this complex (n=98 (28.2%); t064; SCC*mec* type I-*pls*) clustered together with strain E2125/ATCC BAA-38 (t051; SCC*mec* type I). Another major complex, RSA16 (n=90 (25.7%); t012; SCC*mec* type II/IIB) possessed a single-locus variant (SLV) *spa* type and the same or a SLV SCC*mec* types as EMRSA-16 (t018; SCC*mec* type II). The third major complex, RSA03 (n=74 (21.2%); t037; SCC*mec* type III/IIIE), had similar *spa* and SCC*mec* types to control strain

ANS46 (t037; SCC*mec* type III). One MRSA and twelve MSSA isolates were also identified as carrying genes for the toxin Panton-Valentine leukocidin, which was confirmed by DNA nucleotide sequencing.

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I want to thank the **organising committee** of the **joint 17th ECCMID/25th ICC (31st March-3rd April 2007)** for accepting my **abstract** and giving me the opportunity to present my work. I would also like to thank them for conferring **The ISC Tom Bergan Memorial Award** upon me for my poster.

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LIST OF ABBREVIATIONS AND ACRONYMS

6-APA	6-aminopenicillanic acid
µg	Microgram
µl	Microlitre
µM	Micromolar
A	Adenine
bd	Twice daily
BHI	Brain heart infusion
bp	Base pairs
C	Cytosine
CA-MRSA	Community-associated methicillin-resistant *Staphylococcus aureus*
CC	Clonal complex
ccr	Cassette chromosome recombinase
CHEF	Contour-clamped homogenous electric field
CLSI	Clinical Laboratory Standards Institute
CMID	Clinical Microbiology and Infectious Diseases
CSF	Cerebrospinal fluid
CT	Clonal type
d.H_2O	Distilled water
dcs	Downstream coding sequence
DLV	Double locus variant
DMP	Diagnostic Media Products

DNA	Deoxyribonucleic acid
EC	Eastern Cape province
EDTA	Ethylenediaminetetraacetic acid
EMRSA	Epidemic methicillin-resistant *Staphylococcus aureus*
ESBL	Extended-spectrum beta-lactamase
ET	Epidermolytic toxins
FIGE	Field-inversion gel electrophoresis
FS	Free State province
G	Guanine
g	Grams
GP	Gauteng province
HA-MRSA	Hospital-associated methicillin-resistant *Staphylococcus aureus*
HCl	Hydrochloric acid
HOD	Head of Department
hVISA	Hetero-vancomycin intermediately-resistant *Staphylococcus aureus*
HVR	Hyper-variable region
ICU	Intensive care units
IS	Insertion sequence
kb	Kilobases
KZN	KwaZulu-Natal province
l	Litre
LP	Limpopo province
M	Molar
Mb	Megabases

mg	Milligram
MIC	Minimum Inhibitory Concentration
min	Minute
ml	Millilitre
MLST	Multi-locus sequence typing
MM	Murein monomer
mM	Millimolar
MMP	Murein monomer precursor
MP	Mpumalanga province
MRA	Macro-restriction analysis
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-susceptible *Staphylococcus aureus*
NaAOc	Sodium acetate
NaCl	Sodium chloride
NAG	*N*-acetylglucosamine
NAM	*N*-acetylmuramic acid
NaOH	Sodium hydroxide
NASF	National Antibiotic Surveillance Forum
NC	Northern Cape province
ng	Nanogram
NHLS	National Health Laboratory Service
nM	Nanomolar
NWP	North West province
od	Once daily

ORF	Open reading frame
PABA	Para-aminobenzoic acid
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PCR-RFLP	Polymerase chain reaction-restriction fragment length polymorphism
PEARLS	Pan-European Antimicrobial Resistance Using Local Surveillance
PFGE	Pulsed-field gel electrophoresis
PFT	Pulsed-field type
PG	Peptidoglycan
pls	Plasmin-sensitive surface protein gene
PMN	Polymorphonuclear leucocyte
PVL	Panton-Valentine leukocidin
RAPD	Random amplified polymorphic DNA
REA	Restriction enzyme analysis
RFLP	Restriction fragment length polymorphisms
SCC*mec*	Staphylococcal cassette chromosome *mec*
SDS	Sodium dodecyl sulphate
SE	Staphylococcal enterotoxins
sec	Second
SLV	Single locus variant
SSC	Saline sodium citrate
SSR	Short sequence repeat
ST	Sequence type
T	Thymine

TE	Tris-EDTA buffer
TG	Transglycosylase
Tn	Transposon
Tris	Alpha,alpha,alpha-Tris-(hydroxymethyl)-methylamin
TSST	Toxic shock syndrome toxin
UK	United Kingdom
UPGMA	Unweighted-pair group matching analysis
USA	United States of America
VISA	Vancomycin intermediately-resistant *Staphylococcus aureus*
vol	Volume
VRE	Vancomycin-resistant enterococci
WC	Western Cape province
WITS	University of the Witwatersrand, Johannesburg
wt	Weight

CHAPTER 1

1 LITERATURE REVIEW

1.1 Antimicrobial Chemotherapy

Due to the ability of many micro-organisms to infect man and cause disease, it is essential that we utilise ways to control or prevent their growth. Antibiotics are secondary metabolites produced by some bacteria or moulds that are able to inhibit the growth of, or kill, other susceptible bacteria. They can act in numerous ways such as inhibiting bacterial cell wall synthesis, protein synthesis, nucleic acid synthesis or by disrupting certain metabolic pathways. A few antibiotics, like polymyxin B, are able to interfere with cell membrane integrity, leading to cell leakage and death. However, due to the misuse of antibiotics, many susceptible bacteria have successfully adapted to the antibiotics. This misuse also places many bacteria under selective pressure to develop different mechanisms of resistance against certain classes of antibiotics.

1.1.1 Mode of action of antibiotics

1.1.1.1 Inhibition of cell wall synthesis

The cell wall determines the shape of bacteria, whereas mycoplasmas and certain Archaebacteria have cell membranes and not cell walls. The cell wall is usually a rigid

structure, maintaining cell rigidity and preventing osmotic lysis. Cell wall structures of some bacteria are; (1) recognised by the human immune system; and (2) can contribute to bacterial pathogenesis. The cell wall also serves as a barrier, preventing the entry and exit of most molecules. Many key enzymes involved in cell wall synthesis are inhibited by antibiotics.

Inhibitors of the cell wall usually have a very high therapeutic index. This is due to the presence of a unique structure in the bacterial cell wall not found in eukaryotic cells, called peptidoglycan (PG) (Figure 1.1), which also aids in the maintenance of bacterial cell wall rigidity. PG is composed of an alternating series of two major subunits, *N*-acetylmuramic acid (NAM) and *N*-acetylglucosamine (NAG). NAM and NAG are joined covalently, forming a glycan chain. A string of four amino acids, a tetrapeptide chain, is attached to each NAM molecule. The formation of cross-linkages between tetrapeptide chains joins adjacent glycan chains together to form a single molecule. The tetrapeptide chains are joined directly in Gram-negative bacteria, while in Gram-positive bacteria, they are usually joined indirectly though a peptide interbridge.

The cell walls of Gram-positive bacteria (Figure 1.2) are usually characterised by a relatively thick layer of peptidoglycan that is fully permeable to many substances, such as sugars, amino acids and ions. Attached to the PG through covalent bonds to NAM are teichoic acids. They are composed of chains of a common subunit, either ribitol-phosphate

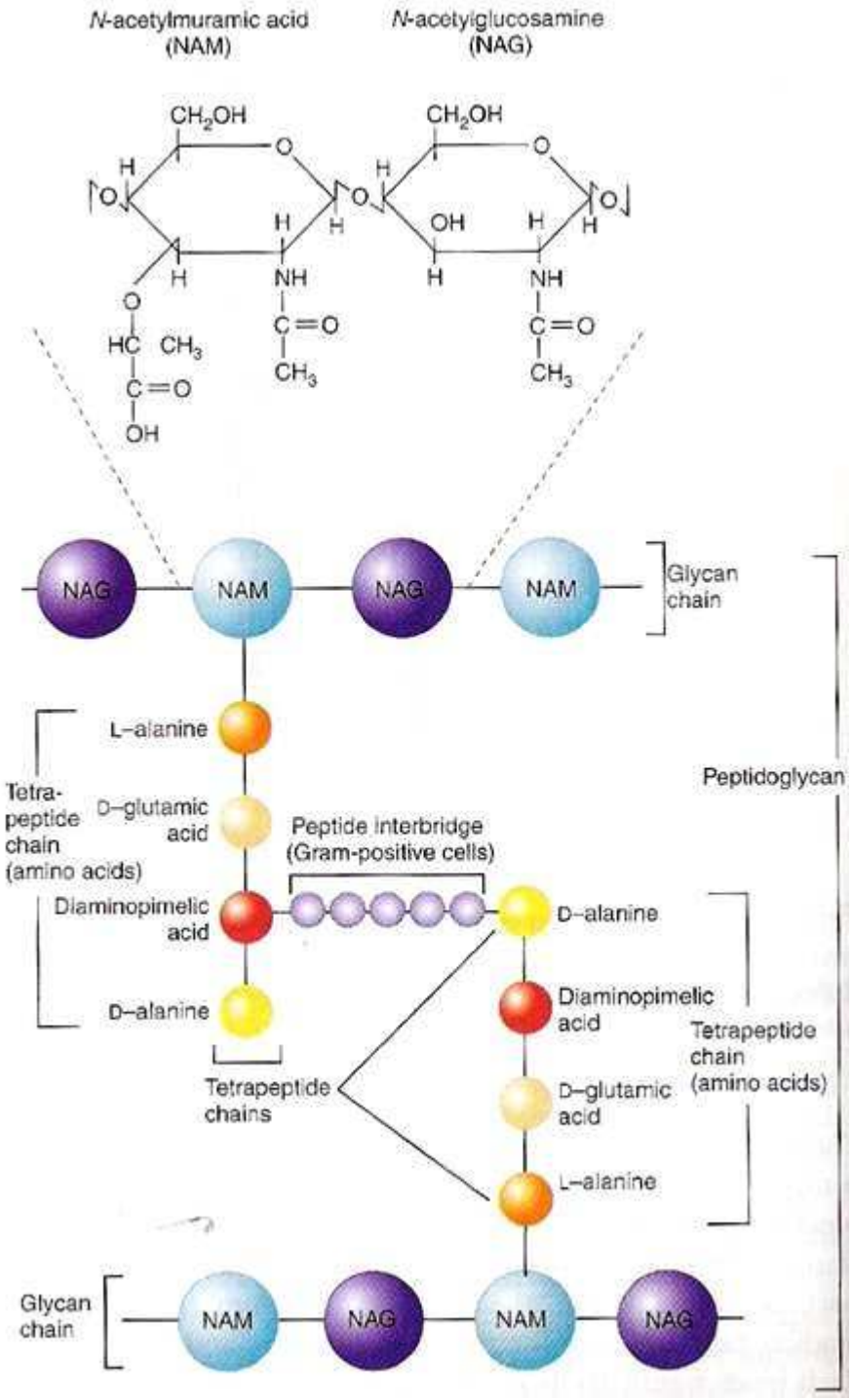


Figure 1.1: Components and structure of bacterial peptidoglycan[39]**.** The chemical structures of NAG and NAM have glucose as a ring structure.

or glycerol-phosphate, and various sugars or *D*-alanine are usually attached to them. Some of these are linked to the cytoplasmic membrane and are called lipoteichoic acids. Gram-negative bacteria have a far more complex cell wall (Figure 1.3). Only a thin layer of PG is present, covered on the outside by the outer membrane, a unique lipid bi-layer in which lipopolysaccharides proteins, such as porins, are embedded.

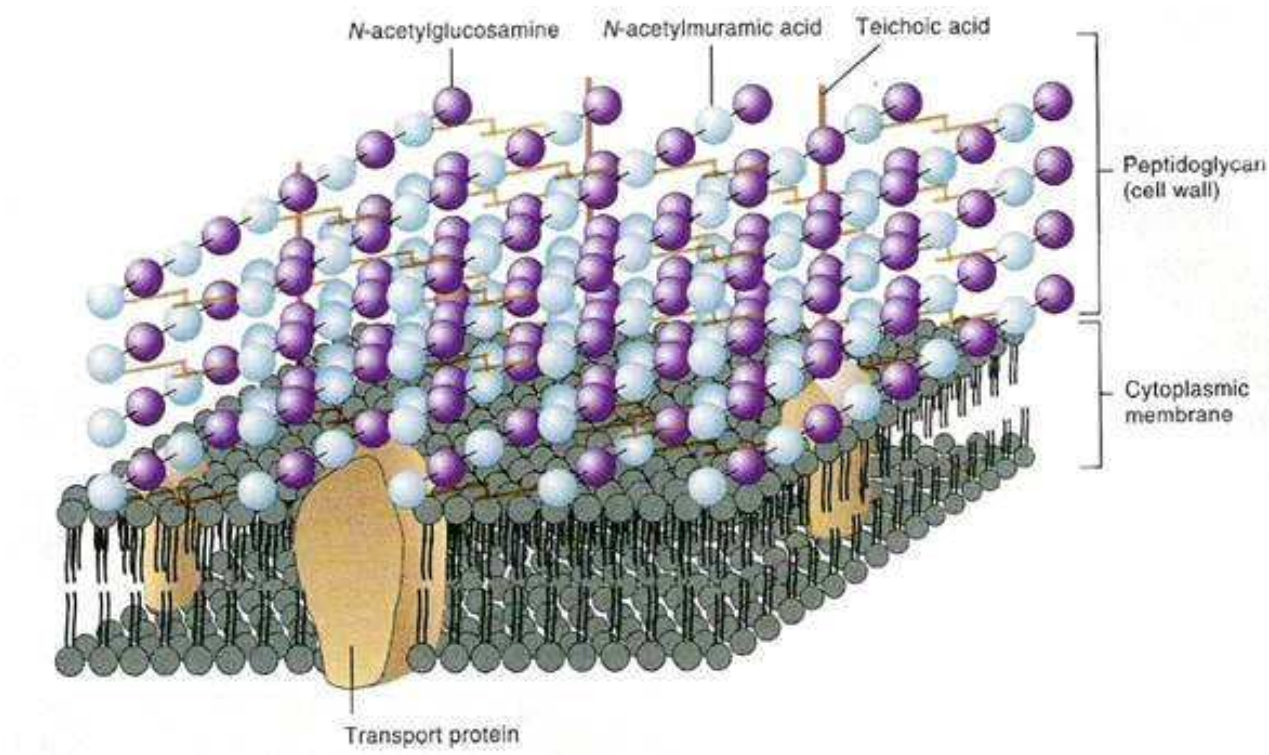


Figure 1.2: Graphic representation of a Gram-positive cell wall[39]. It is composed of many sheets of interconnected glycan chains, which are interconnected to form the thick peptidoglycan molecule.

Murein monomer precursor (MMP) is produced inside the cell, attached to the cytoplasmic membranes' lipid carrier undecaprenol, and modified by the sequential addition of five glycines and one amino-sugar molecule, NAM, and by amidation at the *D*-glutamic acid residue. The completed murein monomer (MM) is then transferred to the outer surface of

the cytoplasmic membrane to form a linear nascent PG chain. This is achieved through the polymerisation of 10-20 MMs between their sugar residues by the enzyme transglycosylase (TG). The PG is then converted into a meshwork by the transpeptidase, penicillin-binding protein (PBP), by cross-bridging nascent polypeptide chains. The two amino acid residues *D*-alanyl-*D*-alanine at the tip of MM are recognised by PBP, which cleaves between them. The penultimate *D*-alanine is ligated to the pentaglycine chain of the nearby PG chain (cross-bridge formation)[21].

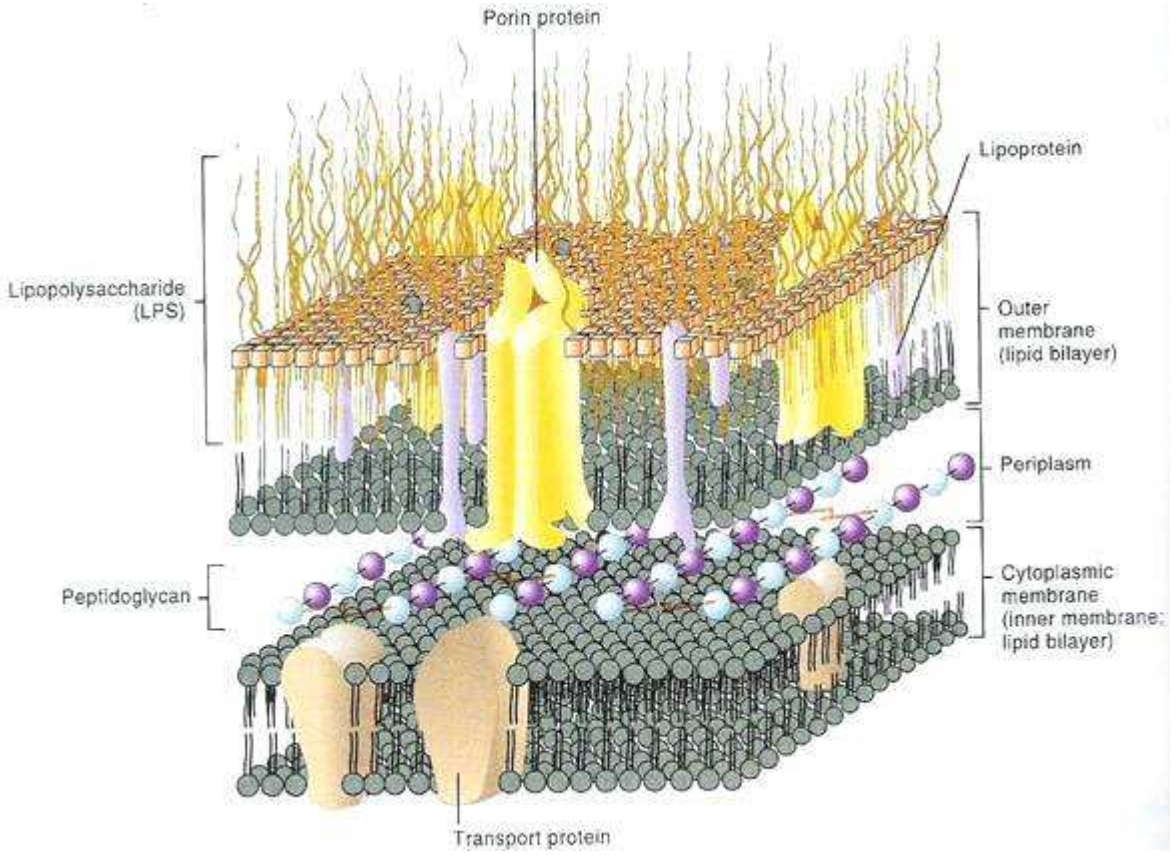


Figure 1.3: Schematic representation of the Gram-negative cell wall[39]. Only one or two sheets of interconnected glycan chains make up the peptidoglycan molecule. The PG layer is surrounded by the outer membrane, which is a typical phospholipid bilayer.

1.1.1.2 Inhibition of protein synthesis

The prokaryotic 70S ribosome, composed of a 30S and a 50S subunit, differs enough in structure from the eukaryotic 80S ribosome to allow numerous antibiotics to inhibit prokaryotic protein synthesis. The 30S ribosomal subunit is the target for the aminoglycosides and the tetracyclines. Aminoglycosides bind irreversibly to the subunit, causing it to distort and malfunction. The initiation of transcription is blocked and misreading of mRNA occurs. The tetracyclines bind reversibly to the subunit and block tRNA attachment to the ribosome, thus preventing protein synthesis progression. The macrolides, chloramphenicol, lincosamides, oxazolidinones and streptogramins all target the 50S ribosomal subunit. The continuation of protein synthesis is also inhibited by the macrolides, by reversibly binding to the subunit. Chloramphenicol acts through preventing the formation of peptide bonds, leading to protein synthesis blockage. The lincosamides inhibit the continuation of protein synthesis, while it is thought that the oxazolidinones inhibit protein synthesis initiation[39].

1.1.1.3 Disruption of metabolic pathways

The folate inhibitors, sulphonamide and trimethoprim inhibit different steps in the pathway that initially lead to folic acid synthesis and finally to the synthesis of a coenzyme required for nucleotide biosynthesis. Sulphonamide is structurally similar to a substrate in the folic acid biosynthesis pathway, para-aminobenzoic acid (PABA). This results in the competitive inhibition of the enzyme that normally binds PABA. The enzyme that

catalyses a metabolic step following the one inhibited by sulphonamides is inhibited by trimethoprim[39].

1.1.1.4 Inhibition of nucleic acid synthesis

The supercoiling of closed circular DNA within the bacterial cell is maintained by a group of enzymes called the topoisomerases. These enzymes are inhibited by ciprofloxacin and ofloxacin, which are fluoroquinolones. During replication and transcription, the strain caused by the localized unwinding of DNA is relieved through the action of the enzyme DNA gyrase, or topoisomerase II, by breaking the DNA strands and then rejoining them. Prokaryotic RNA is blocked from initiating transcription by the rifampicins[39].

1.1.1.5 Disruption of the plasma membrane

Polymyxin B, a common ingredient in first-aid skin ointments, damages the bacterial membranes. After binding to the membranes of Gram-negative bacteria, their permeability is altered, which leads to the leakage of cellular content and eventually cell death[39].

1.1.2 Mechanisms of antimicrobial resistance

Spontaneous mutations in existing genes or the acquisition of new genes can lead to antimicrobial resistance. Point mutations can lead to the alteration of the drug target, thus rendering the antimicrobial compound inactive. However, it is more difficult for an organism to develop resistance if the antimicrobial compound has more than one target. Several different mutations are required to render the drug ineffective. Resistance genes can be acquired e.g. through the transfer of a plasmid from one organism to the other or the acquisition of a transposon. Many of these plasmids and transposons confer resistance to more than one antibiotic. Resistance occurs either through the production of drug inactivating enzymes, alterations in the target molecule, decreased uptake of the drug or increased elimination of the drug[39].

1.1.2.1 Drug-inactivating enzymes

A drug can be chemically modified by a specific enzyme in such a way as to render it ineffective. Bacteria that synthesise the enzyme penicillinase are resistant to the antibiotic penicillin, while the enzyme chloramphenicol acetyltransferase chemically alters the antibiotic chloramphenicol[39].

1.1.2.2 Alteration in the target molecule

A specific target molecule inside the bacterium is usually recognised and bound by an antimicrobial drug, interfering with its function. A mutation can lead to minor structural changes in the target, which can prevent the drug from binding. For example, changes in the ribosomal RNA prevent macrolides from interfering with ribosomal functioning[39].

1.1.2.3 Decreased uptake of drug

Small hydrophobic molecules are selectively allowed to enter a cell through porin proteins in the outer membranes of Gram-negative bacteria. Certain drugs can be prevented from entering the cell through alterations in these proteins. The effect of the drug is avoided by preventing entry of the drug[39].

1.1.2.4 Increased elimination of the drug

Efflux pumps transport hazardous molecules out of a cell. The overall capacity of an organism to eliminate a drug can be altered through the increased expression of these pumps, thus allowing the organism to withstand higher concentrations of the drug. The array of drugs that can be actively pumped out can also be influenced by structural changes of the pumps, potentially enabling the organisms to become resistant to several different drugs[39].

1.1.3 Emergence of resistance

The fact that numerous different bacterial species, including those that are responsible for causing disease in humans, have the ability to resist the inhibitory action of antimicrobial substances has become a problem of global concern. Resistance not only continues to spread in the hospital setting but also in the community setting. The pathways by which resistance has come about play an important role in determining the appropriate control measures. However, these pathways differ between different organisms and settings[64]. During the past few decades, there has been a worldwide increase in the frequency and spectrum of infections caused by antibiotic resistant micro-organisms. A combination of microbial characteristics, the selective pressure of antimicrobial use and social and technical changes enhancing the transmission of resistant organisms can lead to an increase in resistance. The acquisition of resistance-encoding genetic material, transferred from other bacteria, or mutational changes lead to the acquisition of resistance. The excessive use of antibiotics in human health-care and animal feeds, increased use of invasive devices and procedures, an increased number of susceptible hosts and lapses in infection control practices have led to the development and spread of antibiotic resistant organisms[14].

Genes coding for resistance mechanisms, which are integrated by recombination into a number of classes of naturally occurring gene expression cassettes, are then disseminated in the microbial population through transduction, transformation and conjugation, i.e. horizontal gene transfer mechanisms. The widespread use of antimicrobial agents within the hospital and for immunocompromised patients have selected for multidrug-resistant organisms, such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE) and extended-spectrum beta-lactamase (ESBL) producing

Gram-negative bacilli. The application of strict prevention strategies should be enforced to prevent the emergence of multidrug-resistant bacteria and the spread of resistance genes. These strategies include alterations of antibiotic treatment regimes, infection prevention and control, and the control of horizontal transmission of organism, both in the hospital and community setting[14].

The problem of resistance has two main components: (1) the antibiotic, which inhibits susceptible organisms and selects for resistant organisms, (2) the specific genetic resistance determinant present in the organism, which is selected by the antibiotic. Only when these two components come together in a specific environment or host does drug resistance emerge. More than fifteen classes of antibiotics exist and their targets are involved in essential physiological or metabolic functions of bacteria. Not one of these classes has been able to escape a resistance mechanism. The use of millions of kilograms of antimicrobials yearly drives the problem of resistance through the elimination of susceptible strains and selection of the resistant ones[31].

1.2 β-Lactam Antibiotics

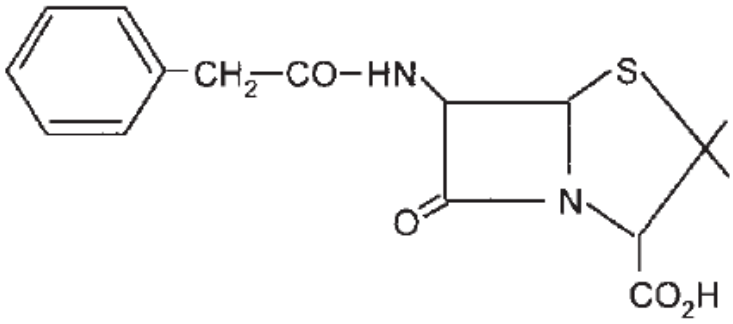
The most varied and widely used group of antimicrobials is the β-lactam antibiotics. Since the discovery of benzylpenicillin in 1928 by Alexander Flemming and its first clinical use in 1940, a vast number of both natural and synthetic compounds have been described based upon penicillins' active structure, the β-lactam ring. Fifty percent of all systemic antimicrobial use is accounted for by the β-Lactams. Their low toxicity and the availability of so many derivatives have contributed to their success[31]. The penicillins and cephalosporins are the most commonly used β-lactam drugs, which also includes the monobactam and the carbapenems. These drugs share the same chemical structure, namely the β-lactam ring. The β-lactam ring, bearing structural similarity to the normal substrates of PBPs, binds to the PBPs, which are consequently competitively inhibited[39].

1.2.1 Penicillin

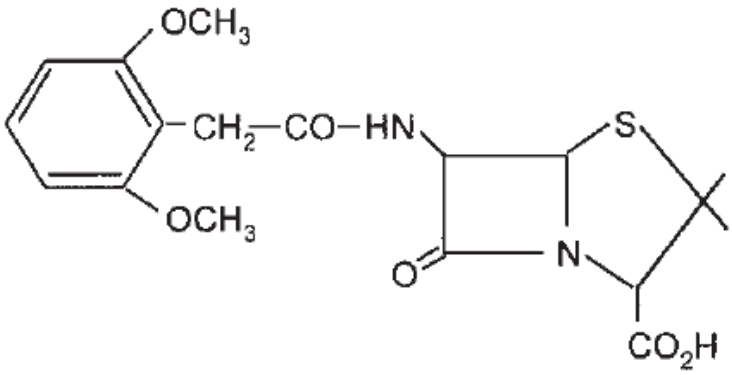
Each member that is a part of the penicillin family shares a common moiety, namely 6-aminopenicillanic acid (6-APA). By changing the side chains in the laboratory, derivatives with different characteristics can be created. Natural penicillins are the penicillins produced by the mould *Penicillium chrysogenum*. These are narrow spectrum antibiotics, effective against Gram-positive bacteria and some Gram-negative cocci. These include penicillin G (benzylpenicillin) (Figure 1.4) and penicillin V. The latter is more acid-stable and is better absorbed when taken orally[39].

1.2.2 Methicillin

Through the addition of certain side chains to the 6-APA nucleus, numerous penicillin derivatives resistant to staphylococcal β-lactamases have been produced. The first of these, dimethoxybenzylpenicillin, or methicillin (Figure 1.4), requires parenteral administration and is unstable in gastric acid. A number of acid-stable compounds were subsequently developed eg. cloxacillin, oxacillin and flucloxacillin, which remain in widespread use. Staphylococcal β-lactamases are prevented from attaching by the side chains of these drugs. However, these compounds are not useful against species other than staphylococci and have lower inherent activity than benzylpenicillin[34].



Benzylpenicillin



Methicillin

Figure 1.4: The chemical structures of benzylpenicillin and methicillin[59].

1.3 Staphylococci

Members of the genus *Staphylococcus* are Gram-positive cocci, occurring singly, in pairs, tetrads and short chains, but appear predominantly in grape-like clusters. Staphylococci are non-motile, non-spore forming and are usually catalase-positive. They are also typically unencapsulated, or capsule formation is limited. Aerobic conditions favour fast and profuse growth as most species are facultative anaerobes, except for *S. saccharolyticus* and *S. aureus* subsp. *anaerobius*, which are anaerobic and frequently catalase-negative[29]. The skin and mucous membranes of humans and animals are frequently inhabited by staphylococci. The coagulase-positive species *S. aureus* and two coagulase-negative species *S. epidermidis* and *S. saprophyticus* are among the staphylococci most frequently seen in human infections[71].

1.3.1 Staphylococcus aureus

Among the staphylococci, *S. aureus* is the most important human pathogen. It is found to be widespread in nature and colonises the anterior nares of 20-40% of adults. It can also colonise the intertriginous skin folds, the perineum, the axillae and the vagina. *S. aureus* is frequently part of the normal human microflora and can cause significant opportunistic infections apart from the usual infections associated with this bacterium, e.g. folliculitis, furuncles and carbuncles, impetigo, mastitis, meningitis, pericarditis and pulmonary infections[71].

The organism possesses several virulence factors that cause disease. These virulence factors are not found in all *S. aureus* strains. The organism can cause toxic epidermal necrolysis through the production of certain toxins called exfoliatins or epidermolytic toxins (ET-A and ET-B), and it can cause food poisoning by producing enterotoxins (Enterotoxins A through E, H and I). It also produces toxins belonging to a group collectively known as pyrogenic toxin superantigens, namely the staphylococcal enterotoxins (SE) and toxic shock syndrome toxin-1 (TSST-1). These toxins have the ability to stimulate T lymphocyte proliferation without regard for their antigenic specificities. Panton Valentine leukocidin (PVL) and γ-hemolysin, which create pores in the membranes of polymorphonuclear leucocytes (PMNs), are two other toxins that can also be produced[71].

1.4 Methicillin-Resistant *Staphylococcus aureus* (MRSA)

The introduction of methicillin, the first penicillinase-resistant semi-synthetic penicillin, into clinical practice in 1959 and 1960 solved the problem of penicillinase-producing *S. aureus* strains, which were very common in health care institutions at that time. Unfortunately, *S. aureus* strains resistant to methicillin were identified almost immediately in 1961[27]. These were initially regarded as laboratory phenomena of limited clinical significance.

Methicillin resistance was observed in coagulase-negative staphylococci before it was reported for *S. aureus*. Resistance to methicillin was heterogenous in the original strains isolated, but this could be increased several fold by a single passage in methicillin. Unlike the laboratory-resistant strains, which were avirulent and grew poorly, naturally resistant strains showed normal growth and virulence. The first major nosocomial epidemic caused by a methicillin-resistant *S. aureus* strain was described in 1963[8]. This strain, isolated from an infant treated with penicillin caused the death of one child and infected staff and children in different wards.

Five epidemic MRSA (EMRSA) clones have been identified. Reported in 1989 in Spain for the first time, the Iberian clone has also been identified in Portugal, Italy, the United Kingdom, Germany, Belgium, Switzerland and France. The Brazilian clone was first described in Brazil in 1992 and thereafter in Portugal, Argentina, Uruguay and Chile, while the Hungarian clone was identified in Hungary and Taiwan. The New York/Japan clone was described in New York, Pennsylvania and Japan. In 1992, the Paediatric clone was first reported in a paediatric hospital in Portugal, and has since then been found in the

USA, Poland and Argentina. The first MRSA strain isolated was a member of the now extinct Archaic clone. The Iberian clone is a direct descendant of the Archaic clone[43].

1.5 Methicillin Resistance

The gene that confers resistance to methicillin and other penicillinase-resistant β-lactam antibiotics, *mecA*, is carried on a genetic element called staphylococcal cassette chromosome *mec* (SCC*mec*). All the different SCC*mec* types and variants share the same chromosome integration site (*attBscc*) in the ORF X gene (*orfX*) and common structural features , such as conserved terminal inverted repeats, 15bp direct repeats at the integration junction points, conserved genetic organisation around the *mecA* gene, and the presence of cassette chromosome recombinase (*ccr*) genes[21,25]. Differences in the sizes of the types are mainly due to the presence of type-specific DNA regions.

Two essential gene complexes are present on SCC*mec*, the *mec* gene complex and the *ccr* gene complex. The *mec* gene complex is composed of the *mecA* gene and its regulators, *mecRI* and *mecI*. The *ccr* gene complex (*ccrA* and *ccrB* for SCC*mec* type I-IV, or *ccrC* for SCC*mec* type V) is responsible for movement of SCC*mec* through site- and orientation specific integration into and precise excision out of the *S. aureus* chromosome[21,29].

All the Ccr proteins are very basic (pI 10.07-10.49) and share motifs in their N-terminal domains for site-specific recombinases of the invertase/resolvase family. The deduced polypeptide sequences of CcrA and CcrB, of each SCC*mec* type, differ by approximately 30% from one another. However, the catalytic serine residue of the recombination active site is conserved among the Ccr proteins[25]. The sizes of CcrA and CcrB are similar, namely 448/449 amino acids for CcrA and 542 amino acids for CcrB. Each SCC*mec* type is driven by a specific set of recombinases[21].

PBP, a transpeptidase, is one of the bacterial enzymes involved in cell wall synthesis. It cross-bridges a nascent PG chain to pre-existing cell wall PG strands. This cross-bridging function is inhibited by β-lactam antibiotics by binding covalently to the catalytic site of PBP. β-lactam antibiotics are the structural analogues of *D*-alanyl-*D*-alanine. Thus, it is expected that in the presence of β-lactams, PBPs bind a β-lactam molecule at its *D*-alanyl-*D*-alanine recognition site. Thus, the target of β-lactam antibiotics is PBP. The PBPs produced by *S. aureus* have very high binding affinities for β-lactam antibiotics, resulting in inhibition of cell wall synthesis leading to cell death.

mecA encodes an alternative PBP, called PBP2'/PBP2a[21]. Apart from PBP2', MRSA also produces an intrinsic set of four different *S. aureus* PBPs (PBP1-PBP4), of which three (other than PBP4) may be required for cell growth. When compared to the normal PBPs, PBP2' has extremely low binding affinities for β-lactam antibiotics due to the presence of a unique *D*-alanyl-*D*-alanine recognition site. This makes it very difficult to inhibit PBP2' with β-lactam drugs.

1.6 Methicillin Resistance Determinants

Five main SCC*mec* types have been identified to date. SCC*mec* types I, II and III are mainly associated with health-care associated MRSA (HA-MRSA) strains. SCC*mec* type IV is associated with community-associated (CA-MRSA) strains, as well as with the Paediatric clone MRSA strains. SCC*mec* type V is associated with CA-MRSA strains and has only been identified in Australia and New Zealand. Numerous variants of each type have also been described[28,56].

Four allotypes of the ccrA and ccrB genes are found: *ccrA1*, *ccrA2*, *ccrA3* and *ccrA4* for *ccrA*; and *ccrB*, *ccrB2*, *ccrB3* and *ccrB4* for *ccrB*[26]. The combination of the *mec* gene complex class and the *ccr* gene complex type determine the classification of SCC*mec* allotype. See Figure 1.5 for the five core SCC*mec* types.

1.6.1 SCC*mec* type I

SCC*mec* type I is ± 34 000bp in size and was isolated from the first HA-MRSA isolate in England in 1961, NCTC 10442. It has a type 1 *ccr* complex and a class B *mec* complex[25] (Figure 1.6). Only *mecA* is present on this SCC*mec* type. Type-specific open reading frames (ORFs) of unknown functions, except for CE010, are normally found on the left half of this SCC*mec* type. A polypeptide of the Shine-Dalgarno repeat multi-gene family is potentially encoded for by CE010, and is virtually the same as the plasmin-sensitive surface protein (Pls), a large surface protein of *S. aureus*. SCC*mec* type I is representative

of the Archaic clone, while SCC*mec* type IA represents the Iberian clone. The only difference between SCC*mec* type I and type IA is the presence of the linearised plasmid pUB110 in type IA.

1.6.2 SCC*mec* type II

First isolated from a MRSA strain in 1980 in Japan, SCC*mec* type II is associated with HA-MRSA and is ± 53 000bp in size. It has a type 2 *ccr* complex and a class A *mec* complex[25] (Figure 1.6). The plasmid pUB110 is also present on this SCC*mec* type and encodes for resistance to tobramycin and bleomycin. pUB110 is also flanked by copies of the insertion sequence IS*431*. Multiple resistance in this SCC*mec* type (and in SCC*mec* type III) is achieved through the action of copies of IS*431*, known to act as a chromosomal deposit site for multiple resistance genes, downstream of the *mec* complex. pUB110 and pT181 were acquired through homologous recombination events across two IS*431* copies. This is supported by the presence of direct repeats at both ends of both integrated plasmids. The Kdp operon is also present, mostly as pseudogenes, on this SCC*mec* type. The Kdp operon encodes for a potassium-dependant ATPase and its regulators. SCC*mec* type II is representative of the New York/Japan clone[25].

1.6.3 SCC*mec* type III

SCC*mec* type III is ± 66 000bp in size and was first isolated from a MRSA strain in New Zealand in 1985. It is only associated with HA-MRSA and has a type 3 *ccr* complex and a class A *mec* complex[25] (Figure 1.6). The plasmid pT181, bracketed by copies of IS*431* and encoding resistance to tetracycline is found on this SCC*mec* type. ΨTn*554*, encoding cadmium resistance and Tn*554*, which encodes erythromycin and spectinomycin resistance are also commonly found. Z059 is also exclusive to SCC*mec* type III. The deduced amino acid sequence shows high resemblance to HsdR of *Klebsiella pneumoniae* and *Salmonella enterica*. It is also flanked by IS*431*. HsdR is a catalytic sub-unit of the restriction-modification system, although, when compared to intact HsdR, 300 amino acid residues appear to be deleted at the N-terminus[25].

Type III SCC*mec* can also contain: (1) an additional unit of *ccr* complex (Ψ*ccr* complex); and (2) flanked between two copies of IS*431*, the 15bp direct repeat sequence found at the right end of SCC*mec*. This suggests that SCC*mec* type III is composed of two separate SCC*mec* elements. SCC*mec* type III is representative of the Hungarian clone and SCC*mec* type IIIA represents the Brazilian clone, which lacks the pT181 insertion[25].

1.6.4 SCC*mec* type IV

SCC*mec* type IV was first seen in two CA-MRSA strains in 2002. This SCC*mec* type is much smaller (21-24kbp) than the SCC*mec* types associated with HA-MRSA and only carries the *mecA* gene. This SCC*mec* type has a type 2 *ccr* gene complex (also found in

SCC*mec* type II) and a class B *mec* complex[36] (also found in SCC*mec* type I) (Figure 1.6). The presence of these *ccr* and *mec* gene complexes, also found in other SCC*mec* types, indicates that genetic recombination events may have taken place, leading to the emergence of SCC*mec* type IV[12]. No virulence factors or other resistance genes are found. The lack of resistance genes is consistent with CA-MRSA, i.e. strains are susceptible to a variety of non-β-lactam antibiotics.

Apart from the genes required for the movement (*ccr* genes) of the element and resistance to methicillin (*mecA*), the lack of function of this SCC*mec* type may indicate that the element has gone through evolutionary refinement to carry DNA resulting in methicillin resistance. This lack of surplus function can make the element more fit than other SCC*mec* types as a *S. aureus* mobile genetic element in the community.

Numerous open-reading frames (ORFs) found on SCC*mec* type I and II are unnecessary for the benefit of the host cell. Most are mutated, partially deleted or inactive and some can even be detrimental to the host cell, as the plasmin-sensitive surface protein encoded on SCC*mec* type I can interfere with fibrinogen- and fibronectin-binding properties of the host cell. SCC*mec* elements associated with HA-MRSA are usually large due to the acquisition of numerous resistance genes, which may be required for survival in the nosocomial setting where numerous antibiotics and antiseptics provide selective pressure. In the community setting, higher growth rates and the ability to better colonise humans are more advantageous than a multi-drug resistant phenotype[36].

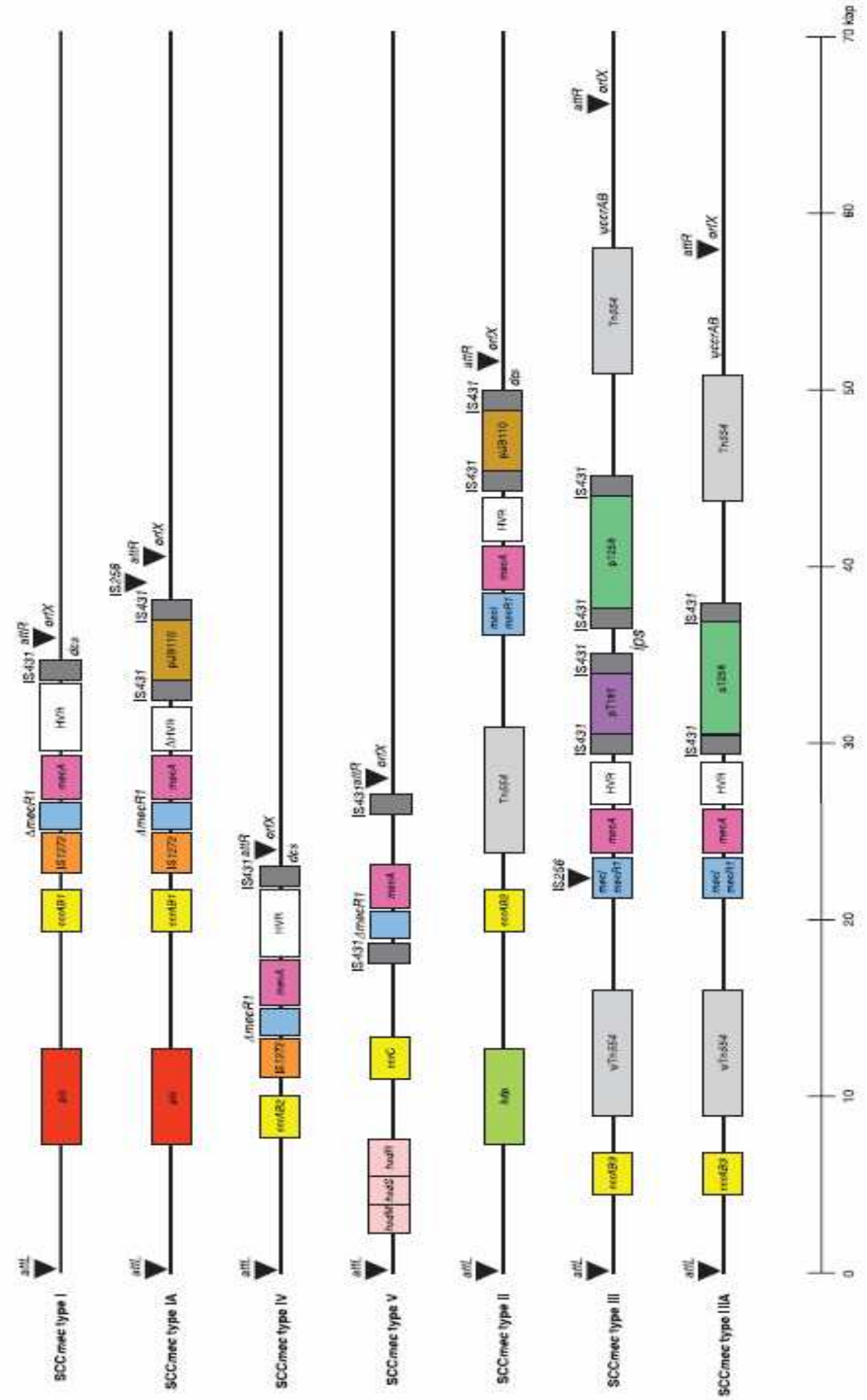


Figure 1.5: Schematic of SCC*mec* type elements I-V[18]. Selected variants are also shown.

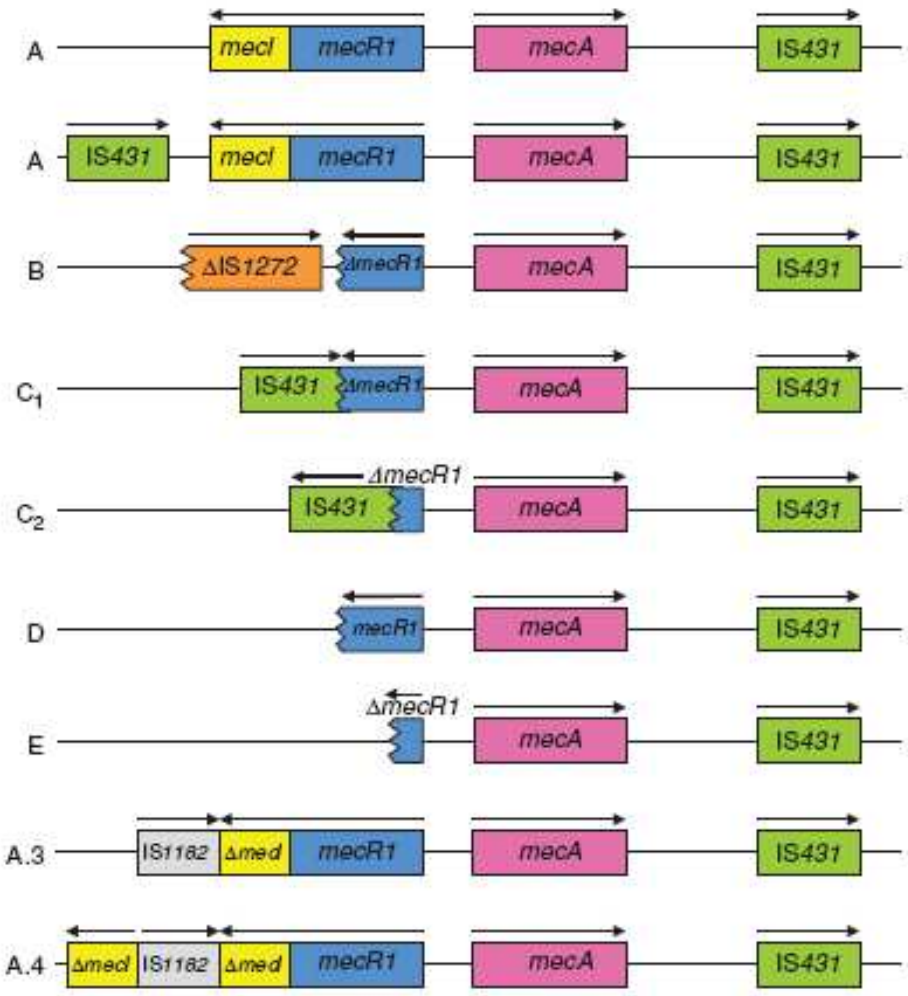


Figure 1.6: Schematic of the different classes of the *mec* gene complex present in staphylococci[18].

CA-MRSA isolates are associated with multiple genetic backgrounds[12]. The mechanism of transfer of SCC*mec* type IV remains unknown, but the presence of this SCC*mec* type in the vast majority of CA-MRSA strains indicates the promiscuous manner by which this element spreads. In addition, its smaller size might facilitate transfer to recipient strains by means of a plasmid or bacteriophage.

1.6.5 SCC*mec* type V

This SCC*mec* type was discovered in a CA-MRSA strain isolated in Australia and its description was published in 2004[26]. It carries an IS*431* flanked *mecA* (class C2 *mec* complex) (Figure 1.6) and a single copy gene homologue that encodes *ccr*, designated *ccrC* instead of the usual *ccrA* and *ccrB* genes. Numerous coagulase-negative staphylococci, especially *S. haemolyticus*, have been found to carry the class C2 *mec* gene complex, along with the type 5 *ccr* gene complex (*ccrC*). *S. haemolyticus* and *S. epidemidis* strains have also been identified that carry the class C2 *mec* gene complex. This is also a small element (± 28kbp) and carries no other resistance genes, except for *mecA*[26].

SCC*mec* type V also possesses a set of foreign genes encoding a restriction-modification system, which may aid in stabilising the element on the chromosome[26]. The deduced amino acid sequence of one fourth of the NH_2 terminus, containing the serine residue that catalyses DNA strand exchange, is well conserved among CcrB and CcrC variants. It seems that the activities of Ccr proteins are not affected much by the absence of some amino acids in the COOH terminus. One aspect distinguishing SCC*mec* type V from SCC*mec* elements I-IV is the missing characteristic inverted repeat sequences found at the extremities of SCC*mec*.

Analysis of the genome sequences of the seven sequenced *S. aureus* strains, NCTC 8325, MSSA 476, COL, MRSA 252, MW2, Mu50 and N315, revealed the presence of *hsdS* and *hsdM* genes in each of the two genomic islands (Gislands), νSaα and νSaβ. It seems as if the stability of certain regions can be affected by the restriction-modification system[26].

hsdR$_{aur,}$ which encodes the restriction function, is frequently found on the seven *S. aureus* genomes at the same locus, far from the two Gislands. This suggests that the Gislands, carrying enterotoxins and exotoxin-like genes, are protected from deletional loss through co-repression of the modification system. A complete set of genes involved in the type I restriction-modification system (*hsdR*, *hsdS* and *hsdM*) is carried on SCC*mec* type V. SCC*mec* V is the only SCC*mec* element in *S. aureus* that carries genes for a restriction-modification system. A SCC in strain MSSA 476 (SCC_{476}) also possesses a complete set of genes involved in the restriction modification system. Data obtained from phylogenetic trees and codon usage patterns showed that the restriction-modification systems encoded by SCC_{476} and SCC*mec* type V are different from those encoded by genes *hsdR*$_{aur}$, *hsdS* and *hsdM* on the *S. aureus* genome, suggesting that those elements evolved in different species and were transferred to *S. aureus* by horizontal transfer[26].

1.7 Origins and Evolution of Methicillin-Resistant *Staphylococcus aureus*

1.7.1 Origins of methicillin-resistant *Staphylococcus aureus*

MRSA arose through the acquisition of the staphylococcal cassette chromosome *mec* (SCC*mec*). Within SCC*mec*, many ORFs are found with atypical codon-usage patterns and skewed G+C content at the 3rd codon positions, which is indicative of the relatively recent acquisition of SCC*mec* compared to other genes on the *S. aureus* chromosome. The location of the integration site, being so close to the origin of replication, might have strategic significance. This allows for useful antibiotic resistance genes, acquired from other bacterial species, to be quickly taken advantage of by *S. aureus*. These genes would have a bigger likelihood of being present in numerous copies due to the uncoupled nature of DNA replication and cell division. The inefficient translation of gene products, linked to atypical codon-usage patterns, is partly compensated for by the high copy number[20].

Conserved among all types of SCC*mec* elements is the *ccr* gene complex, composed of the *ccrA* and *ccrB* genes and surrounding ORFs. Three homologues for each *ccr* gene are found and 3 corresponding SCC*mec* elements have been identified for HA-MRSA, each one carrying a typical combination of the *mec* and *ccr* gene complexes. Type I SCC*mec* has a type 1 *ccr* complex and a class B *mec* complex (*mecI* and 3' region of *mecRI* deleted; truncated copy of IS*1272* integrated downstream of deletion). Type II and III SCC*mec* elements have class A *mec* complexes with type 2 and 3 *ccr* complexes, respectively. In type III SCC*mec* another truncated *ccr* gene is found together with a 15bp direct repeat between two IS*431* elements, suggesting that type III SCC*mec* arose from two SCC

elements that fused together[20]. The antibiotic resistance determinants of the three SCC*mec* elements are different as well.

It is known that the SCC elements are disseminated widely in staphylococci, including *S. aureus* but their origin remains unknown. SCC*mec* has never been found in a strain of any other genera. Due to the occurrence of IS*1272*, prevalent in *Staphylococcus haemolyticus*, in the class B *mec* complex, it has been proposed that SCC*mec* type I was transferred from *S. haemolyticus* to *S. aureus* in the past. *Staphylococcus sciuri* possess an intrinsic PBP to which PBP2' has the greatest similarity (87.8% amino acid identity). No other bacterial species has been found in which PBP2' is part of the intrinsic set of PBPs[20].

1.7.2 Evolution of methicillin-resistant *Staphylococcus aureus*

The acquisition of SCC*mec*, which allowed for *S. aureus* to become fully resistant to β-lactam antibiotics, was the first common genetic event. Regarding the evolution of clonotype II-A, the dominant clone in Japanese hospitals in the 1980s, at least two more genetic events were involved. The class A *mec* complex is found in this clone. The *mec* regulatory gene system does not respond well to many β-lactam antibiotics and it has been hypothesised that this is due to the inadequate ligand-binding specificity of the penicillin-binding domain of MecRI[20].

In the 1980s numerous clinical staphylococcal strains, with class A *mec* complexes, were isolated in Japan. These isolates, called pre-methicillin-resistant *S. aureus* (pre-MRSA), contained intact *mecI* genes and repressed methicillin-resistance. This state is thought to

be the naïve precursor of modern MRSA. It has been observed that most strains with type II or III SCC*mec* complexes (containing the class A *mec* complex) have mutations in either the *mecI* gene or in the operator region of the *mecA* gene (where binding occurs with the MecI repressor protein).

Hetero-MRSA was dominant in Japan in the early 1980s. By the end of the 1980s most hetero-MRSA strains disappeared and the clonal expansion of homo-MRSA has since become evident. In the early 1980s the number of MRSA strains with diverse genetic backgrounds increased drastically. The third generation cephalosporins were launched and widely used in Japan. The overuse of these cephalosporins was stopped and other β-lactam antibiotics, with low MIC values against hetero-MRSA, were used against MRSA infections. These antibiotics were not potent enough against clonotype II-A strains and the clonotype was converted into homo-MRSA, which allowed for its clonal dissemination all over Japan and it remains the dominant clone in Japan today[20].

It was found in the United Kingdom (UK) that early MRSA isolates had similar genetic and phenotypic properties (phage group, antibiotypes, PFGE pattern, *spaA* type and MLS type) as a group of early MSSA blood isolates. This suggested that the methicillin-susceptible *Staphylococcus aureus* (MSSA) isolates examined were the descendents of a strain that served as the first recipient of *mecA* in Europe. The majority of MSSA isolates had similar PFGE-, *spaA*- and multi-locus sequence type (MLST) as the Iberian clone. Two other MSSA isolates showed PFGE-, *spaA*- and MLS types similar to the Paediatric and the New York clones[24], suggesting that these clones received the *mecA* gene independently.

1.8 Molecular Characterisation of Micro-Organisms

Due to the increase in the frequency of infections caused by MRSA in both nosocomial and community settings, fast and precise characterisation of isolates is required. Enough information must be generated to be able to distinguish and reliably group epidemiologically related from unrelated strains in the case of an outbreak, to permit the implementation of appropriate infection control measures[65].

Numerous typing techniques are available to the epidemiologist for the discrimination of MRSA strains. Performance criteria (efficacy) and convenience criteria (efficiency) can be used to evaluate any typing technique. Performance criteria include typeability, reproducibility, discriminatory power and agreement between typing techniques. Versatility, rapidity, ease of execution and interpretation are examples of convenience criteria.

1.8.1 Macro-restriction analysis

Macro-restriction analysis (MRA) was developed by Schwarz and Cantor[52,65] and is based on the restriction of bacterial DNA with endonucleases. These endonucleases recognise a small number of sites on the bacterial chromosome only, therefore they are infrequent cutters. This generates large DNA fragments (10-800kb), which cannot be separated effectively by conventional electrophoresis. Therefore, the electric field orientation across the gel is periodically changed, allowing for the effective separation of fragments

according to size. This allows for the comparison of simpler profiles and all bacteria can theoretically be typed using this technique.

Special care must be taken to avoid mechanical shearing of DNA and therefore intact DNA is required for pulsed-field gel electrophoresis (PFGE). The bacterial cells are embedded in low melting temperature agarose, protecting the DNA but also allowing for the free flow of solutions required for cell lysis, digestion of cell proteins and DNA restriction. After restriction, the agarose plug is subjected to PFGE. Before migrating to the cathode, the fragments have to be aligned in the direction of the electric current. The time required for the current to align is dependent on the molecular mass of the DNA, and therefore alteration of the direction of the electric current during electrophoresis results in a sharper resolution of fragments.

Larger fragments require more time to reorientate themselves in the new direction of the electric field and migrate slower, leaving them closer to the origin, while smaller fragments migrate more quickly due to faster re-orientation towards the electric field. The electric field intensity, angle of the current and frequency with which the current alternates, length of run time and electrophoresis temperature are all variables that can affect fragment separation.

To separate DNA fragments, numerous techniques are available involving a pulsed field system. The most common ones are FIGE (field-inversion gel electrophoresis) and CHEF (contour-clamped homogenous electric field), the latter involving the alternating of the angle of the electric current. FIGE is simpler, less expensive and best for the separation of fragments from 0.1 to 200kb. The electric field is alternated between forward and reverse

directions where the forward pulse lasts three times longer than the reverse pulse. CHEF is best for the separation of fragments up to 3Mb. The system consists of a hexagonal arrangement of electrodes, which is used for the generation of uniform electric field at angles of 120º. The fragments thus move in a straight line with little or no distortion. PFGE is currently the "gold-standard" for MRSA typing and has been compared to numerous other techniques. The discriminatory power is equal to and in many cases superior to phenotypic techniques as well as to other genotypic techniques such as ribotyping, RAPD (random amplified polymorphic DNA), PCR-RFLP and inter-IS*256* PCR[65]. Results obtained are also more reliable than those obtained by REA (restriction enzyme analysis), as there is very little or no interference from plasmid DNA. The fragments generated after digestion of the plasmid are far too small to affect the profile. A number of restriction endonucleases have been tested, but *Sma*I has been shown to provide good results. All strains are typeable using this enzyme and standard strains are reproducible after extensive sub-culturing[70].

However, there are a number of disadvantages associated with PFGE, namely the long time it takes to obtain a result, the technique's labour intensiveness and the high cost of reagents. Specialised electrophoretic equipment is also required, which is also expensive. Inter-laboratory comparisons can also be quite difficult, as slight differences in electrophoretic conditions can alter the distance travelled by fragments. This can complicate the comparison between isolates run under different electrophoretic conditions or on different gels[70]. Computer-aided analysis and the use of a control organism or molecular weight marker on each gel assist with comparisons and a standardised interpretation scheme has been published to determine the genetic relatedness between strains[65].

If the restriction patterns of isolates have the identical number of bands and the bands migrate the same distance, the isolates can be regarded as genetically indistinguishable. The isolates are considered to represent the same strain. Isolates whose PFGE pattern differs by 2 to 3 bands are considered to be closely related, as theses changes are consistent with a solitary genetic event such as a point mutation or an insertion or deletion of DNA. If the isolates' PFGE pattern differs by changes consistent with 2 autonomous genetic events, resulting in 4 to 6 band differences, they are considered to be possibly related. Changes consistent with 3 or more independent genetic events will usually result in 7 or more band differences. The isolates are considered to be unrelated if this is the case[62]. These criteria have been simplified in Table 1.1 below.

Table 1.1: PFGE pattern interpretation criteria according to Tenover *et al*[62].

Category	Number of fragment differences
Indistinguishable	0
Closely related	2-3
Possibly related	4-6
Unrelated	≥7

1.8.2 *spa* typing

DNA sequencing has the advantages of speed, unambiguous data interpretation, ease of use, ease of interpretation, standardisation and simplicity of large-scale database creation and is a powerful approach to strain typing.

A highly polymorphic sequence within the X region, composed of repeats of 24 base pairs, is found within the protein A gene of *S. aureus*. The function of this region might be that a

longer X region could result in better exposure of the Fc-binding domains of protein A. This would facilitate the colonisation of host surfaces and could contribute to the epidemic phenotype associated with some MRSA strains[55].

Most epidemic clones usually have more than seven repeats, whereas most non-epidemic clones usually have seven or fewer repeats[15]. The *spa* gene harbours a number of functionally distinct regions and is composed of approximately 2150bp. The regions are the Fc-binding domain and the X region (Figure 1.7). The X region is divided into the X_r region (short sequence repeat region) and the X_c region, a cell wall attachment sequence.

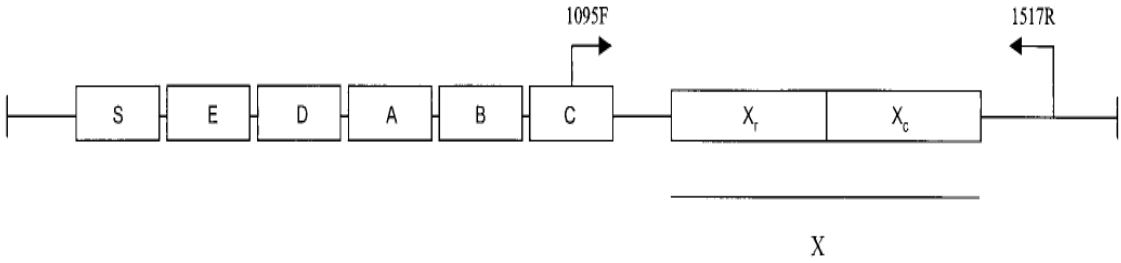


Figure 1.7: Map of the *S. aureus* Protein A gene. The boxes represent gene segments encoding the signal sequence (S), the immunoglobulin G-binding regions (A-D), a region homologous to A-D (E) and the COOH terminus (X)[55].

DNA sequencing of the polymorphic X_r or short sequence repeat (SSR) region of the *spaA* gene (protein A), or *spa* typing, is currently employed as an alternative typing technique[57]. Deletions and duplication of repetitive units and point mutations within a repeat are the main ways from which diversity of the SSR arise. Well-conserved regions flank the X region coding sequence in the *spaA* gene. This allows for direct sequence typing after

amplification with PCR. *spa* typing is very rapid and convenient for outbreak investigation in both the hospital and community setting since only a single locus is involved[55].

The X region of protein A has a variation rate (clock speed) that despite its high degree of polymorphism is slow enough to provide discrimination suitable for outbreak investigation, or for strain collections gathered within a short period of time, restricted to one location[40]. However, for long-term surveillance or studies comparing distant lineages, *spa* typing should be complemented with other typing techniques, such as macro-restriction analysis[38].

Ridom StaphType (Ridom GmbH, Würzburg) is a software tool that allows for rapid repeat determination, data management and retrieval and, following automatic quality control of DNA sequence chromatograms, an Internet-based assignment of new *spa* types. The repeats are assigned an alphanumeric code and the order of the specific repeats determines the *spa* type. A considerable degree of *spa* gene repeat number variations are found within a given sequence type (ST) by multi-locus sequence typing (MLST), which suggests that in certain instances, *spa* typing provides greater resolution than MLST.

However, the consensus is still that PFGE is superior to *spa* typing and MLST in terms of the discriminatory power. *spa* typing cannot be considered a portable tool, because the technique is hampered by the lack of generally available software tools for repeat identification and by the lack of consensus on new repeat and *spa* type assignment[19]**.** The three modules of Ridom StaphType (Figure 1.8) are a sequence editor, a database and a report generator. After the input sequence has been provided, the software assigns a quality value to each base called, which corresponds to the sequence error probability.

Thereafter the *spa* repeats are automatically detected and the *spa* type assigned. A versatile graphic user interface allows for the manual editing of sequences where required. The software searches for signature sequence, both at the 5' and 3' positions, at the correct distance to ensure leading and ending repeats are not excluded in *spa* type assignment. *spa* type results and any epidemiologically relevant information can be stored in relational database systems. Any information can be exported in tab-delineated files after retrieval with Boolean searches. Different configurable reports can also be created that are stored internally as read-only files[19].

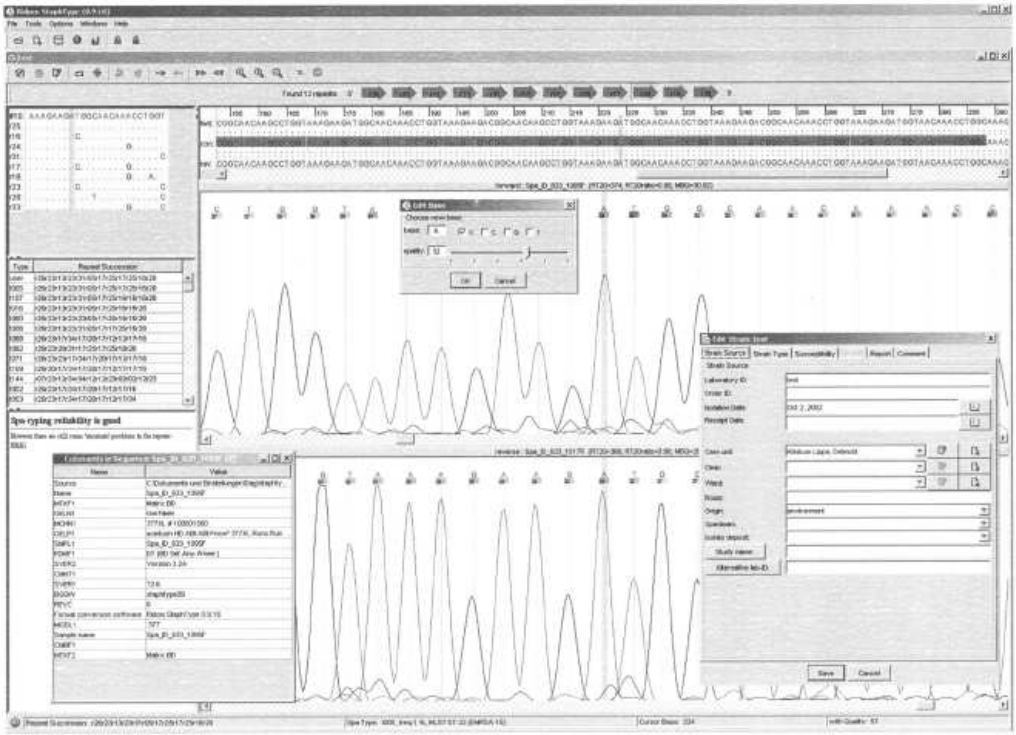


Figure 1.8: Screen shot of the Ridom StaphType software. A nucleotide base quality-based sequence editor, a database and a report generator (not shown) module are featured[19].

Ridom StaphType employs numeric *spa* repeat and type codes. The software synchronises with an associated website, functioning as the active source for all new *spa* repeat/type codes, directly via the http protocol or via a file-based system (e.g. E-mail). This ensures uniform code terminology usage. To allow for the final designation of new /unidentifiable *spa* types/repeats, all new *spa* repeats/types that meet quality criteria (5'/3' signatures are unambiguously detected; *spa* types are deduced from chromatograms) can be transferred to the server during synchronisation.

Repeats and types detected by others are transferred to the Ridom StaphType client software, from the time when the last synchronisation occurred (http://www.ridom.de/spaserver/). *spa* repeat sequences (FASTA format) and types can be downloaded from the website (http://www.ridom.de/spaserver/) and are accessible to any person. It is possible to submit chromatograms of new *spa* repeats/types for inclusion in the reference database; therefore, users not working with Ridom StaphType have access to the same uniform terminology[19].

1.9 MRSA in South Africa

During 1985, MRSA was isolated from 18% of 2 681 pus swabs and 25% of blood cultures at Tygerberg Hospital, Cape Town[44]. In the Republic of South Africa (RSA), the National Antibiotic Surveillance Forum (NASF) observes, provides and publishes information regarding antimicrobial resistance patterns. Data is obtained from academic teaching hospitals and private diagnostic microbiology laboratories. Thirty-six percent of 1566 *S. aureus* samples tested at the Johannesburg Hospital in 2003 were methicillin/oxacillin resistant (personal communication, Prof. A.G. Dusé, HOD CMID).

Baseline data established by The Pan-European Antimicrobial Resistance Using Local Surveillance (PEARLS) study during 2001-2002 of MRSA and other antibiotic resistant bacterial species showed that South Africa had a MRSA incidence of 33.3%[6]. Analysis of epidemic MRSA strains from 20 countries identified 5 distinct MRSA clones globally[21]. After studying a very limited number of samples, data obtained have shown that the South African isolates belonged to the clonotype I-A group, the oldest MRSA strain, which dates back to the 1960s. This clonotype is represented by NCTC 10442 (Archaic clone). However, only one South African MRSA strain was subjected to analysis.

It is very important to determine clonal dominance in a country over time, as virulence and pathogenic factors are specific to each clonotype. In a study by Zinn *et al.* in 1996, methicillin resistance in *Staphylococcus aureus* in South Africa was found to be 39%[73]. Several of the clones were identified as international epidemic MRSA by pulsed-field gel electrophoresis.

Twenty-six percent (61/233) of *S. aureus* isolates collected from 14 health institutions in KwaZulu-Natal (KZN) province were resistant to methicillin, oxacillin and cefoxitin[53]. The *mecA* gene was also detected in all isolates, confirming resistance. Forty-eight of the MRSA isolates (78.7%) were recovered from wound samples, 9.8% (n=6) from sputum, 3.3% (n=2) from otitis media and 1.6% (n=1) each from blood sample, urine, eye-related infection and endotracheal aspirate. The MRSA isolates were categorised into 12 antibiotypes with 41% (n=25) of the isolates sharing the same antibiotype (penicillin^{R}, gentamicin^{R}, erythromycin^{R}, tetracycline^{R}, trimethoprim^{R} and rifampicin^{R}). After *Alu*I digestion of the coagulase gene PCR product, 5 main RFLP patterns were identified for the 61 MRSA isolates, of which the majority (67.2%) of the isolates were represented by a single pattern. It was noted that isolates having the same RFLP pattern shared a common antibiotype.

The first report on the molecular characterisation and confirmation of mannitol-negative MRSA in South Africa was published in 2006[54]. Publications on mannitol-negative MRSA are rare and the prevalence is unknown in South Africa. The isolates were confirmed to be *S. aureus* by PCR targeting the *nuc* gene. SCC*mec* typing also revealed that all five isolates carried a SCC*mec* IV. All isolates were resistant to penicillin, oxacillin, gentamicin, tetracycline, trimethoprim-sulfamethoxazole and rifampicin.

In 2005, a 67-year old male presented with relapse 14 days after treatment with vancomycin for a MRSA ventriculitis. At the time of relapse, MRSA (with a vancomycin MIC of 4mg/l by E-test) was cultured from CSF samples. Therapy with linezolid and intraventricular vancomycin was initiated. The isolate was found by the E-test to have sub-populations with a vancomycin MIC of 8mg/l and a teicoplanin MIC of 12mg/l. The

strain was also found to have a population analysis profile very similar to that of MU3, a hetero-vancomycin-intermediate resistant *Staphylococcus aureus* (hVISA) strain. The patient made a successful recovery. It is believed that this was the first documented case of clinical infection by a hVISA strain in South Africa[3]. However, no molecular information was obtained for this strain.

No comprehensive study has been completed so far to investigate and determine the molecular epidemiology and clonal prevalence of MRSA in South Africa. Only 54 isolates were tested for methicillin resistance during the PEARLS study. A moderate number of samples (n=236) were tested for methicillin resistance in the Zinn study. Only two samples were subjected to MRA. No generalised conclusions could be drawn and the data available is not representative of the true nature of MRSA in South Africa.

A nationwide study with sufficient samples would allow for the molecular elucidation of clonotypes of each province, permitting both national and international comparisons. It is imperative to build up a continuous national surveillance database because *S. aureus* is a global public health problem. The molecular epidemiology is usefull in establishing how the organism is spread and how it can be controlled.

1.10 Other Virulence Factors

Numerous bacterial surface components (capsular polysaccharides and protein A), including those molecules that recognize adhesive matrix molecules (clumping factor and fibronectin binding protein), together with extracellular proteins (coagulase, hemolysins, enterotoxins, toxic-shock syndrome toxin (TSST), exfoliatins and Panton-Valentine leukocidin (PVL)) are involved in the pathogenicity of *Staphylococcus aureus* infections[32]. However, it is difficult to assess the exact role of these different virulent factors in invasive infections.

1.10.1 Panton-Valentine leukocidin (PVL)

Panton-Valentine leukocidin (PVL) is a toxin found mainly in CA-MRSA and it is estimated that less than 5% of all *S. aureus* strains carry the genes encoding for PVL production. There is no published record of *S. aureus* strains from South Africa that encode the different subunits of PVL. PVL is part of the family of synergohymenotropic toxins, which also hosts γ-hemolysin and other leukocidins such as bovine leukocidin R, and is able to damage the membranes of erythrocytes and host defence cells[32]. Two non-associated classes of secretory proteins, S and F, act synergistically to cause membrane damage. *LukS-PV* and *lukF-PV*, the S and F class proteins specific to PVL, are produced by all PVL-producing strains. Purified PVL is leukotoxic to rabbit and human polymorphonuclear cells and macrophages by means of pore production. When injected intra-dermally in rabbits, it also induces severe inflammatory lesions, leading to capillary dilatation, chemotaxis, PMN infiltration and karyorrhexis and skin necrosis.

The production of PVL is usually associated with strains causing direct invasion and tissue destruction, including primary community-associated pneumonia, severe necrotic skin infections, furuncles and cutaneous abscesses. Strains causing secondary infection after skin injury, hospital-associated pneumonia, infective endocarditis, and mediastinitis, urinary tract infections, TSS or enterocolitis normally lack gene for PVL, and therefore PVL is typically associated with CA-MRSA strains and not HA-MRSA strains.

The role of PVL in the pathogenesis of CA-MRSA was tested recently in mice infection models, which was used to compare the virulence of PVL-positive with PVL-negative strains. However, mice sepsis models (to investigate virulence) showed the PVL-negative strains were significantly more virulent than a PVL-positive genetically similar strain. A mouse skin-infection model was used to test the ability to cause abscesses and/or dermonecrosis, and showed that a mouse infected with a PVL-negative strain had a greater average abscess volume when compared to a mouse infected with a PVL-positive strain. After infection with PVL-positive strains, more mice had dermonecrotic abscesses than mice infected with PVL-negative strains. There was virtually no difference in the virulence of isogenic *luk-S/F-PV* knockout strains (Δ*pvl*). It seems as if the genetic makeup of each strain plays a bigger role in virulence, rather than PVL as a single toxin[69].

CHAPTER 2

2 STUDY AIM AND OBJECTIVES

2.1 Aim of the Study

This study was undertaken to elucidate the molecular epidemiology and clonality of methicillin-resistant *Staphylococcus aureus* (MRSA) strains in South Africa, using macro-restriction analysis (MRA) with pulsed-field gel electrophoresis (PFGE) and *spa* (protein A gene) typing. The isolates were collected between August 2005 and November 2006.

2.2 Study Specific Objectives:

1. Obtain MRSA isolates from centres across South Africa.
2. Distinguish between health-care-associated- and community-associated MRSA isolates, based on molecular characterisation.
3. Perform molecular fingerprinting of isolates using; (1) MRA with PFGE; and (2) *spa* typing.
4. Compare the genomic profiles of the MRSA strains to determine their relatedness and to establish which clone(s) is/are dominant.
5. Compare the genomic profiles of South African MRSA strains (obtained by PFGE and *spa* typing) with available profiles of internationally important clones.

6. Screen all isolates by the polymerase chain reaction (PCR) technique for the toxin Panton-Valentine leukocidin (PVL).

CHAPTER 3

3 MATERIALS AND METHODS

3.1 Sample Collection

Various diagnostic laboratories across the country were approached to assist with the collection of isolates. A complete list of all the laboratories, including the number of isolates collected is shown in Table 3.1. Sample collection commenced on 1 August 2005 and ended on 30 November 2006. All laboratories were requested to collect only one isolate per patient in order to avoid repeats. The laboratories were supplied with semi-solid agar vials, which were stab-inoculated with the various strains as they were isolated. Each semi-solid agar vial was assigned a specific number. The laboratories completed an information sheet, corresponding to the number assigned for each semi solid agar vial. The information sheet is given in Appendix B (p. 123). Ethical clearance (M01-05-07) was obtained from the University of the Witwatersrand Human Research Ethics Committee (Medical) to (1) collect patient isolates (M050457), and (ii) obtain patient age and gender (M010507). Refer to Appendix L (p. 179) for a map of South Africa.

3.2 Chemicals, Reagents, Media and Kits

All chemicals and reagents used in this study were of analytical or molecular grade. The composition of all media is given in Appendix A (p. 120). All media used for bacterial

growth was obtained from Diagnostic Media Products (DMP) (NHLS, Sandringham, South Africa). Lists of the composition of all buffers are given in section 3.10.

Table 3.1: Centres across South Africa that participated in the collection of *S. aureus* isolates.

Province	Centre name	City/Town	Number collected	
			Total	MRSA
Gauteng	Tshwane Academic Hospital, NHLS	Pretoria	15	13
	New Johannesburg Complex, NHLS	Johannesburg	15	12
	Van Drimmelen	Johannesburg	15	14
	Ampath	Pretoria	14	14
Limpopo	NHLS	Polokwane	30	24
	Ampath	Polokwane	9	5
Mpumalanga	NHLS	Witbank	11	10
	NHLS	Nelspruit	10	9
	NHLS	Ermelo	10	4
	Ampath	Nelspruit	17	6
Northern Cape	NHLS	Kimberley	39	29
Free State	NHLS	Welkom	11	6
	Universitas Hospital, NHLS	Bloemfontein	16	12
	Ampath	Bloemfontein	18	13
North West	NHLS	Rustenburg	13	10
	NHLS	Klerksdorp/ Tshepong	13	10
	Lancet	Johannesburg	2	1
Eastern Cape	NHLS	Port Elizabeth	30	22
	Ampath	Port Elizabeth	27	25
KwaZulu-Natal	Ampath	Durban	45	34
	RK Khan Hospital	Durban	15	9
Western Cape	NHLS	George	12	11
	Groote Schuur Hospital, NHLS	Cape Town	15	15
	Pathcare	Cape Town	30	29

3.3 Strains and Growth Conditions

The bacterial strains used in this study as controls are listed in Table 3.2. All strains were incubated overnight on 5% blood agar plates and in 10ml brain heart infusion (BHI) broth at 37ºC. Prior to any analysis, bacterial isolates received that were mixed after plating on 5% blood agar plates were plated out on either: (1) blood agar plates supplemented with nalidixic acid and colistin; or (2) Chapman's agar plates (Mannitol salt agar plates).

3.4 Phenotypic Characterisation

All bacterial isolates received were identified according using the DNase test. This test was performed on DNA agar plates according to the guidelines of Koneman[71]. Deoxyribonuclease, or DNase, is an enzyme produced by *S. aureus* that cleaves DNA. The DNA present in the agar is hydrolysed by the DNases if this enzyme is produced by the organism. After incubation of the DNA agar plate, the plate is flooded with 1M HCl, which precipitates any unhydrolysed DNA, producing cloudiness. A zone of clearance is visible where the DNA has been hydrolysed.

Table 3.2: Bacterial strains used as controls during this study.

Designation	Meth$^{R/S}$	SCC*mec* type	*spa* type	Application	Reference(s)
ANS46	R	III	t037	SCC*mec* type III control; PFGE	42, 43, 44
BK2464	R	II	t002	SCC*mec* type II control; PFGE	42, 43, 44
MW2	R	IV	t128	SCC*mec* type IV control; PFGE, PVL PCR (+) control	42, 45
STA61	R	I	t001	SCC*mec* type I control; PFGE; Phenotypic characterisation (DNase); Antibiotic susceptibility testing, *spaA* PCR (+) control.	This study
ATCC BAA-38 (E2125)	R	I	t051	PFGE	43
HPV107	R	IA	t051	PFGE	42, 43, 44
ATCC 6532	S	None	NA	Antibiotic susceptibility testing	This study
NCTC 8325	S	None	NA	Size standard for PFGE	43

Meth = Methicillin; R = Resistant; S = Susceptible; PFGE = Pulsed-field gel electrophoresis; NA = Not available

3.4.1 DNase test

Eight to ten isolates were tested for DNase production per DNA plate by drawing horizontal and vertical lines on the agar plate, creating small squares. A known *S. aureus* strain, STA61, was included with every plate as a positive control. Each square on the plate was inoculated with a single colony of each isolate. The plate was aerobically incubated overnight at 35±2°C. After incubation the plate was flooded with 1M HCl, left to stand for a minute following which excess HCl was discarded. A zone of clearing around the inoculum was taken as a positive result.

3.5 Antibiotic Susceptibility Testing

3.5.1 Kirby-Bauer disk diffusion antibiotic susceptibility method

Resistance to methicillin was tested using the Kirby-Bauer disk diffusion antibiotic susceptibility method according to the guidelines of the CLSI (January 2006)[11]. The test was performed using 1μg oxacillin antibiotic disks (Oxoid, Hampshire, United Kingdom) on Mueller-Hinton agar plates supplemented with 2% NaCl. The interpretive criteria of the CLSI for *Staphylococcus aureus* were used to establish the antibiotic susceptibility of the isolates. Refer to Appendix C (p. 124) for a complete table regarding the zone diameter interpretive standards for *Staphylococcus* spp.

Briefly, a single colony was suspended in 1ml saline, adjusted to a 0.5 MacFarland standard and spread evenly with a sterile cotton swab onto the agar plate surface. After the

surface of the agar plate had dried for about 5 min, the antibiotic disk was placed on the inoculated plate and incubated for 24 hours aerobilcally at 37˚C. The results were interpreted by measuring the zone diameters with the aid of sliding callipers, including the disk diameter.

3.6 DNA Preparations

3.6.1 Total genomic DNA isolation

In order to extract the genomic DNA, a previously published "rapid lysis" procedure was used[67]. Briefly, the bacterial isolates were cultured overnight in 10ml of BHI broth at 37˚C (shaking at 100rpm) (Incubator Shaker Series 25, New Brunswick Scientific Co. Inc, Edison New Jersey, USA). 300µl of the overnight culture was removed and the bacterial cells harvested by centrifugation at 16 000 × *g* for 2minutes (min). Cells were resuspended in lysostaphin (50µl, 100µg/ml) (Sigma-Aldrich, St. Louis, MO. USA) and incubated for 10min at 37˚C. Thereafter, 50µl of Proteinase K (100µg/ml) (Merck KGaA, Darmstadt, Germany) and 150µl Proteinase K buffer were added. Again the cell suspensions were incubated for 10min at 37˚C and then boiled for 5min.

3.7 DNA Amplification, Electrophoresis and Analysis

3.7.1 DNA amplification and electrophoresis

Total genomic DNA was extracted as described in section 3.5.1. All PCR reactions were set up in a PCR laminar flow hood. PCR reactions were performed using a PCR thermal cycler, either an iCycler (Bio-Rad Laboratories, Hercules, CA, USA) or a Hybaid cycler (Hybaid Omnigene, Middlesex, England). A final volume of 25µl was used, containing 1-10ng bacterial DNA template, primer and 1×Promega (Promega, Madison, WI, USA) or Roche PCR Master Mix (Roche Applied Science, Indianapolis, IN, USA), with a final $MgCl_2$ concentration of 3mM. When the Hybaid cycler was used, 30µl of mineral oil was pipetted over the PCR reaction, as this instrument does not have a heated lid. 20µl of the PCR product was analysed by electrophoresis in a Tris-acetate-EDTA (TAE) agarose gel (wt/vol) (100V) containing 5µl ethidium bromide (10mg/ml). A 100bp ladder (Promega) (Fermentas Life Sciences, Hanover, MD, USA) was mainly used as a molecular weight standard and all gels were visualised using a Bio-Rad Gel Doc 1000 system (Bio-Rad Laboratories).

3.7.2 SCC*mec* multiplex PCR

The SCC*mec* structural variant of each MRSA isolate was determined by employing a previously described protocol[41]. Eight loci (A to H) were included in this multiplex PCR, together with an internal positive control, the *mecA* gene. Located downstream of the *pls* gene is locus A, specific to SCC*mec* type I; internal to the *kdp* operon is locus B and is

specific to SCC*mec* type II; locus C is specific to SCC*mec* types II and III and is internal to the *mecI* gene; locus D is present in SCC*mec* types I, II and IV and is internal to the *dcs* region; located in the region between integrated plasmid pT1258 and transposon Tn*554* is locus E and it is specific to SCC*mec* type III; locus F is located in the region between Tn*554* and the chromosomal right junction in *orfX* and is also specific for SCC*mec* III. To distinguish structural variant IA and IIIA, loci G and H were included, respectively. Locus G is the left junction between IS*431* and pUB110, while locus H is the left junction between IS*431* and pT181. The oligonucleotide primer sequences, together with the amplicon sizes are given in Table 3.3.

Total genomic DNA was extracted as described in section 3.6.1. All PCR reactions were set-up as described in section 3.7.1. PCR reactions were performed on an iCycler PCR thermal cycler (Bio-Rad Laboratories). The primer concentrations were as follows: 400nM of primers for loci A, C, F, pUB110 R1 and pT181 R1; 800nM of primers for loci D, *mecA* and IS*431* P4; 200nM of primers for loci B and E.

The cycle conditions used were as follows: initial denaturation at 94˚C for 4min, followed by 30 cycles of 94˚C for 30sec (denaturation), 53˚C for 30sec (annealing) and 72˚C for 1min (extension). 4min at 72˚C was used as the final extension time. The PCR products were subjected to electrophoresis as described in section 3.7.1 in a 3% TAE agarose gel.

Table 3.3: Oligonucleotide primer sequences used for the multiplex PCR to determine the SCC*mec* type of each MRSA isolate[18].

Locus	Primer	Sequence (5'-3')	Amplicon size (bp)	Specificity
A	CIF2 F2 CIF2 R2	TTCgAgTTgCTgATgAAgAAgg ATTTACCACAAggACTACCAgC	495	I
B	KDP F1 KDP R1	AATCATCTgCCATTggTgATgC CgAATgAAgTgAAAgAAAgTgg	284	II
C	MECI P2 MECI P3	ATCAAgACTTgCATTCAggC gCggTTTCAATTCACTTgTC	209	II, III
D	DCS F2 DCS R1	CATCCTATgATAgCTTggTC CTAAATCATAgCCATgACCg	342	I, II, IV
E	RIF4 F3 RIF4 R9	gTgATTgTTCgAgATATgTgg CgCTTTATCTgTATCTATCgC	243	III
F	RIF5 F10 RIF5 R13	TTCTTAAgTACACgCTgAATCg gTCACAgTAATTCCATCAATgC	414	III
G	IS*431* P4 pUB110 R1	CAggTCTCTTCAgATCTACg GAgCCATAAACACCAATAgCC	381	IA
H	IS*431* P4 pT181 R1	CAggTCTCTTCAgATCTACg gAAgAATggggAAAgCTTCAC	303	IIIA
mecA	MECA P4 MECA P7	TCCAgATTACAACTTCACCAgg CCACTTCATATCTTgTAACg	162	Internal control

3.7.3 PVL PCR and nucleotide sequencing

All MRSA and MSSA isolates collected were examined for the presence of the *lukS-PV* and *lukF-PV* genes, which encode the class S and class F proteins specific for PVL, according to a previously described protocol[32]. Gene specific primers were used, as listed in Table 3.4, which allowed for the co-amplification of *lukS-PV* and lu*kF-PV*. Strain MW2 was used as a positive control. All PCR reactions were set-up as described in section 3.7.1, where a final primer concentration of 0.5µM was used. PCR reactions were performed on an iCycler PCR thermal cycler or a Hybaid cycler.

Table 3.4: Oligonucleotide primer sequences used for the PCR amplification of the *lukS-PV/lukF-PV* genes.

Primer name	Sequence (5'-3')	Amplicon Size (bp)	Reference
luk-PV1	ATCATTAggTAAAATgTCTggACATgATCCA	433	70
luk-PV2	gCATCAASTgTATTggATAgCAAAAgC		70

The cycle conditions used were as follows: initial denaturation at 94˚C for 2min, followed by 30 cycles of 94˚C for 30sec (denaturation), 60˚C for 30sec (annealing) and 72˚C for 1min (extension). 4min at 72˚C was used as the final extension time. The PCR products were subjected to electrophoresis as described in section 3.7.1 in a 1% TAE agarose gel.

PCR amplicons were sequenced as described in section 3.8.2 to determine the specificity of the PCR reaction. The same primers used for PCR amplification were used for the sequencing reactions. All DNA sequences obtained were analysed using the online tool BLAST, available from the NCBI website.

3.8 *spa* Typing

spa typing involves the DNA sequence analysis of the polymorphic X, or short sequence repeat (SSR), region of the protein A (*spaA*) gene. The hyper-variable regions (HVR) of all the MRSA isolates and only the PVL-positive MSSA isolates were amplified by PCR with previously published primer sequences[55] listed in Table 3.5.

Table 3.5: Oligonucleotide primer sequences used for the PCR amplification of the SSR region of the *spaA* gene.

Primer name	Sequence (5'-3')	Reference
1095F	AgACgATCCTTCggTgAgC	32
1517R	gCTTTTgCAATgTCATTTACTg	32

3.8.1 DNA amplification and electrophoresis

Total genomic DNA was extracted as described in section 3.6.1. DNA isolated from STA61 was used as a PCR control strain. All DNA amplifications were the same as those described in section 3.7.1.

The cycle conditions used were as follows: initial denaturation at 95˚C for 4min, followed by 30 cycles of 95˚C for 30sec (denaturation), 60˚C for 30sec (annealing) and 72˚C for 45sec (extension). 10 min at 72˚C was used as the final extension time. The PCR products were subjected to electrophoresis as described in section 3.7.1 in a 1% TAE agarose gel. However, SYBR Green (Molecular Probes, Eugene, OR, USA) (2.5µl/PCR tube (1:100)) was also used. SYBR Green (Molecular Probes) gels was visualised using a DarkReader (Inqaba Biotechnologies, Pretoria, South Africa).

3.8.2 Nucleotide sequencing

The DNA fragments of PCR positive samples were excised and eluted using the QIAquick Gel Extraction Kit (Quigen Inc. Valencia, CA, USA) according to the manufacturers'

recommendations. The eluted products' concentration was estimated using gel electrophoresis with a 1% TAE agarose gel (wt/vol) and 6µl of Promega 100bp ladder as a comparison.

Sequencing reactions were set-up as follows: 100ng of PCR product, 2µl Big Dye Terminator Reaction Mix (Applied Biosystems, Foster City, CA, USA), 1µl 5×sequencing buffer (Applied Biosystems) and 2µl of 5µM forward primer. The reaction was made up to 10µl with distilled H_2O (d.H_2O). The cycle conditions were 96˚C for 1min, followed by 25 cycles of 96˚C for 10sec, 50˚C for 5sec and 60˚C for 4min. The reaction was kept at 4˚C until it was purified using ethanol precipitation.

The reaction was made up to 100µl with d.H_2O, where after 10µl of 3M NaAOc (pH 4.6) (Sigma-Aldrich) and 250µl of 100% ethanol were added. The samples were then centrifuged at 20 800×*g* for 15min. The supernatant was aspirated and 250µl of 70% ethanol was added. The samples were centrifuged at 20 800×*g* for 5min. The supernatant was aspirated and the samples was left to air dry for 15min, following which they were stored at -20˚C until they were analysed with a Genetic Analysis System SCE2410 (SpectruMedix LLC, Pennsylvania, USA). Only the sense strand was sequenced.

3.8.3 Nucleotide sequence analysis

The chromatograph sequence files of the isolates were analysed with the software package Ridom StaphType version 1.4.1 (Ridom GmbH, Würzburg, Germany). After providing the input sequence (FASTA, ABI or SCF format), the software automatically detected the

repeat units and assigned a *spa* type. To ensure that no beginning or end repeat was excluded, the software searched for 5' and 3' signature sequences at the correct distance. With the aid of the software, a database was set-up to allow for easy management of all sequences. After all the sequences had been analysed, a report was created describing each strain and its *spa* ID. This report was viewed in the freely available Adobe Acrobat Reader (www.adobe.com) software, version 7.0. *spa*-Clonal Complexes (*spa*-CC) were also assigned using the Ridom StaphType (Ridom GmbH) software package with the aid of the built-in algorithm BURP (Based Upon Repeat Pattern). BURP is an algorithm that allows clustering of *spa* types into different groups or *spa*-Clonal Complexes (*spa*-CC), where the calculated cost between members of a group was less than or equal to 8. *spa* types shorter than five repeats were excluded from analysis as no reliable deduction about ancestries can be made from these *spa* types.

3.9 Macro-Restriction Analysis (MRA)

In this study, MRA with PFGE was used to obtain a molecular fingerprint, or banding pattern, of digested DNA from each isolate. The CDC ORSA PulseNet protocol was used. The banding pattern was used to establish the relatedness of the isolates. The computer software package GelCompar II version 4.0.0 (BioNumerics, Applied Maths, Sint-Martens-Latem, Belgium) was used to aid in the analysis of the gel images and the construction of UPGMA dendograms.

3.9.1 Agarose plugs preparation

Agarose plugs were prepared for all of the MRSA isolates and only the PVL-positive MSSA isolates. Bacterial isolates were grown overnight in 10ml BHI broth at 37˚C while shaking at 100rpm. The OD_{610} readings were taken and these were used to normalise the volumes of culture used to 1.1. This was done to ensure approximately equal concentrations of cells per plug. The required volume of culture was centrifuged at 20 800×*g* for 4min in 2ml microfuge tubes. After the supernatant was aspirated, the bacterial pellet was resuspended in 300µl Tris-EDTA (TE) buffer and equilibrated at 37˚C for more than 10min. A 1.8% agarose solution (wt/vol) was made in TE buffer, cooled and kept at 55˚C. 3µl lysostaphin, 1mg/ml, (Sigma-Aldrich), together with 300µl 1.8% agarose was added to the bacterial suspension and pipetted into block moulds. The moulds were allowed to set at room temperature for 15min. The blocks were pressed out into 5ml EC lysis buffer and incubated overnight at 37˚C. The EC lysis buffer was replaced with TE buffer (washing buffer) and the tubes were agitated on an orbital shaker for 30min at room temperature. This washing procedure was repeated three times. The blocks were transferred to 2ml TE buffer (storage buffer) and stored at 2-8˚C.

3.9.2 Restriction endonuclease DNA digestion

A 2-3mm slice of an agarose block was cut with a clean scalpel, placed inside a 1.5ml microfuge tube and covered with 200µl 1×Buffer Tango (Fermentas Life Sciences). The plugs were left to equilibrate at room temperature for 30min. The liquid was removed and 200µl enzyme solution was added to cover the block. The enzyme solution contained 20µl

10×Buffer Tango and 30U of *Sma*I restriction endonuclease (Fermentas Life Sciences) and d.H_2O. The plug, completely submerged in restriction enzyme mix was incubated for 3h at 37˚C. Gel electrophoresis was performed immediately after the enzyme digestion.

3.9.3 Pulsed-field gel electrophoresis (PFGE)

A CHEF DR III system (Bio-Rad Laboratories) was used for the electrophoretic separation of the DNA fragments. After the restriction enzyme digestion, the enzyme mixture was aspirated from all tubes. A 1% pulsed-field certified agarose gel (Bio-Rad Laboratories) (wt/vol) with 0.5×Tris-borate-EDTA (TBE) buffer was prepared and left to cool to 50˚C, following which it was poured into the gel casting platform, making sure not to dislodge any of the plug slices. The gel was left to set for 1h and the comb was carefully removed. Two litres of 0.5×TBE was poured into the chamber and allowed to cool to 14ºC an hour before the start of the run. After the comb was removed, the gel was secured in the chamber and the current-switching parameters programmed as specified by the CDC ORSA PulseNet protocol. The parameters were as follows: 200V (6V/cm), 5sec initial switch time, 40sec final switch time, buffer temperature at 14ºC and a total run time of 21h. Following electrophoresis the gel was removed and stained in 500ml d.H_2O containing 50µl ethidium bromide (10mg/ml) for 30min, followed by destaining in d.H_2O for 20min. The gel images were digitally captured using the Bio-Rad Gel Doc 1000 system (Bio-Rad Laboratories).

3.9.4 Analysis of PFGE gel images

All gels were photographed with a Bio-Rad Gel Doc 1000 system (Bio-Rad Laboratories) and saved as TIFF files for analysis with the GelCompar II version 4.0.0 software package (BioNumerics, Applied Maths). The reference standard *S. aureus* NCTC 8325 was included with every gel in the first, middle and last lanes. The Dice coefficient was used to identify percent similarities and clusters were visualised as a dendrogram created by the UPGMA (unweighted-pair group matching analysis) method. The band position tolerance was set at 1.25% and the optimisation at 0.5%. A similarity coefficient of 70%[60] was used to group the strains into clusters.

3.10 Composition of Buffers Used In This Study

Table 3.6: Stock solutions used during this study.

Stock solution	Constituent(s)	Directions
1M Tris-Cl (pH 8.0)	121grams (g) Tris-base d.H_2O	Dissolve Tris and ± 42ml concentrated HCl (required to achieve a pH of 8.0). Add d.H_2O to 1l
0.5M EDTA (pH 8.0)	93.05g EDTA	Dissolve EDTA with ± 10g NaOH in d. H_2O. Use NAOH to adjust pH to 8.0, make up to 500ml with d.H_2O
5M NaCl	292g NaCl	Dissolve NaCl in d.H_2O, make up to 1l
3M NaAOc (pH4.6)	244.8g NaAOc	Dissolve NaAOc in d.H_2O. Adjust pH with glacial acetic acid and make up to 200ml with d.H_2O
TE buffer	10ml 1M Tris-Cl (pH 8.0) 2ml 0.5m EDTA (pH 8.0)	Add and make up to 1l with d.H_2O
50×TAE electrophoresis	96.8g Tris	Add and make up to 400ml

buffer	22.8ml glacial acetic acid 40ml 0.5M EDTA (pH 8.0)	with d.H_2O
10×TBE electrophoresis buffer	43.2g Tris 22g boric acid 16ml 0.5M EDTA(pH 8.0)	Add and make up to 400ml with d.H_2O
5% Brij-58	5g Brij-58	Make up to 100ml with d.H_20. Brij-58 is solid at room temperature, heat to 65˚C
5% Sodium lauroylsarcosine	5g Na-lauroylsarcosine	Dissolve in, and make up to 100ml with d.H_2O
2% Sodium deoxycholate	2g Na-deoxycholate	Dissolve in, and make up to 100ml with d.H_2O
10% SDS	10g SDS	Dissolve at 68ºC. Adjust to pH 7.2 with HCl

Table 3.7: Working solutions used during this study.

Working solution	Constituent(s)	Directions
TE buffer	10ml 1M Tris-Cl (pH 8.0) 2ml 0.5m EDTA (pH 8.0)	Add and make up to 1l with d.H_2O
1×TAE electrophoresis buffer	20ml 50×TAE	Add and make up to 1l with d.H_2O
0.5×TBE electrophoresis buffer	100ml 10×TBE	Add and make up to 2l with d.H_2O
1M HCl	86.2ml concentrated HCl	Make up to 1l with d.H_2O

Table 3.8: Buffers used during CHEF gel electrophoresis experiments.

Buffer	Constituent(s)
EC lysis buffer	6mM Tris-Cl 1M NaCl 100mM EDTA 0.5% Brij-58 0.2% Na deoxycholate 0.5% Na lauroylsarcosine
PFGE wash buffer	10mM Tris-Cl 1mM EDTA (pH 8.0)
PFGE storage buffer	10mM Tris-Cl 1mM EDTA (pH 8.0)
PFGE running buffer	0.5×TBE

Table 3.9: Buffer used during genomic DNA extractions.

Buffer	Constituent(s)
Proteinase K buffer	0.1M Tris (pH 7.5)

CHAPTER 4

4 RESULTS

4.1 Sample Collection

A summary of all isolate specific information, such as collection date, source and molecular characteristics is listed in Appendix D (p. 125). Table 4.1 contains all the relevant information pertaining to the number of samples collected per province. A total of 432 putative *S. aureus* isolates were collected. Only the results for the MRSA isolates (n=337) (positive by the DNase test and resistant to methicillin) and the PVL-positive MSSA (n=12) isolates are provided.

4.2 Patient Demographics

Table 4.2 contains all the patient demographics collected. Only MRSA isolates and PVL-positive MSSA isolates are included. No clinical information was available for three isolates from Mpumalanga and two from the North West Province.

Table 4.1: Number of isolates collected, displayed as number of MRSA, MSSA and DNase-negative (misidentified) isolates per province.

Province	Number isolates collected			
	MRSA	**MSSA**	**DNase (-)**	**Total**
Gauteng	53	3	3	59
Limpopo	29	5	5	39
Mpumalanga	29	7	12	48
Northern Cape	29	1	9	39
Free State	31	8	6	45
North West	21	1	6	28
Eastern Cape	47	6	4	57
Western Cape	55	2	0	57
KwaZulu-Natal	43	9	8	60
Total	**337**	**42**	**53**	**432**

Table 4.2: Distribution by province of the MRSA and PVL-positive MSSA isolates together with patient gender and mean patient age.

Province	Isolates used			Patient					
				Mean age		Gender			
	MRSA	**PVL (+) MSSA**	***N***	**Yrs**	**NA**	**# (%) Male**	**# (%) Female**	**NA**	***N***
Gauteng	53	1	54	41.2	-	27 (50)	27 (50)	-	54
Limpopo	29	-	29	33.3	7	15 (51.7)	13 (44.8)	1 (3.4)	29
Mpumalanga	29	4	33	28.5	5	19 (57.6)	11 (33.3)	3 (9)	33
Northern Cape	29	-	29	35.3	4	22 (75.9)	7 (24.1)	-	29
Free State	31	2	33	44	2	25 (78.1)	7 (21.9)	-	32
North West	21	-	21	23.4	6	7 (33.3)	7 (33.3)	7 (33.3)	21
Eastern Cape	47	1	48	38.4	2	23 (47.9)	23 (47.9)	2 (4.2)	48
Western Cape	55	-	55	43.7	-	38 (69.1)	17 (30.9)	-	55
KwaZulu-Natal	43	4	47	45.8	-	28 (59.6)	18 (38.3)	1 (2.1)	47
Total	**337**	**12**	**349**	**37.1**	**26**	**204 (58.5)**	**130 (37.2)**	**15 (4.3)**	**349**

NA = Not available; *N* = Total; Yrs = Years.

204/349 (58.5%) *S. aureus* isolates were collected from males, while 130/349 (37.2%) isolates were collected from females. The remaining fifteen isolates were collected from patients whose gender was not recorded. The mean patient age was 37.1 years.

The clinical origins of the isolates are shown in Table 4.3. One-hundred-and thirty-four (38.4%) of the isolates collected were obtained from blood culture, which was the largest source for this study. The second largest source was pus swabs, from which 74 (21.2%) isolates were collected. The third largest source was pus, from which 36 (10.3%) isolates were collected. Fifteen isolates were collected from swabs (4.3%), fourteen from tissue (4%) and nine from sputum (2.6%). For the remaining isolates, four (1.1%) each were collected from wound swabs and fluid; three (0.9%) each were collected from cerebrospinal fluid (CSF) and catheter tips; and two (0.6%) each were collected from urine, tracheal aspirate, leg and ulcer. One (0.3%) isolate each was collected from the following sites: abdominal cotton swab, drain fluid, pleural pus, wound abscess, septic back, drainage site, empyema fluid, biopsy, abdomen, bile, nose and ear.

4.3 Phenotypic Characterisation

It was observed that most of the *S. aureus* isolates cultured usually had large (2-4 mm in diameter) colonies that were smooth and slightly raised. The colonies were also pigmented, ranging from cream-yellow to orange.

Table 4.3: Clinical sources of all MRSA and PVL-positive MSSA isolates collected, showing distribution by province and source.

Source	Province									
	Gauteng	**Limpopo**	**Mpumalanga**	**Northern Cape**	**Free State**	**North West**	**Eastern Cape**	**Western Cape**	**KwaZulu-Natal**	**Total (%)**
Blood	38	5	13	1	6	10	24	23	14	**134 (38.4)**
Pus swab	-	14	3	12	3	2	12	23	5	**74 (21.2)**
Pus	-	1	6	11	3	-	1	1	13	**36 (10.3)**
Swab	-	-	-	4	2	-	2	-	7	**15 (4.3)**
Tissue	6	-	-	-	7	-	-	-	1	**14 (4)**
Sputum	1	2	2	-	2	-	2	-	-	**9 (2.6)**
Wound swab	2	-	-	-	1	-	-	-	1	**4 (1.1)**
Fluid	-	1	2	-	1	-	-	-	-	**4 (1.1)**
Catheter tip	-	-	-	-	2	-	1	-	-	**3 (0.9)**
CSF	1	-	1	-	-	-	-	1	-	**3 (0.9)**
Abdominal swab	1	-	-	-	-	-	-	-		**2 (0.6)**
Drain fluid	1	-	-	-	-	-	-	-	-	**2 (0.6)**
Urine	-	1	-	-	-	1	-	-	-	**2 (0.6)**
Tracheal aspirate	-	-	-	1	1	-	-	-	-	**2 (0.6)**
Pleural pus	-	-	-	-	1	-	-	-	-	**2 (0.6)**
Leg	-	-	-	-	-	-	2	-	-	**2 (0.6)**
Wound abscess	-	-	-	-	-	-	1	-	-	**2 (0.6)**
Ulcer	-	-	-	-	-	-	1	-	1	**2 (0.6)**
Septic back	-	-	-	-	-	-	1	-	-	**1 (0.3)**
Drainage site	-	-	-	-	-	-	1	-	-	**1 (0.3)**
Empyema fluid	-	-	-	-	-	-	-	1	-	**1 (0.3)**
Biopsy	-	-	-	-	-	-	-	-	1	**1 (0.3)**
Abdomen	-	-	-	-	-	-	-	-	1	**1 (0.3)**
Bile	-	-	-	-	-	-	-	-	1	**1 (0.3)**
Ear	-	-	-	-	-	-	-	-	1	**1 (0.3)**
Nose	-	-	-	-	-	-	-	-	1	**1 (0.3)**
Not specified	4	6	3	-	3	6	-	6	-	**27 (7.7)**
Not available	-	-	3	-	-	2	-	-	-	**5 (1.4)**
Total	**54**	**29**	**33**	**29**	**32**	**21**	**48**	**55**	**47**	**349**

CSF = Cerebrospinal fluid.

4.3.1 DNase test

The DNase test was performed with every isolate as described in section 3.4.1. Of the 432 isolates collected, 379 had presumptive DNase-positive results, confirming the isolates to be *S. aureus*. Refer to Appendix D (p. 125) for isolate specific information.

4.4 Antibiotic Susceptibility Testing

4.4.1 Kirby-Bauer disk diffusion antibiotic susceptibility method

Susceptibility to methicillin was determined using the Kirby-Bauer disk diffusion antibiotic susceptibility method as described in section 3.4. A table regarding the zone diameter interpretive standards for *Staphylococcus* spp. can be found in Appendix C (p. 124). Antibiotic susceptibility testing was performed on all 379 DNase-positive *S. aureus* isolates. Of the 379 *S. aureus* isolates tested, 337 isolates were identified as MRSA using this technique (Table 4.1). Refer to Appendix D (p. 125) for isolate specific information, where zone diameters are only indicated if any were observed.

4.5 SCC*mec* Typing

The distribution of the different SCC*mec* types is shown in Table 4.4 and is broken down per province as well as per SCC*mec* type and variant(s) identified.

Table 4.4: Distribution by province of the SCC*mec* elements present in *S. aureus* isolates from South Africa.

Province	**SCC*mec* type I**			**SCC*mec* type II**		**SCC*mec* type III**		**SCC*mec* type IV**		**NT**	**Total**
	I	**Iv**	**I-*pls***	**II**	**IIB**	**III**	**IIIE**	**IV**	**IV-*dcs***		
Gauteng	-	1	6	14	7	-	24	-	1	-	**53**
Limpopo	4	-	16	2	-	-	6	1	-	-	**29**
Mpumalanga	3	1	16	2	-	1	6	-	-	-	**29**
Northern Cape	-	-	15	-	-	-	13	-	-	-	**29**
Free State	1	-	14	4	1	-	11	-	-	1	**31**
North West	4	6	5	-	-	-	6	-	-	-	**21**
Eastern Cape	4	6	15	15	6	-	-	1	-	-	**47**
Western Cape	4	-	17	21	11	-	-	2	-	-	**55**
KwaZulu Natal	1	1	16	8	-	1	13	3	-	-	**43**
Sub Total	**21**	**15**	**120**	**66**	**25**	**2**	**79**	**7**	**1**	**1**	**337**
Total (%)	**156 (46.3)**			**91 (27)**		**81 (24)**		**8 (2.4)**		**1 (0.3)**	**337**

pls = Plasmin-sensitive protein; *dcs* = Downstream coding sequence.

4.5.1 SCC*mec* type elements identified in South Africa

The SCC*mec* types of 336 (99.7%) isolates were determined. SCC*mec* type I, associated with the Archaic/Iberian clone was the dominant SCC*mec* type in the country, accounting for 46.2% (n=156) of the isolates. SCC*mec* type II comprised the second largest group of isolates, accounting for 27 % (n=91) of the isolates. This SCC*mec* type is associated with the New York/Japan clone. The third largest group, which contained 24% (n=81) of the isolates, harboured SCC*mec* type III, and this SCC*mec* type is associated with the Brazilian/Hungarian clone. The smallest group was SCC*mec* type IV, which was identified in 2.4% (n=8) isolates. This SCC*mec* type is associated with the Paediatric and community-associated MRSA clones. Fifteen isolates were identified as SCC*mec* type I variant (SCC*mec* Iv). This SCC*mec* type has the same sized PCR amplicons as SCC*mec* type I, except that amplicons for loci E and F are also present. It was designated SCC*mec* type I due to the presence of the amplicon for locus A. Refer to Appendix E (p. 145) for all SCC*mec* PCR gel images.

These results indicate the 97% (n=328) of the isolates collected and screened by SCC*mec* typing are HA-CMRSA strains, and that only 3% (n=8) are CA-MRSA strains.

4.5.2 Distribution of SCC*mec* type elements

The SCC*mec* types of 53 isolates from Gauteng were determined. Twenty-four isolates (45.3%) were SCC*mec* type IIIE. Twenty-one isolates (39.6%) were SCC*mec* type II or a variant thereof. Six isolates (11.3%) were SCC*mec* type I-*pls* and one isolate (1.8%) was

SCC*mec* type IV-*dcs*. For Limpopo province, the SCC*mec* types of 29 isolates were determined. Twenty isolates (69%) were SCC*mec* type I or a variant thereof. SCC*mec* type I-*pls* was the dominant SCC*mec* type in this province (n=16; 55.2%). Six isolates (20.7%) were SCC*mec* type IIIE; two isolates (6.9%) were SCC*mec* type II and one isolate (3.4%) were SCC*mec* type IV. RSA 2/03, the only PVL-positive MRSA isolate identified, carried a SCC*mec* type IV element. Twenty-nine isolates were collected from Mpumalanga province and all were screened to identify their SCC*mec* type element. Twenty isolates (69%) were SCC*mec* type I or a variant thereof. SCC*mec* type I-*pls* was the dominant SCC*mec* type in this province (n=16; 55.2%). Seven isolates (24.1%) were SCC*mec* type III or a variant thereof, and two isolates (6.8%) were SCC*mec* type II.

The SCC*mec* types of 28 (96.6%) isolates from the Northern-Cape province were determined. Fifteen isolates (51.7%) were SCC*mec* type I-*pls*, which was the dominant SCC*mec* type in this province. Thirteen isolates (44.8%) were SCC*mec* type IIIE and one isolate (0.3%) was not-typeable. The SCC*mec* type elements of 31 isolates from the Free-State province were identified. Fifteen isolates (51.7%) were SCC*mec* type I or a variant thereof. The dominant SCC*mec* type, SCC*mec* type I-*pls*, was identified in 45.2% (n=14) of the isolates. Eleven isolates (37.9%) were SCC*mec* type IIIE and five isolates (17.2%) were SCC*mec* type II or a variant thereof. For the North-West province, the SCC*mec* types of 21 isolates were identified. Fifteen isolates (71.4%) were SCC*mec* type I or a variant thereof, and six isolates (28.6%) were SCC*mec* type IIIE.

For the Eastern Cape, the SCC*mec* types of 47 isolates were determined. Twenty-five isolates (53.2%) were SCC*mec* type I or a variant thereof, twenty-one (44.7%) isolates were SCC*mec* II or a variant thereof and one (2.1%) isolate was SCC*mec* type IV. The

SCC*mec* types of 55 isolates were identified for the Western Cape province. Thirty-two isolates (58.2%) were SCC*mec* type II or a variant thereof and twenty-one isolates (38.2%) were SCC*mec* type I or a variant thereof. Two isolates (3.6%) were SCC*mec* type IV. The SCC*mec* types of 43 isolates from the KwaZulu-Natal province were determined. Eighteen isolates (41.9%) were SCC*mec* type I or a variant thereof. Fourteen isolates (32.6%) were SCC*mec* type III or a variant thereof; eight isolates (18.6%) were SCC*mec* type II or a variant thereof and three isolates (7%) were SCC*mec* type IV.

4.6 Prevalence of Panton-Valentine leukocidin (PVL)

4.6.1 PCR detection of *lukS*-PV/*lukF*-PV genes

PVL was detected in one MRSA strain by PCR, namely strain RSA 2/30 (0.3%) which carried a SCC*mec* type IV. PVL was also detected in twelve MSSA isolates. Table 4.5 contains all the relevant information for the PVL-positive isolates, including *spa* typing and PFGE results. Refer to Appendix F (p. 152) for all the gel images obtained after PVL PCR experiments.

4.6.2 DNA nucleotide sequencing of lukS-PV/lukF-PV PCR amplicons

PCR amplicons were subjected to DNA nucleotide sequencing to determine the specificity of the PCR reaction. DNA nucleotide sequences were identified as *lukS*-PV/*lukF*-PV using the BLAST function available on the NCBI website.

Table 4.5: Strain specific information of all the PVL-positive isolates. The geographical distribution and all molecular characterisation results are shown.

Isolate name	Province	SCC*mec* type	*spa*			PFGE	
			Repeat pattern	Type	BURP	Pattern	Clonal type
RSA 2/30	Limpopo	IV	TJEJNCMOMOR	t891	*spa*-CC 032	19.4	RSA19
RSA 1/08	Gauteng	MSSA	I2FKBPE	t174	No founder (CC#6)	14.1	RSA14
RSA 3/44	Mpumalanga	MSSA	I2FKBPE	t174	No founder (CC#6)	14.2	RSA14
RSA 3/49	Mpumalanga	MSSA	XMJH2JH2M	t317	Singleton	21.1	RSA21
RSA 3/50	Mpumalanga	MSSA	XMJH2JH2M	t317	Singleton	21.2	RSA21
RSA 3/53P	Mpumalanga	MSSA	UFMEEBBPB	t1951	Singleton	11.4	RSA11
RSA 5/32	Free State	MSSA	XMJH2JH2M	t317	Singleton	21.3	RSA21
RSA 5/33	Free State	MSSA	WGKKAKAOMQ	t318	*spa*-CC 012	18.1	RSA18
RSA 7/31	Eastern Cape	MSSA	TKJNCMOMOR	t1972	*spa*-CC 032	19.5	RSA19
RSA 9/18	KwaZulu-Natal	MSSA	WGKAKAOMQ	t021	*spa*-CC 012	16.31	RSA16
RSA 9/27	KwaZulu-Natal	MSSA	WGKAKAOMQ	t021	*spa*-CC 012	16.31	RSA16
RSA 9/29	KwaZulu-Natal	MSSA	WGKAKAOMQ	t021	*spa*-CC 012	NT	NT
RSA 9/58	KwaZulu-Natal	MSSA	I2FKBPE	t174	No founder (CC#6)	NT	NT

NT = Not typeable; CC = Clonal Complex; MSSA = Methicillin-susceptible *S. aureus.*

4.7 *spa* Typing

The SSR region of the *spaA* gene of all the MRSA isolates (n=337) and the PVL-positive MSSA isolates (n=12) (*N*=349) was amplified. Refer to Appendix G (p. 157) for all gel images obtained after agarose gel electrophoresis of the PCR amplified SSR region of the *spaA* gene of each isolate. Refer to Appendix J (p. 174) for all the various *spa* type repeat motif alignments and to Appendix K (p. 177) for all the *spa* repeat unit alignments.

The DNA nucleotide sequences of all the MRSA (n=337) and the PVL-positive MSSA (n=12) (*N*=349) isolates could be determined. After analysis of the DNA nucleotide sequences with the computer software package Ridom StaphType version 1.4.1 (Ridom GmbH, Würzburg, Germany), the isolates were assigned to thirty-seven different *spa* types, of which *spa* types t032 and t1468 were the longest (16 repeats) and *spa* type t535 was the shortest (3 repeats). Fourteen *spa* types were identified as novel *spa* types (indicated with an asterisk in Table 4.6) after synchronisation with the Ridom *spa* Server (Ridom GmbH). The *spa* type nomenclature was the same as that used in the Ridom StaphType software (Ridom GmbH) and the Ridom *spa* Server (Ridom GmbH). See Table 4.6 for all *spa* types identified, together with their repeat patterns and the number of isolates identified per *spa* type. Refer to Figure 4.1 for a summary UPGMA dendogram constructed using *spa* typing results. Only one representative strain per *spa* type was used.

One of the major *spa* types in the country was *spa* type t037, with repeat pattern 15-12-16-02-16-02-25-17-24-24. Refer to Appendix H (p. 163) for the DNA nucleotide sequences of each repeat. This *spa* type was identified in 79 (22.6%) isolates originating from all

QWprovinces except for the Western and Eastern Cape. This *spa* type is associated with the Brazilian/Hungarian epidemic clone[9,43] and strain ANS46 carries this *spa* type[43].

Another major *spa* type was t012, with a repeat pattern 15-12-16-02-16-02-25-17-24-24. This *spa* type was identified in 73 isolates (20.9%) collected from six provinces, excluding Mpumalanga, Northern Cape and North West Province. This *spa* type is a single locus variant (SLV) of strain EMRSA-16[9,48] which carries *spa* type t018 (15-12-16-02-16-02-25-17-24-24-24).

The third most prevalent *spa* type was t064 (11-19-12-05-17-34-24-34-22-25). This *spa* type was identified in 71 isolates (20.3%) collected from all provinces. Controls ATCC BAA-38 (E2125) which represents the Archaic clone, and HPV107 which represents the Iberian clone are both *spa* type t051 (11-19-21-12-21-17-34-24-34-22-25)[16.24,43,48]. *spa* type t064 is a double locus variant (DLV) of *spa* type t051.

Table 4.6: Classification of South African MRSA isolates by *spa* typing. Each *spa* type together with its repeat pattern and the number of isolates identified for each *spa* type and distribution by province are shown.

***spa* type**	**Nomenclature**		**Isolates**		**Province(s) detected in**
	RIDOM	**Kreiswirth ID**	**#**	**%**	
t001	26-30-17-34-17-20-17-12-17-16	TO2MBMDMGMK	6	1.7	EC
t002	26-23-17-34-17-20-17-12-17-16	TJMBMDMGMK	-	-	-
t008	11-19-12-21-17-34-24-34-22-25	YHGFMBQBLO	1	0.3	GP
t012	15-12-16-02-16-02-25-17-24-24	WGKAKAOMQQ	73	20.9	GP, LP, FS, EC, WC, KZN
t015	08-16-02-16-34-13-17-34-16-34	XKAKBEMBKB	1	0.3	GP
t018	15-12-16-02-16-02-25-17-24-24-24	WGKAKAOMQQQ	7	2.8	FS, EC, WC
t020	26-23-31-29-17-31-29-17-25-17-28-16-28	TJNF2MNF2MOMOKR	1	0.3	EC
t021	15-12-16-02-16-02-25-17-24	WGKAKAOMQ	7	2	GP, EC, WC, KZN
t032	26-23-23-13-23-31-29-17-31-29-17-25-17-25-16-28	TJJEJNF2MNF2MOMOKR	4	1.1	WC, KZN
t037	15-12-16-02-25-17-24	WGKAOMQ	79	22.6	GP, LP, MP, NC, FS, NWP, KZN
t045	26-17-20-17-12-17-16	TMDMGMK	24	6.9	LP, MP, WC, NWP, EC
t046	08-16-02-16-02-25-17-24-24-24	XKAKAOMQQQ	4	1.1	KZN
t051	11-19-21-12-21-17-34-24-34-22-25	YHFGFMBQBLO	-	-	-
t064	11-19-12-05-17-34-24-34-22-25	YHGCMBQBLO	71	20.3	ALL
t104	11-10-34-22-25	YC2BLO	2	0.6	NC
t128	07-23-23-21-16-34-33-13	UJJFKBPE	-	-	-
t174	14-21-16-34-33-136	I2FKBPE	3	0.9	GP, MP, KZN
t317	08-17-23-18-23-18-17	XMJH2JH2M	3	0.9	MP, FS
t318	15-12-16-16-02-16-02-25-17-24	WGKKAKAOMQ	1	0.3	FS
t399	15-12-16-02-17-24-24	WGKAMQQ	2	0.6	MP
t465	08-23-16-34-13-17-34-16-34	XJKBEMBKB	1	0.3	GP
t535	26-17-16	TMK	1	0.3	MP
t840	15-12-02-16-02-25-17-24-24	WGAKAOMQQ	1	0.3	EC
t891	26-23-13-23-31-05-17-25-17-25-28	TJEJNCMOMOR	1	0.3	LP

t951	11-10-05-17-34-24-34-22-25	YC2CMBQBLO	4	1.1	GP, FS, KZN
t1154	26-20-17-12-17-16	TDMGMK	4	1.1	EC
t1256*	11-19-12-05-17-34-24-34-22-65	YHGCMBQBLS3	1	0.3	EC
t1257*	11-19-34-05-17-34-24-34-22-25	YHBCMBQBLO	31	8.9	GP, LP, MP, NC, WC, KZN
t1443*	11-19-12-05-17-34-24-24-34-22-25	YHGCMBQQBLO	3	0.9	LP, WC
t1468*	26-23-13-23-31-31-29-17-31-29-17-25-17-25-16-28	TJEJNNF2MNF2MOMOKR	1	0.3	WC
t1504*	15-12-16-16-02-25-17-24	WGKKAOMQ	1	0.3	KZN
t1555*	11-19-34-05-17-34-24-34-24-34-22-25	YHBCMBQBQBLO	1	0.3	MP
t1774*	11-19-19-12-05-17-34-24-34-22-25	YHHGCMBQBLO	1	0.3	FS
t1779*	11-19-12-12-05-17-34-24-34-22-25	YHGGCMBQBLO	2	0.6	WC
t1880*	26-13-20-17-12-12-17-16	TEDMGGMK	2	0.6	KZN
t1930*	11-19-12-05-17-34-24-34-22-33-25	YHGCMBQBLPO	1	0.3	LP
t1951*	07-21-17-13-13-34-34-33-34	UFMEEBBPB	1	0.3	MP
t1952*	11-19-12-05-17-34-24-34-16	YHGCMBQBK	1	0.3	FS
t1971*	11-34-05-17-34-24-34-22-25	YBCMBQBLO	1	0.3	KZN
t1972*	26-16-23-31-05-17-25-17-25-28	TKJNCMOMOR	1	0.3	EC

= Number; % = Percentage; * = Novel *spa* type; GP = Gauteng; MP = Mpumalanga; LP = Limpopo; NC = Northern Cape; FS = Free State; NWP = North West; EC = Eastern Cape; WC = Western Cape; KZN = KwaZulu-Natal.

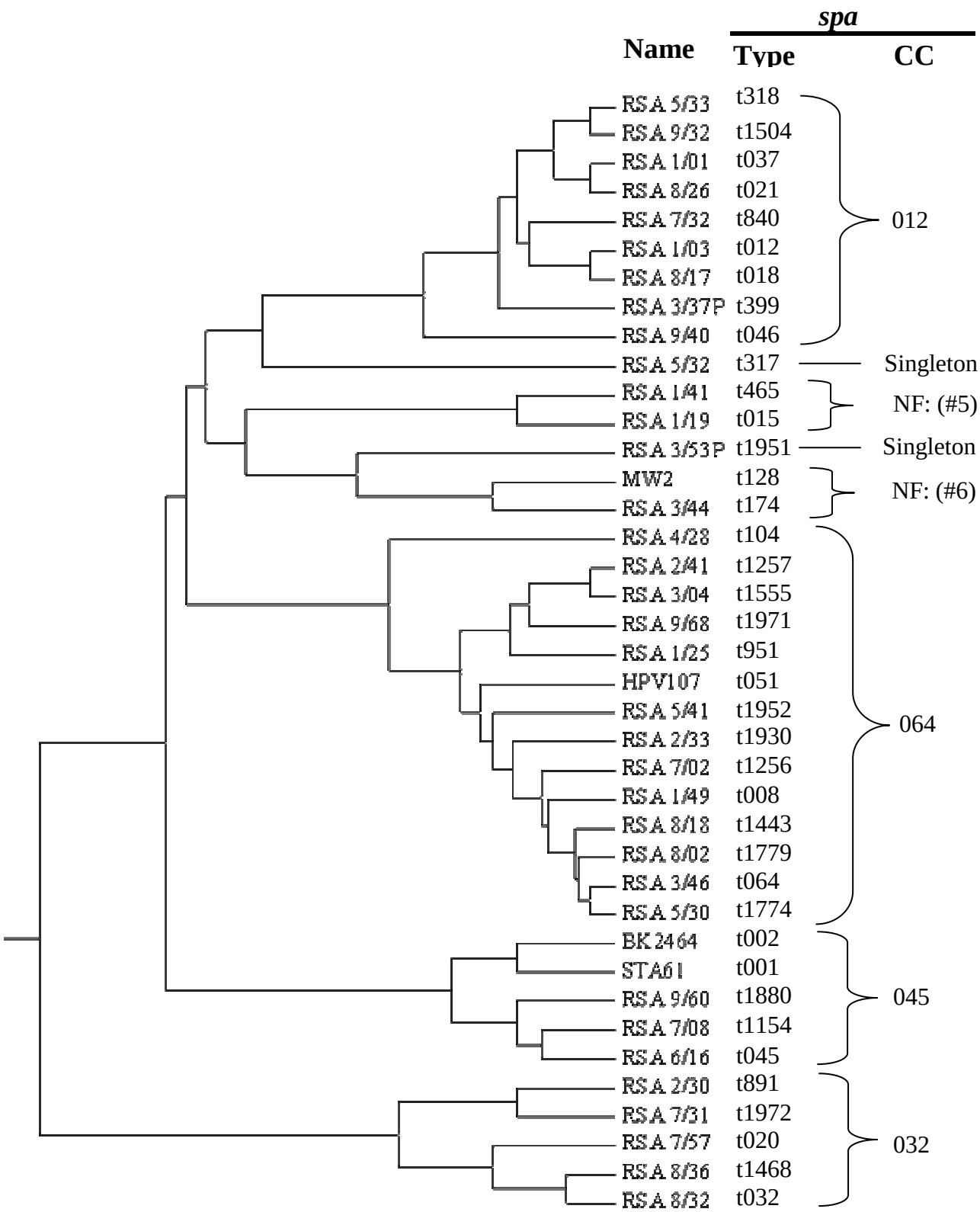


Figure 4.1: UPGMA dendogram of South African MRSA isolates as determined by *spa* typing. Only one representative strain is shown per *spa* type.

4.7.1 BURP cluster analysis

With the aid of the algorithm BURP (implemented in the Ridom StaphType software), *spa* types (including the control strains STA61, HPV107, ATCC BAA-38, BK2464, ANS46 and MW2) were clustered into six different clusters and two so-called singletons. Clustering parameters excluded *spa* types shorter than five repeats, since no reliable deductions regarding ancestries can be made due to the insufficient length of the repeat pattern. One *spa* type, t535, was excluded from BURP clustering (Table 4.7). The control strains were not included in any of the calculations, although they are present in the UPGMA dendograms and the BURP clustering analysis: ANS46 is *spa* type t037; STA61 is *spa* type t001; both ATCC BAA-38/E2125 and HPV107 are *spa* type t051; BK2464 is *spa* type t002; and MW2 is *spa* type t128.

Table 4.7: BURP cluster analysis of the *spa* types identified from South African MRSA isolates.

Cluster #	*spa* types	# isolates	*spa*-CC	# isolates (%)	# *spa* types (%)	Province(s) detected in
1	012	73	012	175 (50.1)	9 (24.3)	All
	018	7				
	021	7				
	037	79 (80)				
	046	4				
	318	1				
	399	2				
	840	1				
	1504	1				
2	008	1	064	120 (34.4)	13 (35.1)	All
	051	0 (2)				
	064	71				
	104	2				
	951	4				
	1256	1				

	1257	31				
	1443	3				
	1555	1				
	1774	1				
	1779	2				
	1930	1				
	1952	1				
	1971	1				
3	020	1	032	8 (2.3)	5 (13.5)	EC, WC, KZN
	032	4				
	891	1				
	1468	1				
	1972	1				
4	001	6 (7)	045	36 (10.3)	4 (10.8)	EC, WC, MP, NWP, KZN
	002	0 (1)				
	045	24				
	1154	4				
	1880	2				
5	015	1	No founder	2 (0.6)	2 (5.4)	GP
	465	1				
6	128	0 (1)	No founder	3 (0.9)	1 (2.7)	GP, MP, KZN
	174	3				
Singletons	317	3	Singletons	4 (1.1)	2 (5.4)	MP, FS
	1951	1				
Excluded	535	1	Excluded	1 (0.3)	1 (2.7)	MP

= Number; % = Percentage; CC = Clonal complex; GP = Gauteng; MP = Mpumalanga; LP = Limpopo; NC = Northern Cape; FS = Free State; NWP = North West; EC = Eastern Cape; WC = Western Cape; KZN = KwaZulu-Natal.

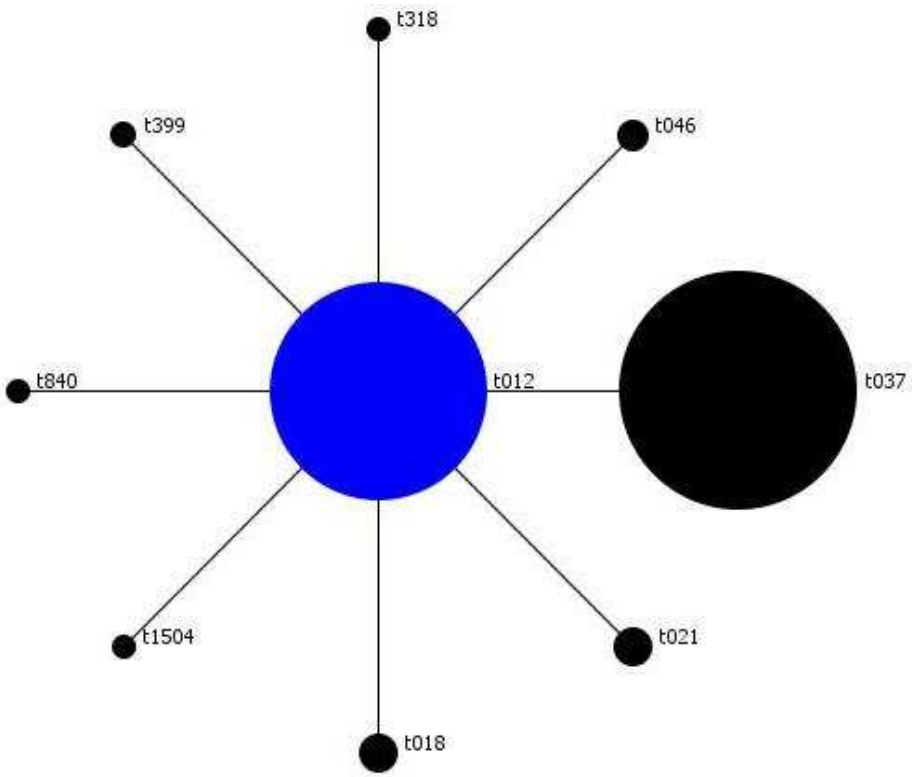


Figure 4.2: Graphical representation of the relatedness of the *spa* types grouped into *spa*-Clonal Complex 012.

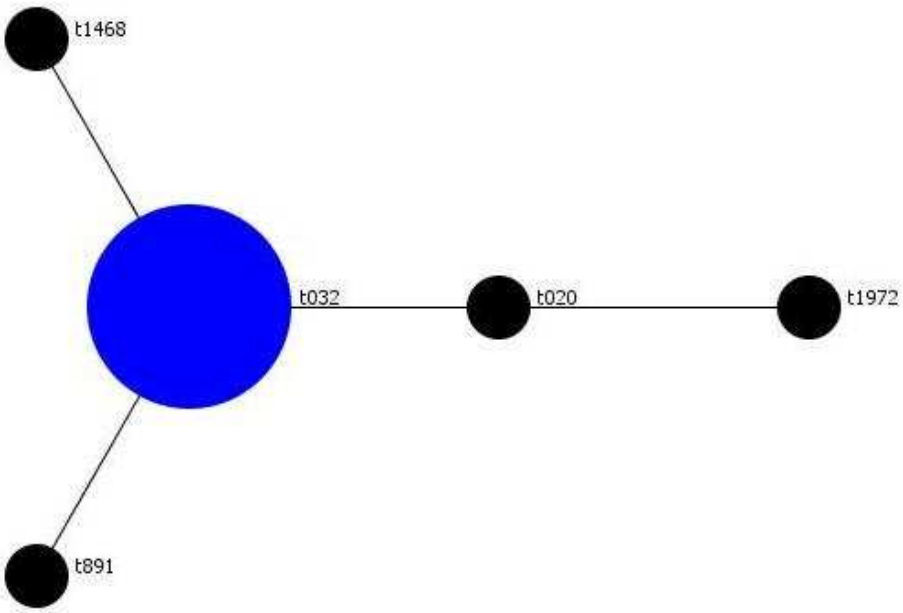


Figure 4.3: Graphical representation of the relatedness of the *spa* types grouped into *spa*-Clonal Complex 032.

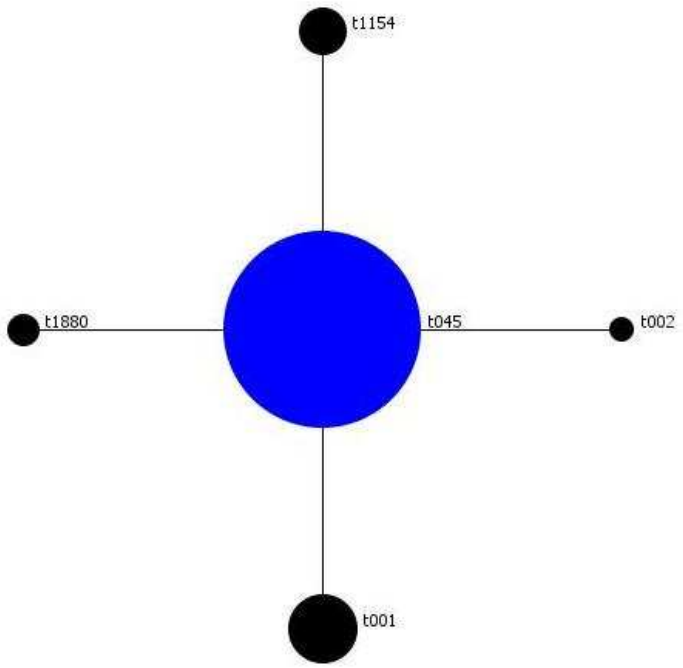


Figure 4.4: Graphical representation of the relatedness of the *spa* types grouped into *spa*-Clonal Complex 045.

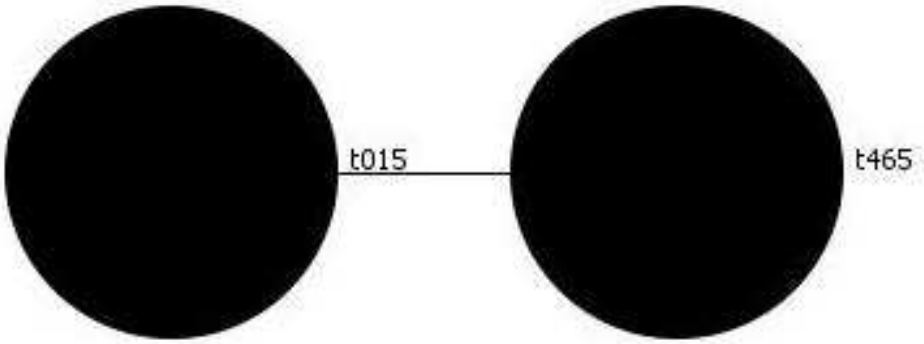


Figure 4.5: Graphical representation of the relatedness of the *spa* types grouped into cluster #5: No founder.

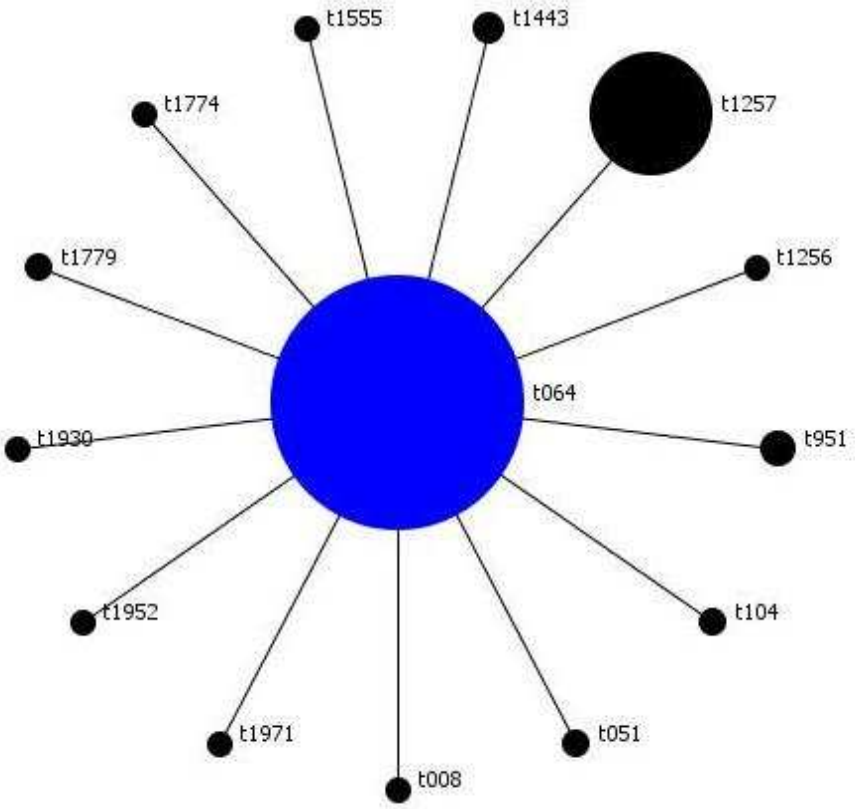


Figure 4.6: Graphical representation of the relatedness of the *spa* types grouped into *spa*-Clonal Complex 064.

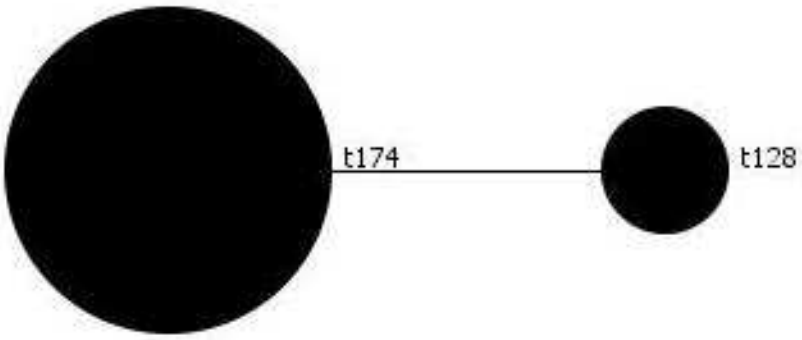


Figure 4.7: Graphical representation of the relatedness of the *spa* types grouped into cluster #6: No founder.

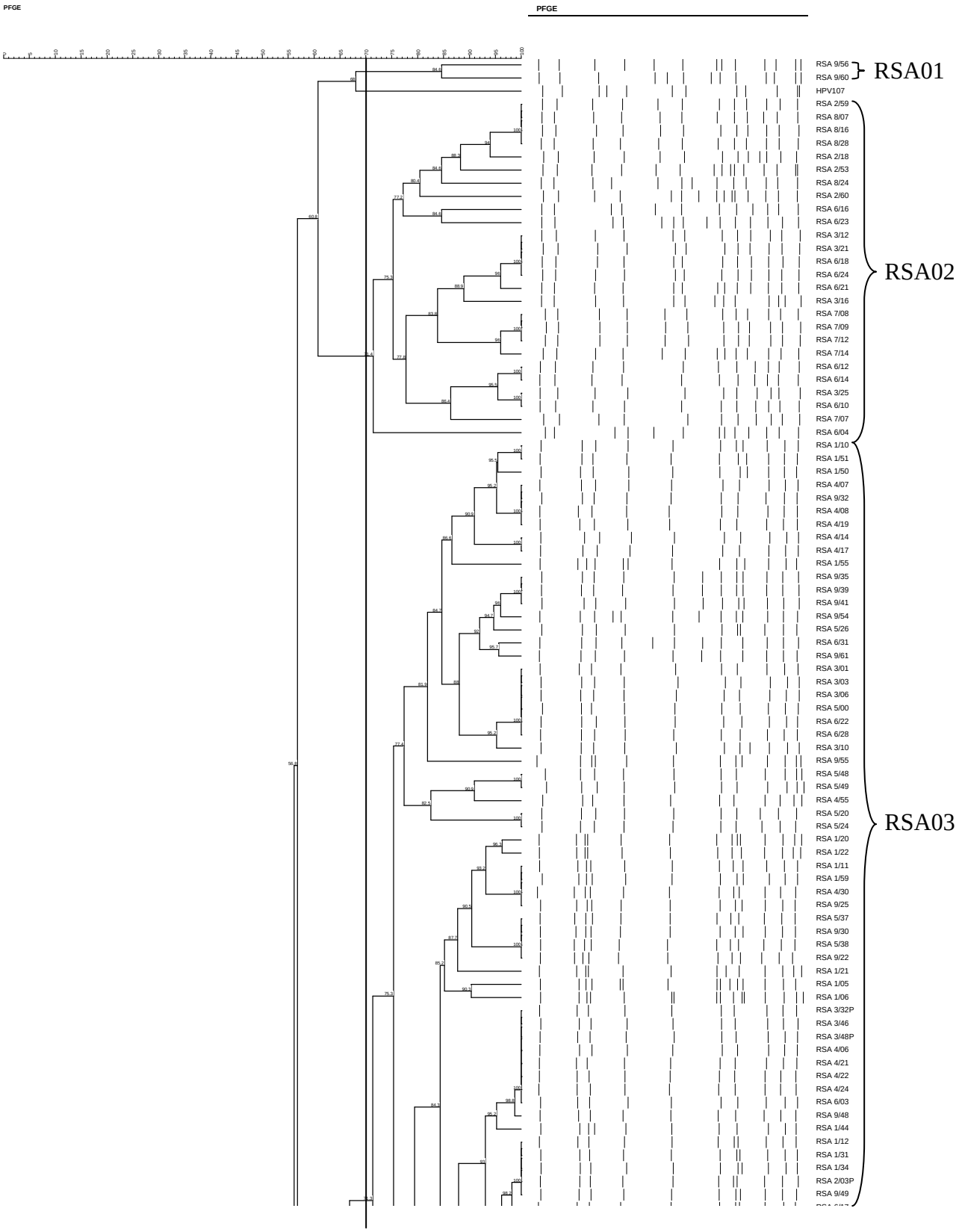
4.8 Macro-Restriction Analysis with Pulsed-Field Gel Electrophoresis

Three hundred and thirty-seven isolates (97%) were typeable by *Sma*I macro-restriction analysis and produced two-hundred-and-five different banding patterns. Employing a cut-off similarity value of 70% in subsequent cluster analysis, the isolates were assigned to twenty-four different PFGE clonal types (CT), designated RSA01-RSA24. Three of the CTs were major lineages, as they each contained more than 20% of the isolates. One CT contained twenty-six isolates; seven CTs contained a single isolate and another seven contained two isolates; three CTs contained five isolates; one CT contained three isolates and another CT contained four isolates. Refer to Figure 4.8 for a complete UPGMA dendogram constructed from PFGE typing results and to Table 4.8 for CT specific information. Refer to Appendix I (p. 167) for all PFGE gel images.

Table 4.8: Classification of South African MRSA isolates by PFGE. The distribution of each PFGE clonal type (CT) by province and the number of isolates identified or each clonal type are shown.

PFGE CT	Isolates		Province(s) detected in
	#	%	
RSA05	98	28.1	All
RSA16	89	25.5	GP, LP, MP, FS, EC, WC, KZN
RSA03	74	21.2	GP, LP, MP, NC, FS, NWP, EC, KZN
RSA02	26	7.4	LP, MP, NWP, EC, WC
RSA19	7	2	LP, EC, WC, KZN
RSA06	5	1.4	FS
RSA08	5	1.4	FS, NWP, KZN
RSA12	5	1.4	EC
RSA11	4	1.1	GP, LP, MP, NC
RSA21	3	0.9	MP, FS
RSA01	2	0.6	KZN
RSA04	2	0.6	GP, KZN
RSA09	2	0.6	EC
RSA10	2	0.6	MP, FS
RSA14	2	0.6	GP, MP
RSA18	2	0.6	FS, WC
RSA22	2	0.6	GP
RSA07	1	0.3	LP
RSA13	1	0.3	GP
RSA15	1	0.3	EC
RSA17	1	0.3	GP
RSA20	1	0.3	KZN
RSA23	1	0.3	FS
RSA24	1	0.3	EC
NT	12	3.4	-

NT = Not typeable; # = Number; % = Percentage; CT = Clonal type; GP = Gauteng; MP = Mpumalanga; LP = Limpopo; NC = Northern Cape; FS = Free State; NWP = North West; EC = Eastern Cape; WC = Western Cape; KZN = KwaZulu-Natal.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PFGE
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RSA 9/60
HPV107
RSA 2/59
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RSA 8/16
RSA 8/28
RSA 2/18
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RSA 2/60
RSA 6/16
RSA 6/23
RSA 3/12
RSA 3/21
RSA 6/18
RSA 6/24
RSA 6/21
RSA 3/16
RSA 7/08
RSA 7/09
RSA 7/12
RSA 7/14
RSA 6/12
RSA 6/14
RSA 3/25
RSA 6/10
RSA 7/07
RSA 6/04
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RSA01
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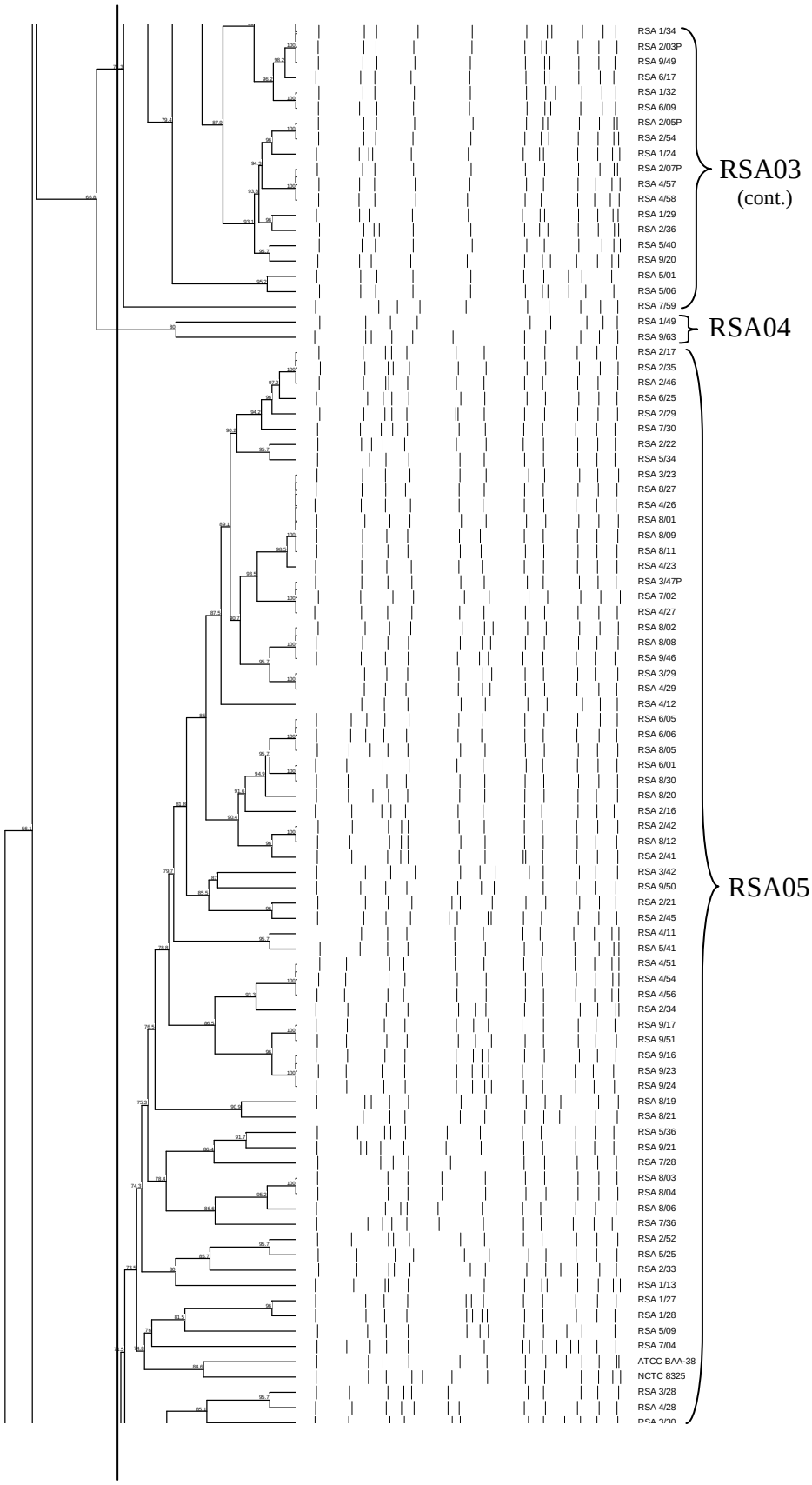
RSA03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RSA 8/20
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RSA 8/04
RSA 8/06
RSA 7/36
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RSA 5/25
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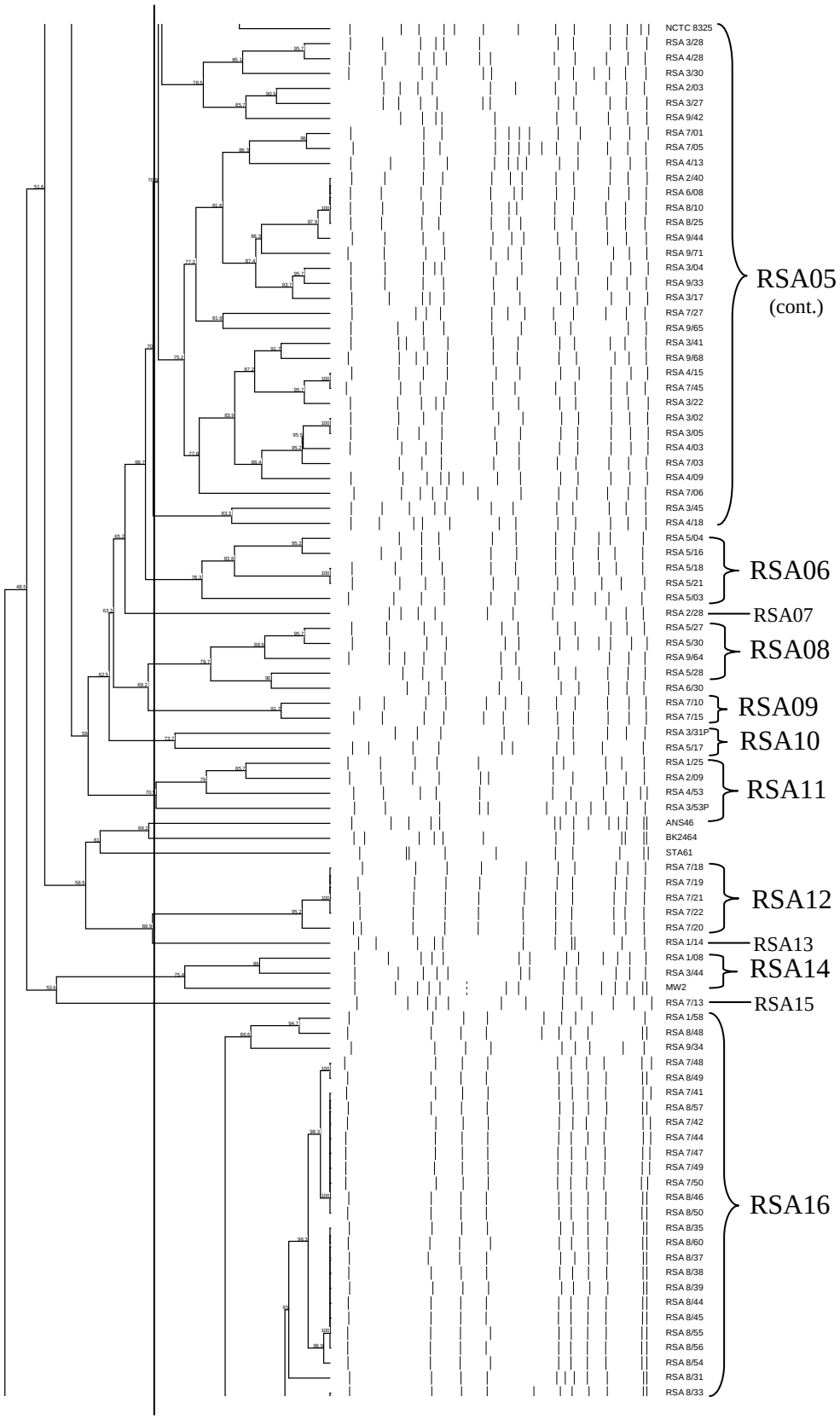
RSA04
RSA05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BK2464
STA61
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RSA 8/56
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RSA 8/31
RSA 8/33
RSA16

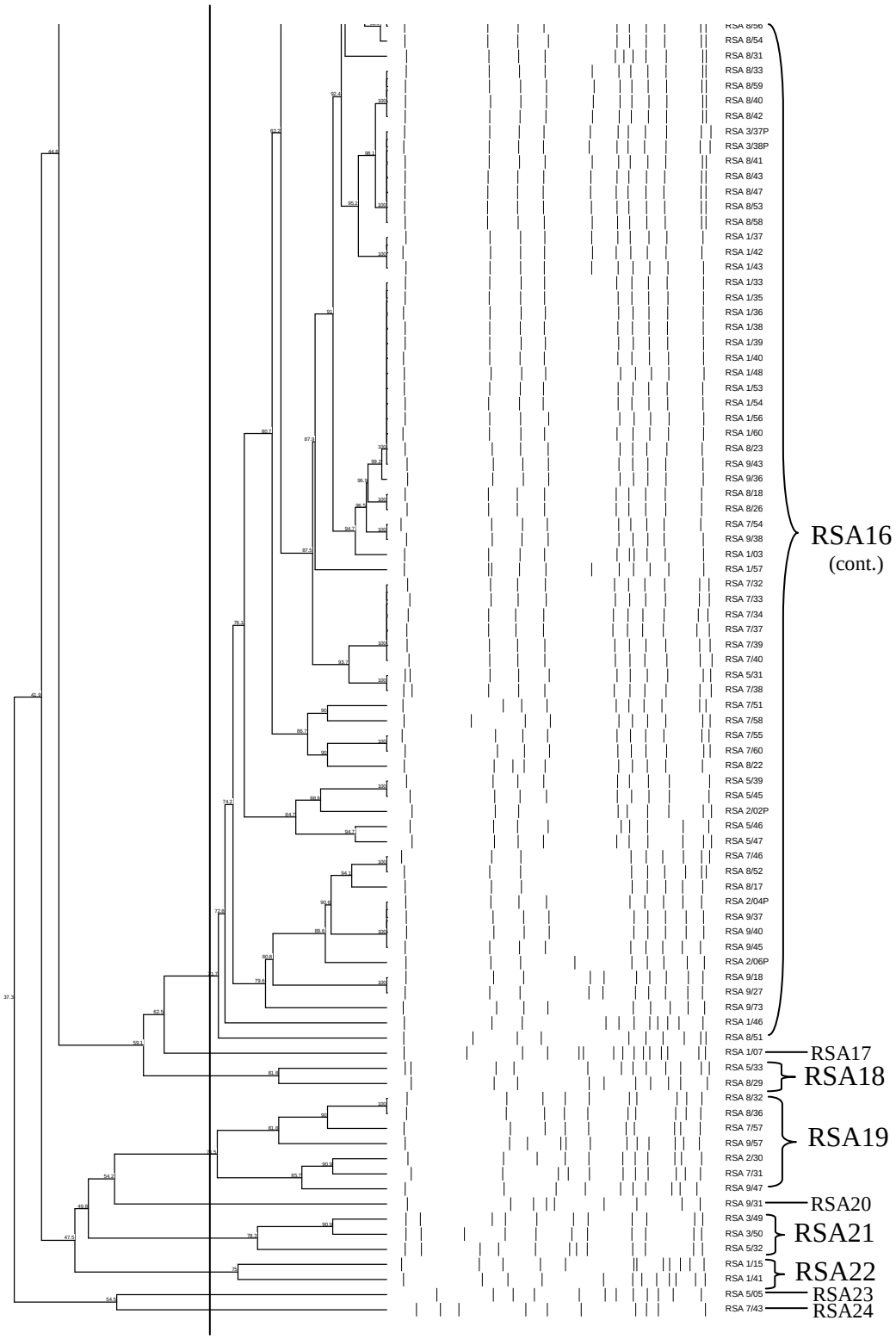

Figure 4.8: UPGMA dendogram of South African MRSA isolates constructed from PFGE typing results.

CHAPTER 5

5 DISCUSSION

5.1 Staphylococcal Cassette Chromosome *mec* Typing

SCC*mec* types I-IV were identified, including variants of each type. The most noteworthy variant identified was SCC*mec* type I-*pls* (n=120). The reason for this was the loss of this SCC*mec* type I specific locus in the plasmin-sensitive protein (*pls*) gene (section 3.7.2, locus A) which yielded a PCR amplification pattern that was similar to SCC*mec* type IV. This resulted in the inability to distinguish SCC*mec* type I-*pls* from SCC*mec* type IV as only locus D (342bp) was amplified during the multiplex PCR experiments. Locus D is present in SCC*mec* type I, II and IV and is internal to the *dcs* (downstream coding sequence) region. Only after completion of the *spa*- and PFGE typing experiments was it deduced that the isolates were in fact SCC*mec* type I-*pls*. This was because: (1) the isolates clustered as *spa*-CC 064 by BURP, which included strain HPV107 (Iberian clone; SCC*mec* type IA) and strain E2125/ATCC BAA-38 (Archaic clone; SCC*mec* type I); (2) the majority of the isolates (RSA05, 94/120, 78%) clustered with strain E2125/ATCC BAA-38 (Archaic clone; SCC*mec* type I) by MRA. The majority of the remaining twenty-six isolates that also carried SCC*mec* type I-*pls* grouped as minor clusters with one to five isolates per cluster.

5.2 *spa* Typing

spa typing, a PCR and DNA sequencing based technique, was used as an additional typing technique to complement PFGE. It has advantages over PFGE in terms of: (1) speed; (2) ease of use and interpretation of data; (3) simplicity of large database creation; and (4) effortless information exchange between laboratories.

When examining the *spa* types belonging to a specific *spa*-CC, there is usually a repeat or several repeats that all the *spa* types have in common. The general hypothesis regarding the evolution of the *spa* types is either through; (1) insertions or deletions of an individual repeat unit or a group of repeat units; or (2) point mutations within a specific repeat unit, thus changing it to a different repeat unit. It is generally accepted that MRSA strains are related, i.e. belong to the same *spa*-Clonal Complex, if the *spa* type repeat motif is related.

5.2.1 spa-Clonal Complex 064

Alignment of the repeat patterns of these *spa* types (Table J1) showed the presence of both motifs 11-19-, 11-10- or 11-34 (start); followed by motif 05-17-34-; followed by motif 24-34-22; and finally repeat r25 (end). *spa* type t051 (MLST CC8[48]) is represented by both strains HPV107 (Iberian clone, SCC*mec* type IA) and E2125/ATCC BAA-38 (Archaic clone, SCC*mec* type I)[16,24,43,48] and is related to *spa* type t064. It appears as if repeat unit r21 (first) was deleted, and the second repeat unit r21 mutated to repeat unit r05 through a point mutation (C→A) at the fifteenth nucleotide position (Table K1). *spa* type t064 has been identified in the USA where it was designated ST 8 ORSA I (part of USA 500 PFT[37])

and has also been associated with persons living with AIDS[17]. *spa* types t008 and t051 have been identified in some strains in Germany and Central Europe[60].

Alignment of repeat units r19 and r10 (Table K1) revealed the same nucleotide sequence except for the last nucleotide (C→T), where alignment of repeat units r19 and r34 (Table L1) revealed the presence of four point mutations. Alignment of repeat units r25 and r36 (Table K2) disclosed the same nucleotide sequence, except for the last nucleotide (C→A), and alignment of repeat units r25 and r16 (Table K3) showed the same nucleotide sequence, except for the ninth nucleotide (T→C).

5.2.2 spa-Clonal Complex 032

The graphic representation of the relatedness of strains comprising *spa*-CC 032 showed that *spa* types t020, t891 and t1468 evolved from *spa* type t032 (EMRSA-15), while *spa* type t1972 evolved from *spa* type t020. Alignment of the repeat patterns of these *spa* types (Table J2) identified motif 26-23- (start; excludes t1972) and repeat unit 28- (end). The motif 17-25-17-25- was identified in all these *spa* types. Motif 31-29-17-31-29- was identified in 60% of the *spa* types of *spa*-CC 032 and is found in strains EMRSA-15 and the Barmin clone[48]. Strain EMRSA-15 has also been detected in hospitals in Kuwait[66]. The only PVL-positive MRSA strain identified, RSA 2/30, and one PVL-positive MSSA strain, RSA 7/31, clustered in *spa*-Clonal Complex 032. All of the MRSA strains carried SCC*mec* type IV.

5.2.3 spa-Clonal Complex 045

Alignment of the repeat patterns of these *spa* types (Table J3) revealed repeat unit r26 (start) and motif 17-16- (end). Motif 20-17-12-17-16 was identified in 80% of the *spa* types of *spa*-CC 045. Alignment of the nucleotide sequences of repeat units r17 and r13 (Table K6) showed that three point mutations occurred. *spa* type t045 (MLST CC-5[48]; SCC*mec* type I) is related to strain EMRSA-3[48] (*spa* type t001). *spa* type t001 was previously identified in the USA, designated ST5 ORSA I (part of USA 800 PFT[32]) and in Korean MRSA isolates collected during 2001-2004[5]. *spa* types t001 and t045 have been identified in some strains prevalent in Germany and Central Europe[60].

5.2.4 spa-Clonal Complex # 5: No founder

Since *spa*-CC # 5 has no founder, is it impossible to predict the evolution of strains within this clonal complex. Alignment of the repeat patterns of these *spa* types (Table J4) revealed repeat unit r08 (start) and motif 16-34-13-17-34-16-34 (end). Alignment of the nucleotide sequences of repeats r16 and r23 (Table K7) revealed the same nucleotide sequences, except for the last nucleotide (T→C/C→T). Previous studies have suggested that *spa* type t015 (SCC*mec* type IV-*dcs*) is part of the MLST CC-4[48].

5.2.5 spa-Clonal Complex # 6: No founder

spa-CC #6 also has no founder. This complex was formed only because strain MW2 (CA-MRSA clone) was included in the BURP cluster analysis. If strain MW2 were to be excluded, *spa* type t172 would be re-classified as a Singleton. All three isolates classified as *spa* type t172 were PVL-positive MSSA strains. Alignment of the repeat patterns of these *spa* types (Table J5) revealed motif 21-16-34-33-13 (last five repeat units). The first repeat unit of *spa* type t128 was identified as repeat unit r07, while *spa* type t172 carried repeat unit r14. Alignment of repeats r07 and r14 (Table K8) revealed identical sequences, except for the last nucleotide (T→C/C→T).

5.2.6 spa-Clonal Complex 012

It is generally accepted that MRSA strains are related if the *spa* type repeat motif is related. With all of the *spa*-CCs identified in this study, the *spa* types are related to each other and generally evolved either through deletions or duplications of repeat units or through point mutations within the nucleotide sequences of any repeat unit. This principle was noted after investigation of the alignment of the *spa* type repeat patterns of the *spa* types in *spa*-CC 012. Alignment of the repeat patterns of the *spa* types in *spa*-CC 012 revealed motif 15-12-16-02 in 55.6% of the *spa* types; motif 02-16-02-25 in 66.7% of the *spa* types; and motif 17-24 in all the *spa* types. Repeat unit r24 was present more than once with some of the *spa* types: two *spa* types (t018 and t046) possessed three repeats of unit r24 at the end, while three *spa* types (t012, t399 and t840) possessed two repeats of unit r24.

Previous studies identified *spa* type t037 as being associated with the Brazilian/Hungarian clone (SCC*mec* type III), while *spa* type t018 is associated with strain EMRSA-16 (SCC*mec* type II). In this study, more than 95% of the strains within a *spa*-CC were only associated with a single SCC*mec* type, whereas *spa*-CC 012 was associated with the Brazilian/Hungarian clone (n=79; *spa* type t037; SCC*mec* type III) and with strain EMRSA-16, or a SLV thereof (n=80; *spa* types t018/t012; SCC*mec* type II). This phenomenon has been described before[60], where the divergent *spa* types t037/t030 were identified in MRSA isolates of ST239 (CC8) and these clustered with *spa* types associated with CC30. This has previously been explained by the recombinative replacement of a large stretch of chromosomal DNA in CC8 MRSA isolates by a stretch originating from CC30, which included the *spaA* gene[49].

After re-investigation of the *spa* type repeat pattern alignment, it was noticed that this *spa*-CC could be divided into 2 groups. Group 1 was composed out of *spa* types related to t018 (EMRSA-16), whereas group 2 was composed out of *spa* types related to t037 (Brazilian/Hungarian). Motif 15-12-16-02-16-02-25-17-24 was identified in three *spa* types, including *spa* types t018 (EMRSA-16; SCC*mec* type II) and t012 (SLV of EMRSA-16; SCC*mec* type II). Motif 15-12-16-02-25-17-24 was identified in *spa* type t037 (SCC*mec* type III) (Brazilian/Hungarian). The only difference between the two groups is the duplication of the motif 16-02- with *spa* types t018 (EMRSA-16), t012, t021 and the other related *spa* types. This correlated well with the SCC*mec* types. However, the applicability of this observation to other studies needs to be confirmed.

Alignment of repeat units r08, identified in *spa* type t046 only, and r15 (Table K9) showed the same nucleotide sequence, except for the last nucleotide (C→T). *spa* type t037 (MLST

CC8[48]), also carried by strain ANS46 (SCC*mec* type III), is associated with the Brazilian/Hungarian epidemic clone[9]. *spa* type t037 has been found in the USA, however, the PFT remained unnamed because only three isolates were identified carrying this *spa* type (together with SCC*mec* type III). It was identified under the name ST239/240ORSA III[32]. *spa* type t037 was also identified in Korean MRSA isolates collected between 2001 and 2004[5] and in some strains prevalent in Germany and Central Europe[60]**.** *spa* types t037 has previously been identified at two hospitals in Miami Florida (USA)[9] in 1998 and also in MRSA strains from Germany, collected between 1994 and 1998[72]. *spa* type t037 has also been reported in various Asian countries, such as Korea[7,30], China[2], Japan[61] and India[4] and also in Argentina[58] and Portugal[1]**.**

spa type t018, which is associated with strain EMRSA-16 (SCC*mec* type II)[9] was identified in the USA as one of the eight major PFTs. It was designated USA200 ORSA II[37]. *spa* type t021 has previously been associated with MSSA and MRSA strains carrying the *lukS-lukF* determinants[60]. Three of the seven isolates identified as *spa* type t021 were PVL-positive and all three were methicillin susceptible. The only isolate identified as *spa* type t318 was also a PVL-positive MSSA strain, strain RSA5/33. *spa* types t012, t018 and t021 and were previously identified in some strains prevalent in Germany and Central Europe[60]**.** *spa* types t012 and t018 (SCC*mec* type II) of the EMRSA-16 epidemic clonal complex have previously been identified at two hospitals in Miami, Florida (USA)[9] in 1998. *spa* type t018 has also been identified in MRSA strains from Germany, collected between 1994 and 1998[72].

5.2.7 Singleton *spa* types

spa types t317 and t1951 were the only *spa* types that were not grouped in any specific clonal complex after BURP clustering. What makes these *spa* types unusual is the fact that all four of the isolates (three for *spa* type t317; one for *spa* type t1951) were PVL-positive MSSA isolates, but their respective repeat patterns do not align (Table J7).

5.2.8 Excluded *spa* types

The only *spa* type that was excluded from BURP cluster analysis was *spa* type t535. This *spa* type contains three repeat units only and because of its deficient length, no reliable deductions can be made regarding the ancestry/ancestries of this *spa* type. Visual inspection of the repeat pattern of *spa* type t535 (26-17-16) showed some similarity with three of the *spa*-CC identified in this study. *spa* types that are part of *spa*-CC 045 all start with the repeat unit r26 and end with the motif 17-16. *spa* type t001 (EMRSA-3) has seven other repeat units located between unit r26 and motif 17-16, while *spa* type t1154 have three other units between unit r26 and motif 17-16. It is possible that the repeat units between unit r26 (start) and motif 17-16 (end) have been deleted, yielding *spa* type t535. *spa* type t1154 only has three other repeat units between unit r26 and motif 17-16.

spa types that are part of *spa*-CC 032 also begin with repeat unit r26. A very common motif among these *spa* types towards the end of the *spa* type repeat pattern is 17-25-17-25-16-28. Repeat unit r17 is also present in the middle of the repeat patterns of three of these *spa* types. It is probably unlikely that so many repeat units (ten to thirteen) would have

been deleted to yield *spa* type t535. Repeat units r17 and r16 are also present in the *spa* types that are part of *spa*-CC #5: NF. The motif 16-34-13-17-34-16-34 is present in both *spa* types that are part of this *spa*-CC.

5.3 Macro-Restriction Analysis with Pulsed-Field Gel Electrophoresis

Of the twenty-four PFGE CTs (clonal types) identified, three were major CTs (RSA05 (n=98); RSA16 (n=89); RSA03 (n=74)). Each of these three CTs contained more than 20% of the isolates. RSA05 contained 28.1% (n=98) of the isolates collected in Gauteng, Limpopo, Mpumalanga, Free State, Eastern Cape, Western Cape and KwaZulu-Natal provinces. Ninety-three (95%) of these isolates were part of *spa*-CC 064 and carried a SCC*mec* type I-*pls* (Table 5.1).

Table 5.1: Strain composition of CT RSA05, displayed as *spa* type and repeat pattern, *spa*-Clonal Complex and SCC*mec* type.

# of isolates	***spa* type**	***spa* type repeat pattern**	***spa*-CC**	**SCC*mec* type**
53	t064	11-19-12-05-17-34-24-34-22-25	*spa*-CC 064	I-*pls*
2	t1779	11-19-12-12-05-17-34-24-34-22-25		
29	t1257	11-19-34-05-17-34-24-34-22-25		
1	t1443	11-19-12-05-17-34-24-24-34-22-25		
2	t951	11-10-05-17-34-24-34-22-25		
1	t1952	11-19-12-05-17-34-24-34-16		
2	t104	11-10-34-22-25		
1	t1930	11-19-12-05-17-34-24-34-22-33-25		
1	t1555	11-19-34-05-17-34-24-34-24-34-22-25		
1	t1971	11-34-05-17-34-24-34-22-25		

RSA16 contained 25.5% (n=89) of the isolates collected in Gauteng, Limpopo, Mpumalanga, Free State, Western Cape, Western Cape and KwaZulu-Natal provinces. Eighty-eight (99%) of these isolates were part of the *spa*-CC 012 and carried a SCC*mec* type II or type IIB (Table 5.2).

Table 5.2: Strain composition of CT RSA16, displayed as *spa* type and repeat pattern, *spa*-Clonal Complex and SCC*mec* type.

# of isolates	***spa* type**	***spa* type repeat pattern**	***spa*-CC**	**SCC*mec* types**
70	t012	15-12-16-02-16-02-25-17-24-24	*spa*-CC 012	II/IIB
6	t018	15-12-16-02-16-02-25-17-24-24-24		II/IIB
5	t021	15-12-16-02-16-02-25-17-24		II
2	t399	15-12-16-02-17-24-24		II
4	t046	08-16-02-16-02-25-17-24-24-24		II
1	t840	15-12-02-16-02-25-17-24-24		II

RSA03 contains 21.2% (n=74) of the isolates collected in Gauteng, Limpopo, Mpumalanga, Northern Cape, Free State, North West, Eastern Cape and KwaZulu-Natal provinces. Seventy (95%) of these isolates were part of the *spa*-CC 012 and carried a SCC*mec* type III or type IIIE (Table 5.3).

Table 5.3: Strain composition of CT RSA03, displayed as *spa* type and repeat pattern, *spa*-Clonal Complex and SCC*mec* type.

# of isolates	***spa* type**	***spa* type repeat pattern**	***spa*-CC**	**SCC*mec* types**
69	t037	15-12-16-02-25-17-24	*spa*-CC 012	III/IIIE
1	t1504	15-12-16-16-02-25-17-24		III

The CA-MRSA isolates, as defined by SCC*mec* and *spa* typing, together with some of the PVL-positive MSSA isolates, grouped into four small clusters. The first cluster, RSA14, contained two PVL-positive MSSA strains, which grouped with control strain MW2 (representative of CA-MRSA). The second cluster, RSA18, also contained two PVL-positive MSSA isolates. The third cluster comprised seven isolates, all of which were part of *spa*-CC 032 and carried SCCmec type IV. *spa* type t032 is part of this *spa*-CC and is associated with EMRSA-15 (SCC*mec* type IV). The fourth cluster, RSA21, contained three PVL-positive MSSA isolates.

The biggest difference regarding the clonal composition of MRSA in South Africa and other countries is the prevalence of the Archaic clone in South Africa. Elsewhere, especially in most parts of Europe, the Archaic clone has been replaced by the Iberian clone, which is a direct descendant of the Archaic clone. The Iberian clone has previously been identified in the USA as one of the eight prevalent PFTs. Strain EMRSA-16 was the dominant HA-MRSA strain in the UK in the recent past, and it was also identified as one of eight prevalent clones in the USA during 2003. In South Africa, this CT appears to be one of the predominant clones. There was no difference between the number of strains clustering in RSA05 (Archaic clone; SCC*mec* type I-*pls*) and RSA16 (EMRSA-16; SCC*mec* type II/IIB). Another common clone identified was the Brazilian/Hungarian clone. This clone is quite common in some parts of the world, like Brazil, Argentina, Portugal, Hungary and some Asian countries. On the other hand, only three isolates characterised in the USA were identified as the Brazilian/Hungarian clone. The prevalence of the Archaic clone in South Africa correlates with results previously obtained by Hiramatsu[21]. A typing study by him showed that a South African MRSA isolate belonged to the Archaic clonal type, by way of MRA.

The surveillance and molecular analysis of the population structure of a specific organism, like MRSA, allows for direct comparisons of strains isolated from different geographical settings. This is especially useful in the case of an outbreak, as numerous epidemic strains are spread globally. Usually, there are certain specific strains circulating within a geographical location. It can thus be determined whether the outbreak strain has been imported from another geographical area. Depending on the techniques used, is it possible to distinguish related from unrelated strains.

Molecular information on different MRSA types provides important information regarding antibiotic resistance patterns. Apart from the *mecA* gene, SCC*mec* can also carry resistance genes for other antibiotics and heavy metals. The resistance genes present on SCC*mec* vary among the different SCC*mec* types. For example, Tn*554*, which encodes resistance to spectinomycin and erythromycin is commonly found on SCC*mec* type II and type III. The integrated plasmid pUB110, encoding tobramycin and bleomycin resistance is common amongst SCC*mec* type II elements. However, pUB110 is only associated with SCC*mec* type IA (Iberian clone) and not with SCC*mec* type I (Archaic clone). SCC*mec* type IV and type V usually only carry the *mecA* gene and are not as multi-resistant as HA-MRSA strains.

A well structured and up-to-date database of PFGE patterns, also containing reference strains, allows one to better distinguish between HA-MRSA and CA-MRSA strains. This can prove to be valuable clinically, as CA-MRSA strains are usually not as multi-drug resistant as HA-MRSA strains. This information can also be useful from an infection control perspective in that CA-MRSA strains can produce numerous toxins (such as PVL, toxic shock syndrome toxin (TSST), γ-haemolysin and several staphylococcal

enterotoxins) that are not normally produced to such a large extent by HA-MRSA strains. These toxins are normally not produced to such a large extent by HA-MRSA strains. Many of these toxins can be fatal to a patient and in general, CA-MRSA strains have a higher mortality rate then HA-MRSA strains.

5.4 Panton-Valentine leukocidin

PVL-producing *S. aureus*, typically associated with CA-MRSA strains that cause skin and soft-tissue infections, has been associated with outbreaks in Australia, France[13,50] the USA[51,63], Japan[45], Greece[68], south-eastern Germany[33], Switzerland[5,46], Taiwan[10] and Singapore[22]. Strains carrying genes for PVL production have also been isolated from companion animals[47].

5.5 Differences Between Individual Typing Techniques

Numerous genotypic typing techniques are available for the successful elucidation of the molecular epidemiology of methicillin-resistant *S. aureus*. The band-based and the sequence-based techniques are most often used. Sequence-based techniques, such as *spa* typing or MLST have the advantage over band-based techniques of: (1) ease of use and data interpretation; (2) transportability; (3) reproducibility; (4) simplicity of comparing results and large database set-up.

Band-based techniques, such as PFGE have the advantage over other techniques of having the highest discriminatory power. However, this method has the disadvantages of: (1) being expensive; (2) labour intensive; (3) requiring specialised electrophoretic equipment; and (4) exchange of information between laboratories is difficult due to the lack of an international standardised protocol. More specifically, 10 European countries use now the HARMONY protocol whereas the Americans, Canadians and Japanese use different protocols. PFGE is currently the "gold-standard" for MRSA typing and is a valuable and suitable method for outbreak investigation, especially in the nosocomial setting, as it can distinguish related from unrelated strains. PFGE can also be utilised to distinguish HA-MRSA strains from CA-MRSA strains. PFGE has previously been applied in studies where different ORSA lineages were delineated, which circulated in Europe, South America and the USA. Each lineage was assigned a name, such as the Archaic/Iberian clone; the New York/Japan clone; the Brazilian/Hungarian clone and the Paediatric clone. Similar studies have been conducted in numerous countries. For example, in Canada, four ORSA lineages were delineated and designated CMRSA 1-4[37]. PFGE data sharing between laboratories was extremely complicated in the past. Even the typing results of similar strains from different laboratories often lacked concordance. Due to the advancement of recent gel analysis software programs, large-scale databases can be created and stored. These are usually composed of normalised PFGE fragment patterns. The use of *S. aureus* NCTC 8325 (an established size standard) for fragment normalisation has aided tremendously in the exchange and transfer of PFGE strain typing data and epidemiological information among different laboratories[37].

During this study, the computer software package Ridom StaphType version 1.4.1 (Ridom GmbH, Germany) was utilised to aid with sequence analysis. Individual repeat units were

identified and the specific *spa* type was deduced and assigned depending on the order of the repeat units. Signature sequences occurring before the first repeat unit and after the last repeat unit were also identified to ensure that no repeat unit was left out during *spa* type assignment. The nucleotide sequences of novel repeat units or *spa* types identified could be synchronised with a server, which ensured uniform terminology usage. Repeats and *spa* types submitted by other researcher were also transferred to the local client software after each synchronisation. *spa* repeat sequences and types could also be downloaded from the Ridom website (http://www.ridom.de/spaserver/), which is accessible to any person. Thus, users who do not work with the Ridom StaphType software are able to submit chromatograms of new *spa* repeats and/or types and therefore have access to the same uniform terminology. Although this software aided tremendously with sequences analysis and interpretation, it is costly especially for laboratories from developing countries.

As with *spa* typing, a specialised computer software package was also required to assist with PFGE banding pattern analysis and comparisons. This software formed an integral part of the analysis, since fingerprint patterns on different gels had to be compared (inter-gel comparisons). During this study, the software package GelCompar II version 4.0.0 (BioNumerics, Belgium) was utilised. However, to allow for inter-gel comparisons, all PFGE patterns had to be normalised to an established size standard before a database of fingerprint patterns could be constructed. Therefore, NCTC 8325 was included in the first, middle and last lanes of every PFGE gel. Normalisation against NCTC 8325 greatly facilitated the interpretation of the banding patterns as two-hundred-and-five different PFGE patterns were identified during this study. Visual inspection is only possible to a certain extent, as two bands can be very close to each other, which can result in the

misinterpretation of the banding patterns. Although this software aids tremendously with banding pattern analysis and interpretation, it is costly.

As only thirty-seven *spa* types were identified, compared to the two-hundred-and-five distinct PFGE banding patterns, it is clear that MRA with PFGE was more discriminatory than *spa* typing. Researchers using *spa* typing must be aware that certain *spa* types can yield ambiguous results, as was seen in this study with *spa* types t037 (SCC*mec* type III/IIIE) and t012 (SCC*mec* type II/IIB), which were both grouped in the same *spa*-CC (*spa*-CC 012) by BURP. However, isolates identified as these *spa* and SCC*mec* types were grouped into separate PFGE CTs, thereby distinguishing them as two separate lineages. This correlated well with results obtained by SCC*mec* typing.

CHAPTER 6

6 CONCLUSIONS

This work describes the molecular epidemiology of methicillin-resistant *Staphylococcus aureus* through MRA, SCC*mec* and *spa* typing, allowing us to delineate the major South African MRSA lineages. This was done by establishing a database of PFGE fingerprints of South African MRSA isolates collected from both state and private diagnostic laboratories from across the country. However, given the complexity of PFGE pattern interpretation, computer aided analysis of the PFGE patterns was required to reduce misclassification and misinterpretation.

MRA proved to be a more discriminatory method than *spa* typing as strains grouped within *spa*-Clonal Complex 012 could be separated into distinct clusters after MRA. This was because of the high degree of similarity between *spa* types t012 and t037, which are associated with different epidemic clones.

We have demonstrated that the population structure of South African MRSA strains is composed of a variety of epidemic clones. The data suggest that the Archaic clone is the dominant clone, followed by EMRSA-16, or a SLV thereof and both clones were similar in prevalence. The Brazilian/Hungarian epidemic clone was also identified, together with a small number of the Iberian epidemic clone. Some isolates were also identified that possessed the same *spa* and SCC*mec* types as strain EMRSA-15, which was a prevalent CA-MRSA strain in Europe in the past.

To our knowledge, this is the first record of both MSSA and MRSA strains from South Africa carrying genes for the toxin Panton-Valentine leukocidin. Interestingly, more MSSA isolates were identified to be PVL-positive than MRSA isolates. This study was not aimed specifically at collecting CA-MRSA, and only eight isolates were identified that carried SCC*mec* type IV. Isolates were collected directly from diagnostic laboratories, which may not be representative of community strains, and epidemiological information such as previous hospitalisation and treatment history of the patient and lifestyle, were not collected. A study that collects isolates from the community with more detailed epidemiological information would be able to identify the CA-MRSA types in South Africa.

CHAPTER 7

7 REFERENCES

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CHAPTER 8

8 APPENDICES

8.1 APPENDIX A: Composition of Media

1. 5% Blood Agar plate

Columbia agar (Oxoid CM0331)	39g
Horse blood (SAVP 052)	50ml
Deionised water	make up to 1l

Autoclave

Store at 4ºC

2. DNA Agar plate

Tryptose	20g
Deoxyribonucleic acid	2g
Sodium chloride	5g
Agar	12g
Deionised water	make up to 1l

pH 7.3 ± 0.2

Autoclave

Store at 4ºC

3. Chapman's Agar plate (Mannitol-Salt Agar plate)

Mannitol-Salt agar	111g
Deionised water	make up to 1l

pH 7.4 ± 0.2

Autoclave

Store at 4ºC

4. Mueller-Hinton Agar plate supplemented with 2% NaCl

Beef, dehydrated infusion	300g
Casein hydrolysate	17.5g
Starch	1.5g
Agar	17g
Sodium chloride	2g

pH 7.4 ± 0.2

Autoclave

Store at 4ºC

5. Blood Agar plate supplemented with Nalidixic Acid and Colistin

CNA Columbia agar	42.5g
Horse Blood	50ml
Deionised water	make up to 1l

pH 7.3 ± 0.2

Autoclave

Store at 4ºC

6. Semi-Solid Agar vials

Nutrient broth N2 (Oxoid CM67)	25g
Bitek Agar (Difco 214530)	9g
Deionised water	make up to 1l

Autoclave

Store at 4ºC

7. Brain Heart Infusion broth (BHI)

Brain heart infusion (Difco 0037-17)	37g
Deionised water	make up to 1l

pH 7.4

Autoclave

Store at 4ºC

8. Normal Saline

Sodium chloride	8.5g
Deionised water	make up to1l

Autoclave

Store at 4ºC

8.2 APPENDIX B: Isolate Information Form

Information Form
Methicillin-resistant *S. aureus* Blood Stream Isolate

CLINICAL MICROBIOLOGY AND INFECTIOUS DISEASES (CMID)

WITS SCHOOL OF PATHOLOGY AND NHLS, JOHANNESBURG

(TEL) +27 11 717 2326 (FAX) +27 11 489 8530
(E-MAIL) oosthuysenw@hsc.pg.wits.ac.za

LABORATORY INFORMATION

Regional Laboratory Specimen Number: _____

Hospital Name: (where patient is admitted) _____

Province: North West Province

Laboratory Name: _____

Laboratory Contact Person: _____

Laboratory Tel: _____

ISOLATE INFORMATION

Isolation/Collection Date: _____ (dd/mm/yyyy)

Organism Isolated Identified As: ☐ *Staphylococcus aureus*

Resistant to Methicillin/Cloxacillin: ☐ Yes

Isolate Source: ☐ Blood

Type of Infection ☐ Single infection ☐ Multiple infection

PATIENT INFORMATION

Age: _____

Gender: ☐ Male ☐ Female

FOR OFFICE USE ONLY

Isolate Number: _____

8.3 APPENDIX C: CLSI Guidelines for Zone Diameter Interpretive Standards for *Staphylococcus* spp.

Antimicrobial agent	Disk content	Zone diameter (mm)		
		R	I	S
Oxacillin	1µg	10	11-12	13

R = Resistant; I = Intermediate resistant; S = Susceptible; mm = Millimetres

8.4 APPENDIX D: Isolate Specific Information Tables

The following tables (one table per province) contain all the isolate specific information, including patient demographics and molecular characterisation results.

Table D1: Patient demographics and molecular characterisation results of typed isolates collected in Gauteng province.

Isolate	Centre	City/ Town	Isolation Date	$Ox^{R/S*}$	Source	Patient		SCC*mec* type	*spa*		PFGE		PVL
						Age#	Gender		Type	CC	Pattern	CT	
RSA 1/01	Tshwane Academic Complex, NHLS	Pretoria	18/11/2005	R	Blood	0	M	IIIE	037	012	NT	NT	-
RSA 1/03			19/11/2005	R	NA	33.6	F	IIB	012	012	16.16	16	-
RSA 1/05			23/11/2005	R	Blood	42	F	IIIE	037	012	3.22	03	-
RSA 1/06			23/11/2005	R	Blood	41	M	IIIE	037	012	3.23	03	-
RSA 1/07			24/11/2005	R	NA	37	F	IIB	012	012	17.1	17	-
RSA 1/08			02/12/2005	S (17)	Blood	18.1	M	MSSA	174	NF:6	14.1	14	+
RSA 1/09			05/12/2005	R	Blood	0	F	I-*pls*	1257	064	NT	NT	-
RSA 1/10			05/12/2005	R	Blood	23.5	M	IIIE	037	012	3.1	03	-
RSA 1/11			09/12/2005	R	Blood	62.8	F	IIIE	037	012	3.19	03	-
RSA 1/12			05/12/2005	R	Blood	42.3	F	IIIE	037	012	3.27	03	-
RSA 1/13			12/12/2005	R	Blood	20	F	I-*pls*	064	064	5.40	05	-
RSA 1/14			14/11/2005	R	NA	23	F	IIIE	037	012	13.1	13	-
RSA 1/15			14/11/2005	R	NA	0.1	F	IIIE	037	012	22.1	22	-
RSA 1/16	New Johannesburg Hospital, NHLS	Johannesburg	28/09/2005	R	Blood	18	M	IIIE	037	012	NT	NT	-
RSA 1/18			10/10/2005	R	Blood	25	M	Iv	037	012	NT	NT	-
RSA 1/19			10/10/2005	R	Blood	0.7	M	IV-*dcs*	015	NF:5	NT	NT	-
RSA 1/20			15/10/2005	R	Blood	31	M	IIIE	037	012	3.17	03	-
RSA 1/21			16/10/2005	R	Blood	70	F	IIIE	037	012	3.21	03	-
RSA 1/22			23/11/2005	R	Blood	28	F	IIIE	037	012	3.18	03	-
RSA 1/23			25/11/2005	R	Drain fluid	33	M	IIIE	037	012	NT	NT	-
RSA 1/24			24/11/2005	R	Tissue	8	M	IIIE	037	012	3.31	03	-
RSA 1/25			26/11/2005	R	Blood	31	F	I-*pls*	951	064	11.1	11	-
RSA 1/26			30/11/2005	R	Sputum	55	M	IIB	012	012	NT	NT	-

RSA 1/27			12/09/2005	R	Blood	1	F	I-*pls*	064	064	5.41	05	-
RSA 1/28			12/09/2005	R	Blood	1	F	I-*pls*	064	064	5.42	05	-
RSA 1/29			14/09/2005	R	Blood	47	M	IIIE	037	012	3.33	03	-
RSA 1/31	Drs. Du Buisson, Bruinette & Kramer (Ampath)	Pretoria	16/09/2005	R	Blood	75	F	IIIE	037	012	3.27	03	-
RSA 1/32			09/01/2006	R	Blood	67	M	IIIE	037	012	3.29	03	-
RSA 1/33			14/01/2006	R	Blood	73	M	II	012	012	16.12	16	-
RSA 1/34			15/01/2006	R	Blood	61	M	IIIE	037	012	3.27	03	-
RSA 1/35			17/01/2006	R	Blood	51	M	II	012	012	16.12	16	-
RSA 1/36			01/04/2006	R	Blood	82	M	II	012	012	16.12	16	-
RSA 1/37			25/05/2006	R	Blood	75	M	II	012	012	16.11	16	-
RSA 1/38			22/06/2006	R	Blood	82	M	II	012	012	16.12	16	-
RSA 1/39			08/07/2006	R	Blood	61	F	II	012	012	16.12	16	-
RSA 1/40			22/07/2006	R	Blood	77	F	IIB	012	012	16.12	16	-
RSA 1/41			25/07/2006	R (9)	Blood	60	F	IIIE	465	NF:5	22.2	22	-
RSA 1/42			27/10/2006	R	Blood	27	M	II	012	012	16.11	16	-
RSA 1/43			28/10/2006	R	Blood	27	M	II	012	012	16.11	16	-
RSA 1/44			31/10/2006	R	Blood	56	M	IIIE	037	012	3.26	03	-
RSA 1/46	Van Drimmelen (Ampath)	Johannesburg	07/11/2005	R	Blood	79	M	IIB	012	012	16.33	16	-
RSA 1/47			05/12/2005	R	Blood	73	M	IIB	012	012	NT	NT	-
RSA 1/48			09/01/2006	R	Blood	1.2	F	II	012	012	16.12	16	-
RSA 1/49			11/01/2006	R	Tissue	46	M	I-*pls*	008	064	4.1	04	-
RSA 1/50			07/02/2006	R	Blood	46	M	IIIE	037	012	3.2	03	-
RSA 1/51			02/05/2006	R	Blood	16	F	IIIE	037	012	3.1	03	-
RSA 1/53			18/05/2006	R	Tissue	36	F	II	012	012	16.12		-
RSA 1/54			18/05/2006	R	CSF	48	F	II	012	012	16.12		-
RSA 1/55			29/05/2006	R	Tissue	48	F	IIIE	037	012	3.5	03	-
RSA 1/56			20/06/2006	R	Wound swab	70	F	II	012	012	16.12	16	-
RSA 1/57			20/06/2006	R	Wound swab	45	F	IIB	012	012	16.17	16	-

RSA 1/58			11/06/2006	R	Abdominal swab	31	M	II	012	012	16.1	16	-
RSA 1/59			11/06/2006	R	Tissue	34	F	IIIE	037	012	3.19	03	-
RSA 1/60			11/06/2006	R	Tissue	87	F	II	021	012	16.12	16	-

Ox = Oxacillin; R = Resistant; S = Susceptible; NA = Not available; CT = Clonal type; * = Zone diameters (if present), given in millimetre; # = Age given in years; - = Negative; + = Positive; M =Male; F = Female; PVL = Panton-Valentine leukocidin; NF = No founder.

Table D2: Patient demographics and molecular characterisation results of typed isolates collected in Limpopo province.

Isolate	Centre	City/ Town	Isolation Date	$Ox^{R/S*}$	Source	Patient		SCC*mec* type	*spa*		PFGE		PVL
						Age#	Gender		Type	CC	Pattern	CT	
RSA 2/03	NHLS	Polokwane	24/05/2006	R	Sputum	41	M	I-*pls*	064	064	5.48	05	-
RSA 2/09			25/05/2006	R	Pus swab	37	M	I-*pls*	1443	064	11.2	11	-
RSA 2/16			14/10/2005	R	Blood	NA	F	I-*pls*	064	064	5.16	05	-
RSA 2/17			28/03/2006	R	Pus	35	F	I-*pls*	1257	064	5.1	05	-
RSA 2/18			07/11/2005	R	Blood	NA	M	I	045	045	2.2	02	-
RSA 2/21			17/07/2006	R	Pus swab	36	F	I-*pls*	064	064	5.21	05	-
RSA 2/22			23/04/2006	R	Blood	1.5	F	I-*pls*	1257	064	5.5	05	-
RSA 2/28			15/04/2006	R	Pus swab	13	M	I-*pls*	064	064	7.1	07	-
RSA 2/29			14/03/2006	R	Pus swab	50	M	I-*pls*	1257	064	5.3	05	-
RSA 2/30			23/02/2006	R	Pus swab	18	M	IV	891	032	19.4	19	+
RSA 2/33			14/07/2006	R	Sputum	68	M	I-*pls*	1930	064	5.39	05	-
RSA 2/34			26/04/2006	R	Pus swab	NA	M	I-*pls*	064	064	5.26	05	-
RSA 2/35			27/02/2006	R	Pus swab	51	M	I-*pls*	1257	064	5.1	05	-
RSA 2/36			23/02/2006	R	Pus swab	54	F	IIIE	037	012	3.34	03	-
RSA 2/40			04/05/2006	R	Fluid	39	M	I-*pls*	1257	064	5.54	05	-
RSA 2/41			10/10/2005	R	Blood	NA	F	I-*pls*	1257	064	5.18	05	-
RSA 2/42			28/02/2006	R	Pus swab	21	F	IIIE	037	012	5.17	05	-
RSA 2/45			17/07/2006	R	Pus swab	35	F	I-*pls*	064	064	5.22	05	-
RSA 2/46			05/05/2006	R	Pus swab	28	M	I-*pls*	1257	064	5.1	05	-
RSA 2/52			15/05/2006	R	Pus swab	NA	F	I-*pls*	045	045	5.37	05	-
RSA 2/53			28/02/2006	R	Pus swab	20	F	I	045	045	2.3	02	-
RSA 2/54			21/02/2006	R	Pus swab	54	F	IIIE	037	012	3.30	03	-
RSA 2/59			14/01/2006	R	Blood	NA	M	I	045	045	2.1	02	-
RSA 2/60			01/04/2006	R	Urine	44	M	I	1257	064	2.5	02	-

RSA 2/02P	NHLS	Polokwane	NA	R	NA	NA	NA	II	012	012	16.25	16	-
RSA 2/03P			11/09/2006	R	NA	18	F	IIIE	037	012	3.27	03	-
RSA 2/04P			14/08/2006	R	NA	51	M	II	012	012	16.30	16	-
RSA 2/05P			08/09/2006	R	NA	8	M	IIIE	037	012	3.30	03	-
RSA 2/07P			23/03/2006	R	NA	11	F	IIIE	037	012	3.32	03	-

Ox = Oxacillin; R = Resistant; S = Susceptible; NA = Not available; CT = Clonal type; * = Zone diameters (if present), given in millimetre; # = Age given in years; - = Negative; + = Positive; M =Male; F = Female; PVL = Panton-Valentine leukocidin.

Table D3: Patient demographics and molecular characterisation results of typed isolates collected in Mpumalanga province.

Isolate	Centre	City/ Town	Isolation Date	$Ox^{R/S*}$	Source	Patient		SCC*mec* type	*spa*		PFGE		PVL
						Age#	Gender		Type	CC	Pattern	CT	
RSA 3/01	NHLS	Witbank	06/04/2006	R	Blood	0.5	M	IIIE	037	012	3.11	03	-
RSA 3/02			05/04/2006	R	Pus	39	M	I-*pls*	064	064	5.66	05	-
RSA 3/03			05/04/2006	R	Pus	0.5	M	IIIE	037	012	3.11	03	-
RSA 3/04			16/05/2006	R	Pus	27	M	I-*pls*	1555	064	5.57	05	-
RSA 3/05			16/05/2006	R	Pus	53	M	I-*pls*	064	064	5.66	05	-
RSA 3/06			16/05/2006	R	Pus	2	M	IIIE	037	012	3.11	03	-
RSA 3/10			08/03/2006	R	Blood	0.3	M	IIIE	037	012	3.12	03	-
RSA 3/12			05/03/2006	R	Pus swab	41	F	I	045	045	2.8	02	-
RSA 3/16			08/03/2006	R	Pus swab	29	F	I	045	045	2.10	02	-
RSA 3/17			09/03/2006	R	Pus swab	25	M	I-*pls*	064	064	5.59	05	-
RSA 3/21	NHLS	Nelspruit	27/10/2005	R	Blood	0.1	F	I	045	045	2.8	02	-
RSA 3/22			09/11/2005	R	Blood	0.7	F	I-*pls*	064	064	5.65	05	-
RSA 3/23			16/11/2005	R	Blood	26	F	I-*pls*	064	064	5.7	05	-
RSA 3/24			10/01/2006	R	Blood	56	M	I-*pls*	045	045	NT	NT	-
RSA 3/25			21/01/2006	R	CSF	29	F	Iv	535	Ex	2.14	02	-
RSA 3/27			30/01/2006	R	Blood	46	M	I-*pls*	1257	064	5.49	05	-
RSA 3/28			24/02/2006	R	Blood	40	M	I-*pls*	1257	064	5.45	05	-
RSA 3/29			09/03/2006	R	Blood	25	F	I-*pls*	064	064	5.11	05	-
RSA 3/30			03/06/2006	R	Blood	NA	M	I-*pls*	1257	064	5.47	05	-
RSA 3/41	NHLS	Ermelo	12/10/2005	R	Blood	33	M	I-*pls*	064	064	5.62	05	-
RSA 3/42			16/01/2006	R	Blood	9	F	I-*pls*	064	064	5.19	05	-
RSA 3/44			02/02/2006	S (15)	Fluid	32	F	MSSA	174	NF:6	14.2	14	+
RSA 3/45			03/03/2006	R	Fluid	42	M	I-*pls*	064	064	5.71	05	-
RSA 3/46			14/03/2006	R	NA	28	M	I-*pls*	064	064	3.24	03	-

RSA 3/49			11/07/2006	S (15)	NA	39	F	MSSA	317	S	21.1	21	+
RSA 3/50			11/07/2006	S (15)	NA	NA	F	MSSA	317	S	21.2	21	+
RSA 3/31P	Ampath	Nelspruit	08/02/2006	R	Sputum	35	M	I-*pls*	064	064	10.1	10	-
RSA 3/32P			13/02/2006	R	Pus	19	M	III	037	012	3.24	03	-
RSA 3/37P			27/02/2006	R	Blood	61	M	II	399	012	16.10	16	-
RSA 3/38P			11/03/2006	R	Sputum	61	M	II	399	012	16.10	16	-
RSA 3/47P			NA	R	NA	NA	NA	IIIE	037	012	5.9	05	-
RSA 3/48P			NA	R	NA	NA	NA	IIIE	037	012	3.24	03	-
RSA 3/53P			NA	S (14)	NA	NA	NA	MSSA	1951	S	11.4	11	+

Ox = Oxacillin; R = Resistant; S = Susceptible; NA = Not available; CT = Clonal type; * = Zone diameters (if present), given in millimetre; # = Age given in years; - = Negative; + = Positive; M =Male; F = Female; PVL = Panton-Valentine leukocidin; NF = No founder; S = Singleton; Ex = Excluded.

Table D4: Patient demographics and molecular characterisation results of typed isolates collected in Northern Cape province.

Isolate	Centre	City/ Town	Isolation Date	$Ox^{R/S*}$	Source	Patient		SCC*mec* type	*spa*		PFGE		PVL
						Age#	Gender		Type	CC	Pattern	CT	
RSA 4/03	NHLS	Kimberley	13/02/2006	R	Pus swab	64	M	I-*pls*	1257	064	5.67	05	-
RSA 4/06			27/02/2006	R	Pus swab	42	M	IIIE	37	012	3.24	03	-
RSA 4/07			27/02/2006	R	Pus swab	56	M	IIIE	037	012	3.3	03	-
RSA 4/08			28/02/2006	R	Pus	49	M	IIIE	037	012	3.3	03	-
RSA 4/09			28/02/2006	R	Tracheal aspirate	NA	F	I-*pls*	1257	064	5.69	05	-
RSA 4/11			13/03/2006	R	Pus swab	5.4	F	I-*pls*	064	064	5.23	05	-
RSA 4/12			17/03/2006	R	Pus swab	18	F	I-*pls*	064	064	5.12	05	-
RSA 4/13			28/03/2006	R	Pus swab	59	M	I-*pls*	064	064	5.53	05	-
RSA 4/14			28/03/2006	R	Pus swab	57	M	IIIE	037	012	3.4	03	-
RSA 4/15			29/03/2006	R	Pus	0	M	I-*pls*	1257	064	5.64	05	-
RSA 4/17			06/04/2006	R	Pus swab	13	M	IIIE	037	012	3.4	03	-
RSA 4/18			07/04/2006	R	Pus	72	M	NT	064	064	5.72	05	-
RSA 4/19			29/04/2006	R	Pus	35	M	IIIE	037	012	3.3	03	-
RSA 4/21			29/06/2006	R	Pus	6	M	IIIE	037	012	3.24	03	-
RSA 4/22			29/06/2006	R	Pus swab	76	M	IIIE	037	012	3.24	03	-
RSA 4/23			29/06/2006	R	Pus swab	48	F	I-*pls*	1257	064	5.8	05	-
RSA 4/24			06/07/2006	R	Blood	55	F	IIIE	037	012	3.24	03	-
RSA 4/26			13/07/2006	R	Pus	NA	M	I-*pls*	1257	064	5.7	05	-
RSA 4/27			13/07/2006	R	Swab	NA	M	I-*pls*	1257	064	5.9	05	-
RSA 4/28			13/07/2006	R	Swab	NA	F	I-*pls*	104	064	5.46	05	-
RSA 4/29			13/07/2006	R	Swab	13	M	I-*pls*	064	064	5.11	05	-
RSA 4/30			17/07/2006	R	Swab	13	M	IIIE	037	012	3.19	03	-
RSA 4/51			24/08/2006	R	Pus swab	5	M	I-*pls*	1257	064	5.25	05	-

RSA 4/53	NHLS	Kimberley	23/08/2006	R	Pus swab	22	M	I-*pls*	104	064	11.3	11	-
RSA 4/54			13/10/2006	R	Pus	19	M	I-*pls*	1257	064	5.25	05	-
RSA 4/55			11/10/2006	R	Pus	20	F	IIIE	037	012	3.15	03	-
RSA 4/56			10/10/2006	R	Pus	45	M	I-*pls*	1257	064	5.25	05	-
RSA 4/57			18/10/2006	R	Pus	45	M	IIIE	037	012	3.32	03	-
RSA 4/58			18/10/2006	R	Pus	45	M	IIIE	037	012	3.32	03	-

Ox = Oxacillin; R = Resistant; S = Susceptible; NA = Not available; CT = Clonal type; * = Zone diameters (if present), given in millimetre; # = Age given in years; - = Negative; + = Positive; M =Male; F = Female; PVL = Panton-Valentine leukocidin; NT = Not typeable.

Table D5: Patient demographics and molecular characterisation results of typed isolates collected in Free State province.

Isolate	Centre	City/ Town	Isolation Date	Ox$^{R/S*}$	Source	Patient		SCC*mec* type	*spa*		PFGE		PVL
						Age$^{\#}$	Gender		Type	CC	Pattern	CT	
RSA 5/01	NHLS	Welkom	25/05/2006	R	Wound swab	42	F	IIIE	037	012	3.37	03	-
RSA 5/03			21/08/2006	R	NA	21.3	F	I-*pls*	064	064	6.4	06	-
RSA 5/04			11/09/2006	R	NA	38.5	M	I-*pls*	064	064	6.1	06	-
RSA 5/05			02/10/2006	R	NA	65.8	M	I-*pls*	951	064	23.1	23	-
RSA 5/06			03/04/2006	R	Pus	0	M	IIIE	037	012	3.38	03	-
RSA 5/09			30/05/2006	R	Pus	37	F	I-*pls*	064	064	5.43	05	-
RSA 5/16	NHLS (Universitas)	Bloemfontein	05/10/2005	R	Blood	0	M	I-*pls*	064	064	6.2	06	-
RSA 5/17			28/09/2005	R	Blood	0.2	F	I	045	045	10.2	10	-
RSA 5/18			13/10/2005	R	Blood	43	M	I-*pls*	064	064	6.3	06	-
RSA 5/20			01/12/2005	R	Catheter tip	16	M	IIIE	037	012	3.16	03	-
RSA 5/21			08/12/2005	R	Tissue	26	M	I-*pls*	064	064	6.3	06	-
RSA 5/24			14/12/2005	R	Catheter tip	27	M	IIIE	037	012	3.16	03	-
RSA 5/25			27/12/2005	R	Blood	26	M	I-*pls*	064	064	5.38	05	-
RSA 5/26			30/12/2005	R	Blood	49	M	IIIE	037	012	3.8	03	-
RSA 5/27			23/01/2006	R	Pleural pus	20	M	I-*pls*	064	064	8.1	08	-
RSA 5/28			26/01/2006	R	Tissue	78	F	I-*pls*	064	064	8.4	08	-
RSA 5/30			17/02/2006	R	Fluid	28	M	I-*pls*	1774	064	8.2	08	-
RSA 5/00			17/02/2006	R	Tissue	41	M	IIIE	037	012	3.11	03	-
RSA 5/31	Pathcare	Bloemfontein	08/12/2005	R	Tissue	78	M	IIB	012	012	16.19	16	-
RSA 5/32			23/02/2006	S (20)	Pus swab	45	F	MSSA	317	S	21.3	21	+
RSA 5/33			24/02/2006	S (22)	Pus swab	75	M	MSSA	318	012	18.1	18	+
RSA 5/34			28/02/2006	R	Pus swab	51	F	I-*pls*	064	064	5.6	05	-
RSA 5/36			28/06/2006	R	Sputum	NA	NA	I-*pls*	064	064	5.31	05	-

RSA 5/37	Pathcare	Bloemfontein	10/07/2006	R	Sputum	82	M	IIIE	037	012	3.20	03	-
RSA 5/38			21/07/2006	R	Blood	77	M	IIIE	037	012	3.20	03	-
RSA 5/39			11/10/2006	R	Tracheal aspirate	41	M	II	012	012	16.24	16	-
RSA 5/40			16/10/2006	R	Tissue	43	M	IIIE	037	012	3.35	03	-
RSA 5/41			16/10/2006	R	Pus	4	M	I-*pls*	1952	064	5.24	05	-
RSA 5/45			24/11/2006	R	Tissue	70	M	II	018	012	16.24	16	-
RSA 5/46			24/11/2006	R	Tissue	70	M	II	018	012	16.26	16	-
RSA 5/47			24/11/2006	R	Tissue	70	M	II	037	012	16.27	16	-
RSA 5/48			23/11/2006	R	Swab	50	M	IIIE	037	012	3.14	03	-
RSA 5/49			23/11/2006	R	Swab	50	M	IIIE	037	012	3.14	03	-

PC = Pathcare; BFN = Bloemfontein; Ox = Oxacillin; R = Resistant; S = Susceptible; NA = Not available; CT = Clonal type; * = Zone diameters (if present), given in millimetre; # = Age given in years; - = Negative; + = Positive; M =Male; F = Female; PVL = Panton-Valentine leukocidin; S = Singleton.

Table D6: Patient demographics and molecular characterisation results of typed isolates collected in North West province.

Isolate	Centre	City/ Town	Isolation Date	$Ox^{R/S*}$	Source	Patient		SCC*mec* type	*spa*		PFGE		PVL
						Age#	Gender		Type	CC	Pattern	CT	
RSA 6/01	NHLS	Rustenburg	09/02/2006	R	Ear Swab	24	F	I-*pls*	064	064	5.14	05	-
RSA 6/03			16/02/2006	R	NA	27	M	IIIE	037	012	3.24	03	-
RSA 6/04			NA	R	NA	NA	NA	Iv	045	045	2.16	02	-
RSA 6/05			13/03/2006	R	NA	2	F	I-*pls*	064	064	5.13	05	-
RSA 6/06			13/03/2006	R	NA	2	F	I-*pls*	064	064	5.13	05	-
RSA 6/08			14/03/2006	R	NA	51	M	I-*pls*	064	064	5.54	05	-
RSA 6/09			14/03/2006	R	NA	34	M	IIIE	037	012	3.29	03	-
RSA 6/10			NA	R	NA	NA	NA	Iv	045	045	2.14	02	-
RSA 6/12			07/04/2006	R	Pus swab	25	F	Iv	045	045	2.13	02	-
RSA 6/14			07/04/2006	R	Pus swab	38	F	I	045	045	2.13	02	-
RSA 6/16	NHLS	Klerksdorp	23/09/2005	R	Blood	0	NA	Iv	045	045	2.6	02	-
RSA 6/17			26/09/2005	R	Blood	41	M	IIIE	037	012	3.28	03	-
RSA 6/18			01/02/2006	R	Blood	NA	NA	Iv	045	045	2.8	02	-
RSA 6/21			25/07/2006	R	Blood	NA	NA	I	045	045	2.9	02	-
RSA 6/22			22/11/2005	R	Blood	2	F	IIIE	037	012	3.11	03	-
RSA 6/23			02/05/2006	R	Blood	0	F	Iv	045	045	2.7	02	-
RSA 6/24			29/03/2006	R	Blood	0.1	M	I	045	045	2.8	02	-
RSA 6/25			07/08/2006	R	Blood	NA	NA	I-*pls*	064	064	5.2	05	-
RSA 6/28			22/11/2005	R	Blood	31	M	IIIE	064	064	3.11	03	-
RSA 6/30			29/03/2006	R	Blood	NA	NA	I	045	045	8.5	08	-
RSA 6/31	Lancet	JHB	30/11/2005	R	Urine	74	M	IIIE	037	012	3.9	03	-

JHB = Johannesburg; Ox = Oxacillin; R = Resistant; S = Susceptible; NA = Not available; CT = Clonal type; * = Zone diameters (if present), given in millimetre; # = Age given in years; - = Negative; + = Positive; M =Male; F = Female; PVL = Panton-Valentine leukocidin.

Table D7: Patient demographics and molecular characterisation results of typed isolates collected in Eastern Cape province.

Isolate	Centre	City/ Town	Isolation Date	$Ox^{R/S*}$	Source	Patient		SCC*mec* type	*spa*		PFGE		PVL
						Age#	Gender		Type	CC	Pattern	CT	
RSA 7/01	NHLS	Port Elizabeth	28/10/2005	R	Blood	34.8	F	I-*pls*	064	064	5.51	05	-
RSA 7/02			29/10/2005	R	Blood	0.2	M	I-*pls*	1257	064	5.9	05	-
RSA 7/03			31/10/2005	R	Blood	33	F	I-*pls*	064	064	5.68	05	-
RSA 7/04			15/11/2005	R	Blood	11	M	I-*pls*	064	064	5.44	05	-
RSA 7/05			19/11/2005	R	Blood	37	M	I-*pls*	064	064	5.52	05	-
RSA 7/06			04/12/2005	R	Blood	0.5	M	I-*pls*	064	064	5.70	05	-
RSA 7/07			07/12/2005	R	Blood	30	F	Iv	045	045	2.15	02	-
RSA 7/08			12/12/2005	R	Blood	NA	NA	I	1154	045	2.11	02	-
RSA 7/09			09/01/2006	R	Blood	0	M	I	1154	045	2.11	02	-
RSA 7/10			09/01/2006	R	Blood	2.6	F	I-*pls*	064	064	9.1	09	-
RSA 7/12			02/02/2006	R	Blood	0	F	I	1154	045	2.11	02	-
RSA 7/13			16/02/2006	R	Blood	28	F	I-*pls*	064	064	15.1	15	-
RSA 7/14			28/02/2006	R	Blood	0	NA	I	1154	045	2.12	02	-
RSA 7/15			02/03/2006	R	Blood	3	M	I-*pls*	064	064	9.2	09	-
RSA 7/18			04/04/2006	R	Blood	21	F	Iv	001	045	12.1	12	-
RSA 7/19			06/04/2006	R	Blood	21	F	Iv	001	045	12.1	12	-
RSA 7/20			06/04/2006	R	Blood	21	F	Iv	001	045	12.2	12	-
RSA 7/21			07/04/2006	R	Blood	21	F	Iv	001	045	12.1	12	-
RSA 7/22			07/04/2006	R	Blood	21	F	Iv	001	045	12.1	12	-
RSA 7/27			10/03/2006	R	Blood	NA	F	I-*pls*	064	064	5.60	05	-
RSA 7/28			05/07/2006	R	Blood	1.9	M	I-*pls*	064	064	5.33	05	-
RSA 7/30			12/03/2006	R	Blood	3	F	I-*pls*	064	064	5.4	05	-
RSA 7/31			11/02/2006	S (18)	Pus swab	60	M	MSSA	1972	032	19.5	19	+
RSA 7/32			02/04/2006	R	Pus swab	27	M	IIB	840	012	16.18	16	-

RSA 7/33	Drs. Swart & Maré	Port Elizabeth	03/04/2006	R	Pus swab	58	M	IIB	012	012	16.18	16	-
RSA 7/34			26/06/2006	R	Blood	72	M	IIB	012	012	16.18	16	-
RSA 7/36			25/05/2006	R	Catheter tip	34	M	I-*pls*	064	064	5.36	05	-
RSA 7/37			22/05/2006	R	Pus swab	72	M	II	012	012	16.18	16	-
RSA 7/38			26/06/2006	R	Blood	72	M	IIB	012	012	16.19	16	-
RSA 7/39			12/07/2006	R	Drainage site	73	F	II	012	012	16.18	16	-
RSA 7/40			10/07/2006	R	Pus swab	68	F	II	012	012	16.18	16	-
RSA 7/41			26/07/2006	R	Pus swab	54	F	II	012	012	16.5	16	-
RSA 7/42			30/05/2006	R	Septic back	72	M	II	012	012	16.5	16	-
RSA 7/43			30/05/2006	R	Swab	82	M	IIB	001	045	24.1	24	-
RSA 7/44			28/07/2006	R	Pus swab	53	F	II	012	012	16.5	16	-
RSA 7/45			02/08/2006	R	Pus swab	3.2	M	I-*pls*	064	064	5.64	05	-
RSA 7/46			10/08/2006	R	Pus swab	43	M	II	018	012	16.28	16	-
RSA 7/47			10/08/2006	R	Pus swab	43	F	II	012	012	16.5	16	-
RSA 7/48			12/10/2006	R	Wound abscess	75	F	II	012	012	16.4	16	-
RSA 7/49			30/08/2006	R	Leg	78	F	IIB	021	012	16.5	16	-
RSA 7/50			13/10/2006	R	Sputum	66	M	II	012	012	16.5	16	-
RSA 7/51			29/08/2006	R	Leg	77	F	II	012	012	16.20	16	-
RSA 7/54			28/09/2006	R	Pus swab	66	M	II	021	012	16.15	16	-
RSA 7/55			12/10/2006	R	Pus	56	F	II	012	012	16.22	16	-
RSA 7/57			16/10/2006	R	Swab	62	F	IV	020	032	19.5	19	-
RSA 7/58			23/10/2006	R	Sputum	66	M	II	012	012	16.21	16	-
RSA 7/59			20/10/2006	R	Foot ulcer	40	M	I-*pls*	064	064	3.39	03	-
RSA 7/60			02/08/2006	R	Pus swab	3.2	M	II	012	012	16.22	16	-

Ox = Oxacillin; R = Resistant; S = Susceptible; NA = Not available; CT = Clonal type; * = Zone diameters (if present), given in millimetre; # = Age given in years; - = Negative; + = Positive; M =Male; F = Female; PVL = Panton-Valentine leukocidin.

Table D8: Patient demographics and molecular characterisation results of typed isolates collected in Western Cape province.

Isolate	Centre	City/ Town	Isolation Date	$Ox^{R/S*}$	Source	Patient		SCC*mec* type	*spa*		PFGE		PVL
						Age#	Gender		Type	CC	Pattern	CT	
RSA 8/01	NHLS	George	26/01/2006	R	Blood	0.2	M	I-*pls*	064	064	5.7	05	-
RSA 8/02			07/02/2006	R	Pus	39	M	I-*pls*	1779	064	5.10	05	-
RSA 8/03			10/03/2006	R	NA	39	M	I-*pls*	064	064	5.34	05	-
RSA 8/04			10/03/2006	R	NA	29	F	I-*pls*	064	064	5.34	05	-
RSA 8/05			13/03/2006	R	Blood	34	M	I-*pls*	064	064	5.13	05	-
RSA 8/06			02/05/2006	R	Empyema fluid	50	M	I-*pls*	064	064	5.35	05	-
RSA 8/07			12/10/2006	R	Pus swab	79	M	I	045	045	2.1	02	-
RSA 8/08			11/10/2006	R	Pus swab	20	M	I-*pls*	064	064	5.10	05	-
RSA 8/09			14/10/2006	R	Pus swab	21	M	I-*pls*	064	064	5.7	05	-
RSA 8/10			21/11/2006	R	Pus swab	5	F	I-*pls*	1257	064	5.54	05	-
RSA 8/11			21/11/2006	R	Pus swab	27	M	I-*pls*	1779	064	5.7	05	-
RSA 8/16	Groote Schuur Hospital, NHLS	Cape Town	21/01/2006	R	Blood	0.1	M	I	045	045	2.1	02	-
RSA 8/17			20/01/2006	R	Blood	50	M	II	018	012	16.29	16	-
RSA 8/18			18/01/2006	R	Blood	47	M	I-*pls*	1443	064	16.14	16	-
RSA 8/19			18/01/2006	R	Blood	1.3	M	I-*pls*	064	064	5.29	05	-
RSA 8/20			22/01/2006	R	Blood	1	M	I-*pls*	064	064	5.14	05	-
RSA 8/21			23/01/2006	R	Blood	1.3	M	I-*pls*	064	064	5.30	05	-
RSA 8/22			31/01/2006	R	Blood	39	F	II	012	012	16.23	16	-
RSA 8/23			01/02/2006	R	Blood	82	M	IIB	012	012	16.12	16	-
RSA 8/24			02/02/2006	R	Blood	0	F	I	045	045	2.4	02	-
RSA 8/25			05/02/2006	R	Blood	39	F	I-*pls*	1257	064	5.54	05	-
RSA 8/26			15/02/2006	R	Blood	0	M	II	021	012	16.14	16	-
RSA 8/27			14/02/2006	R	Blood	80	F	IIB	012	012	5.7	05	-

RSA 8/28			17/02/2006	R	Blood	0	M	I	045	045	2.1	02	-
RSA 8/29			22/02/2006	R	Blood	21	F	I-*pls*	064	064	18.2	18	-
RSA 8/30			05/03/2006	R	Blood	25	M	I-*pls*	1443	064	5.14	05	-
RSA 8/31	Pathcare	Cape Town	03/12/2005	R	Blood	50	M	IIB	012	012	16.8	16	-
RSA 8/32			14/12/2005	R	Blood	64	F	IV	032	032	19.1	19	-
RSA 8/33			14/12/2005	R	Blood	57	M	IIB	012	012	16.9	16	-
RSA 8/35			11/02/2006	R	Blood	48	M	IIB	012	012	16.6	16	-
RSA 8/36			14/02/2006	R	Pus swab	81	F	IV	1468	032	19.1	19	-
RSA 8/37			15/02/2006	R	Pus swab	61	F	IIB	012	012	16.6	16	-
RSA 8/38			14/02/2006	R	Pus swab	59	F	IIB	012	012	16.6	16	-
RSA 8/39			23/02/2006	R	Pus swab	75	M	IIB	012	012	16.6	16	-
RSA 8/40			23/02/2006	R	Pus swab	18	M	IIB	012	012	16.9	16	-
RSA 8/41			25/02/2006	R	Pus swab	18	M	IIB	012	012	16.10	16	-
RSA 8/42			24/02/2006	R	Pus swab	60	M	IIB	012	012	16.9	16	-
RSA 8/43			24/02/2006	R	Pus swab	65	F	II	012	012	16.10	16	-
RSA 8/44			02/03/2006	R	Blood	76	F	II	012	012	16.6	16	-
RSA 8/45			03/03/2006	R	Pus swab	19	F	II	012	012	16.6	16	-
RSA 8/46			04/03/2006	R	NA	66	M	II	012	012	16.5	16	-
RSA 8/47			03/03/2006	R	Pus swab	67	M	II	012	012	16.10	16	-
RSA 8/48			23/03/2006	R	NA	43	M	II	012	012	16.2	16	-
RSA 8/49			24/03/2006	R	Pus swab	39	M	II	012	012	16.4	16	-
RSA 8/50			29/03/2006	R	NA	80	M	II	012	012	16.5	16	-
RSA 8/51			04/04/2006	R	Pus swab	90	M	II	018	012	16.34	16	-
RSA 8/52			07/04/2006	R	Pus swab	84	F	II	018	012	16.28	16	-
RSA 8/53			10/04/2006	R	NA	49	M	II	012	012	16.10	16	-
RSA 8/54			11/04/2006	R	Pus swab	79	M	II	012	012	16.7	16	-
RSA 8/55			20/04/2006	R	Blood	75	M	II	012	012	16.6	16	-
RSA 8/56			20/04/2006	R	CSF	31	F	II	012	012	16.6	16	-
RSA 8/57			21/04/2006	R	Pus swab	73	M	II	012	012	16.5	16	-
RSA 8/58			21/04/2006	R	Pus swab	22	F	II	012	012	16.10	16	-

RSA 8/59			20/04/2006	R	Pus swab	49	M	II	012	012	16.9	16	-
RSA 8/60			21/04/2006	R	Pus swab	75	M	II	012	012	16.6	16	-

Ox = Oxacillin; R = Resistant; S = Susceptible; NA = Not available; CT = Clonal type; * = Zone diameters (if present), given in millimetre; # = Age given in years; - = Negative; + = Positive; M =Male; F = Female; PVL = Panton-Valentine leukocidin.

Table D9: Patient demographics and molecular characterisation results of typed isolates collected in KwaZulu-Natal province.

Isolate	Centre	City/ Town	Isolation Date	$Ox^{R/S*}$	Source	Patient		SCC*mec* type	*spa*		PFGE		PVL
						Age#	Gender		Type	CC	Pattern	CT	
RSA 9/16	RK Khan Hospital, KZN Health	Durban	08/02/2006	R	Blood	27	F	I-*pls*	1257	064	5.28	05	-
RSA 9/17			14/02/2006	R	Blood	32	F	I-*pls*	1257	064	5.27	05	-
RSA 9/18			21/02/2006	S (25)	Pus swab	40	NA	MSSA	021	012	16.31	16	+
RSA 9/20			21/02/2006	R	Ulcer	72	F	IIIE	037	012	3.36	03	-
RSA 9/21			24/02/2006	R	Swab	9	F	I-*pls*	951	064	5.32	05	-
RSA 9/22			27/02/2006	R	Tissue	53	M	IIIE	037	012	3.20	03	-
RSA 9/23			02/03/2006	R	Biopsy (burns)	25	M	I-*pls*	064	064	5.28	05	-
RSA 9/24			02/03/2006	R	Wound swab	1	F	I-*pls*	951	064	5.28	05	-
RSA 9/25			02/03/2006	R	Pus swab	43	F	IIIE	037	012	3.19	03	-
RSA 9/27			05/03/2006	S (23)	Swab	1	M	MSSA	021	012	16.31	16	+
RSA 9/29			03/03/2006	S (22)	Pus swab	47	M	MSSA	021	012	NT	NT	+
RSA 9/30			07/03/2006	R	Pus swab	54	F	IIIE	037	012	3.20	03	-
RSA 9/31	Dr. Bouwer & Partners	Durban	29/05/2005	R	Pus	67	M	IV	032	032	20.1	20	-
RSA 9/32			12/09/2005	R	Blood	58	F	IIIE	1504	012	3.3	03	-
RSA 9/33			16/08/2005	R	Pus	39	F	I-*pls*	1257	064	5.58	05	-
RSA 9/34			16/08/2005	R	Blood	35	M	II	012	012	16.3	16	-
RSA 9/35			26/09/2005	R	Pus	65	M	IIIE	037	012	3.6	03	-
RSA 9/36			13/09/2005	R	Blood	85	M	II	012	012	16.13	16	-
RSA 9/37			30/10/2005	R	Blood	71	M	II	046	012	16.30	16	-
RSA 9/38			01/12/2005	R	Blood	85	M	II	012	012	16.15	16	-
RSA 9/39			21/12/2005	R	Blood	49	IIIE	IIIE	037	012	3.6	03	-
RSA 9/40			30/10/2005	R	Blood	71	M	II	046	012	16.30	16	-

RSA 9/41			20/09/2005	R	Pus	91	M	III	037	012	3.6	03	-
RSA 9/42			14/19/2005	R	Pus	57	F	I-*pls*	064	064	5.50	05	-
RSA 9/43	Dr. Bouwer & Partners	Durban	04/11/2005	R	Blood	67	M	II	012	012	16.12	16	-
RSA 9/44			17/11/2005	R	Blood	41	M	I-*pls*	1257	064	5.55	05	-
RSA 9/45			10/08/2006	R	Pus	36	F	II	046	012	16.30	16	-
RSA 9/46			23/08/2006	R	Blood	33	M	I-*pls*	037	012	5.10	05	-
RSA 9/47			16/08/2006	R	Blood	86	F	IV	032	032	19.6	19	-
RSA 9/48			29/08/2006	R	Pus	53	M	IIIE	012	012	3.25	03	-
RSA 9/49			29/08/2006	R	Pus	53	M	IIIE	037	012	3.27	03	-
RSA 9/50			NA	R	Pus	40	F	I-*pls*	1257	064	5.20	05	-
RSA 9/51			28/08/2006	R	Swab	42	M	I-*pls*	1257	064	5.27	05	-
RSA 9/53			14/08/2006	R	Pus	73	M	IIIE	037	012	NT	NT	-
RSA 9/54			14/08/2006	R	Swab	73	M	IIIE	037	012	3.7	03	-
RSA 9/55			09/08/2006	R	Swab	37	M	IIIE	037	012	3.13	03	-
RSA 9/56			04/07/2006	R	Swab	0	M	Iv	1880	045	1.1	01	-
RSA 9/57			17/08/2006	R	Pus swab	47	M	IV	032	032	19.3	19	-
RSA 9/58			16/08/2006	S (17)	Bile	40	M	MSSA	174	NF: 6	NT	NT	+
RSA 9/60			04/07/2006	R	Swab	0	M	I	1880	045	1.2	01	-
RSA 9/61			14/11/2006	R	Pus	84	M	IIIE	037	012	3.10	03	-
RSA 9/63			13/11/2006	R	Ear	28	F	I-*pls*	064	064	4.2	04	-
RSA 9/64			10/11/2006	R	Pus	34	F	I-*pls*	064	064	8.3	08	-
RSA 9/65			31/03/2006	R	Pus	26	F	I-*pls*	1257	064	5.61	05	-
RSA 9/68			13/11/2006	R	Nose	37	F	I-*pls*	1971	064	5.63	05	-
RSA 9/71			08/08/2006	R	Blood	24	M	I-*pls*	1257	064	5.56	05	-
RSA 9/73			25/10/2006	R	Abdomen	20	F	II	046	012	16.32	16	-

Ox = Oxacillin; R = Resistant; S = Susceptible; NA = Not available; CT = Clonal type; * = Zone diameters (if present), given in millimetre; # = Age given in years; - = Negative; + = Positive; M =Male; F = Female; PVL = Panton-Valentine leukocidin; NF = No founder.

8.5 APPENDIX E: Multiplex PCR Gel Images Obtained After Amplification of Each Isolates' SCC*mec* Element

SCC*mec* gel images obtained after agarose gel electrophoresis. The following strains were used as controls: STA61 (SCC*mec* type I); BK2464 (SCC*mec* type II); ANS46 (SCC*mec* type III); MW2 (SCC*mec* type IV); Neg. C = Negative control. MWM = Molecular weight marker.

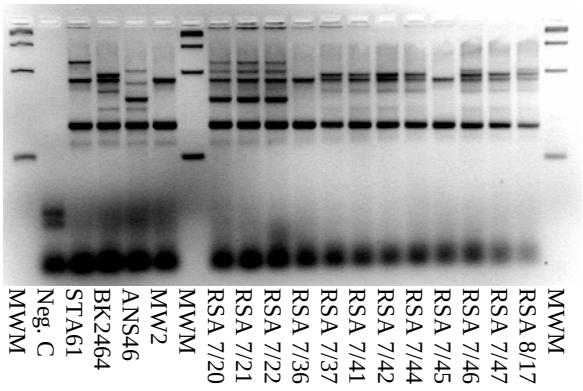

Figure E1: SCC*mec* multiplex PCR gel image #1.

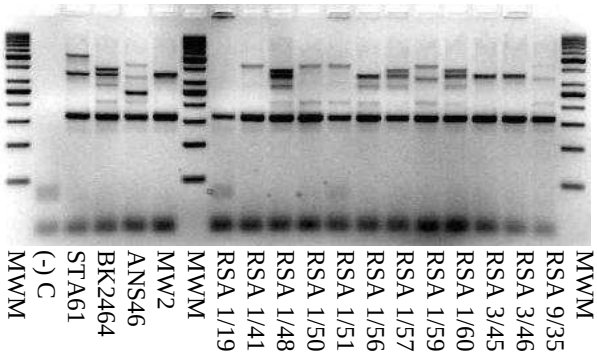

Figure E2: SCC*mec* multiplex PCR gel image #2.

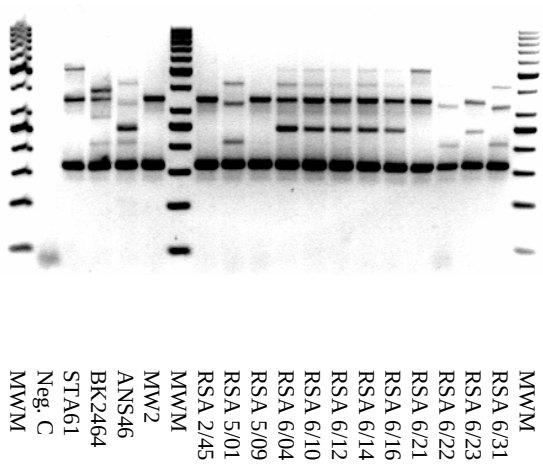

Figure E3: SCC*mec* multiplex PCR gel image #3.

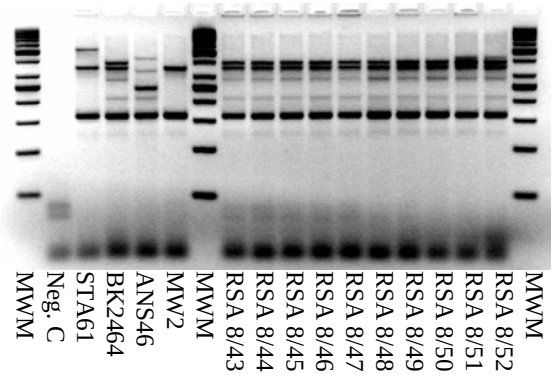

Figure E4: SCC*mec* multiplex PCR gel image #4.

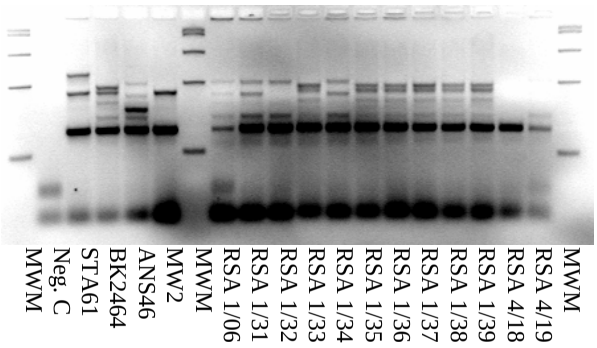

Figure E5: SCC*mec* multiplex PCR gel image #5.

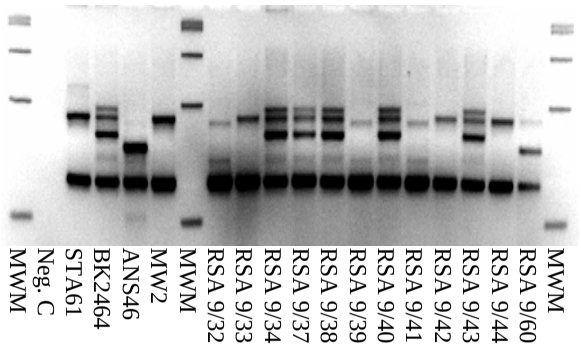

Figure E6: SCC*mec* multiplex PCR gel image #6.

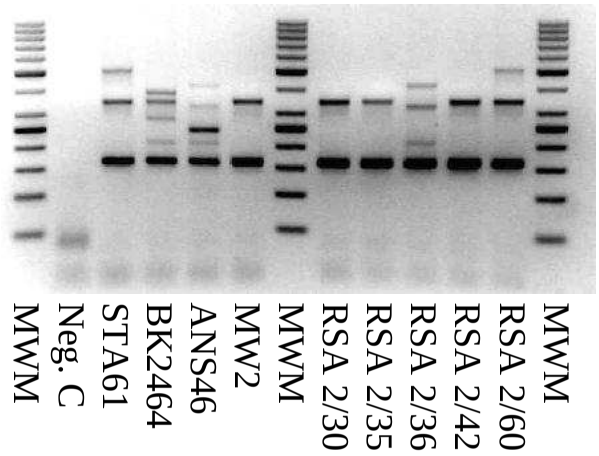

Figure E7: SCC*mec* multiplex PCR gel image #7.

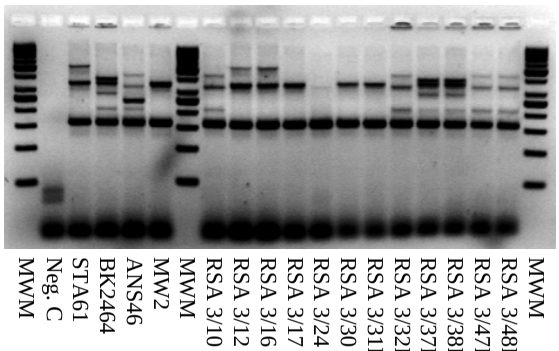

Figure E8: SCC*mec* multiplex PCR gel image #8.

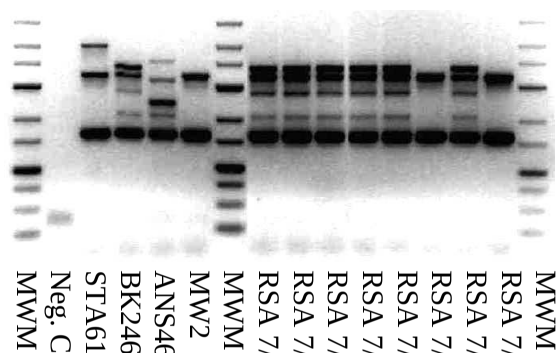

Figure E9: SCC*mec* multiplex PCR gel image #9.

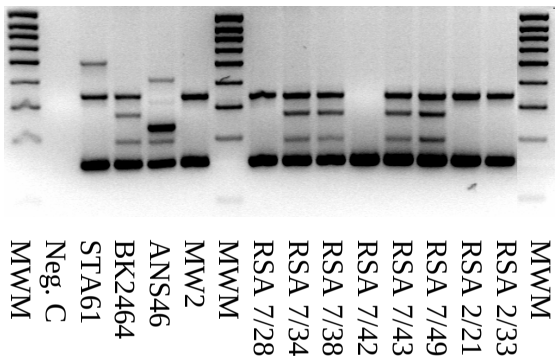

Figure E10: SCC*mec* multiplex PCR gel image #10.

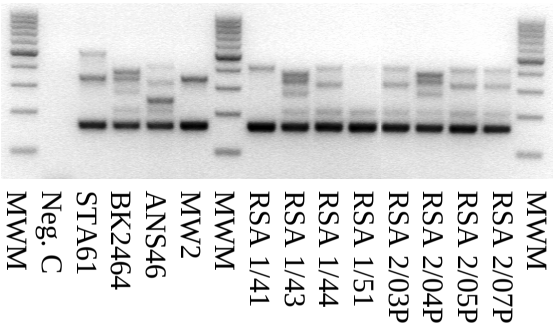

Figure E11: SCC*mec* multiplex PCR gel image #11.

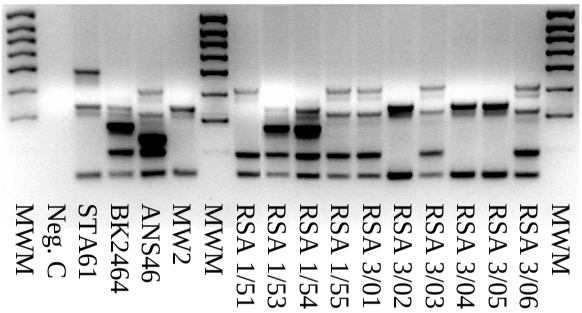

Figure E12: SCC*mec* multiplex PCR gel image #12.

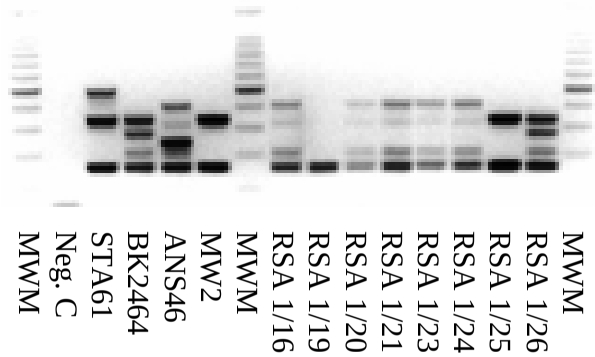

Figure E13: SCC*mec* multiplex PCR gel image #13.

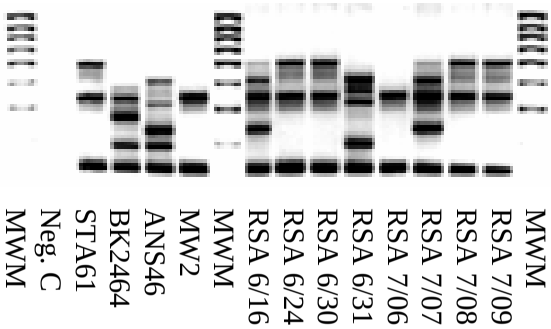

Figure E14: SCC*mec* multiplex PCR gel image #14.

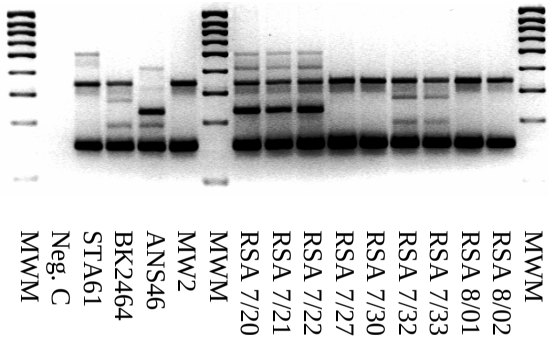

Figure E15: SCC*mec* multiplex PCR gel image #15.

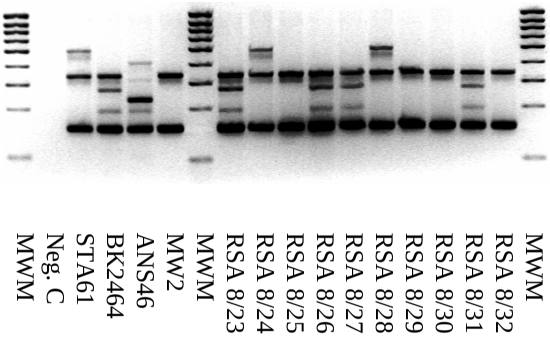

Figure E16: SCC*mec* multiplex PCR gel image #16.

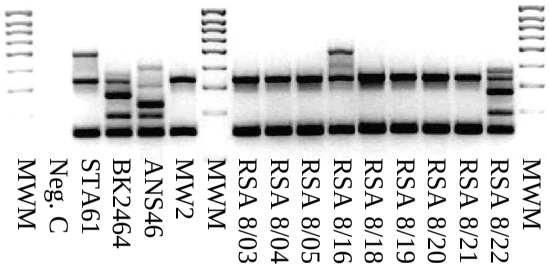

Figure E17: SCC*mec* multiplex PCR gel image #17.

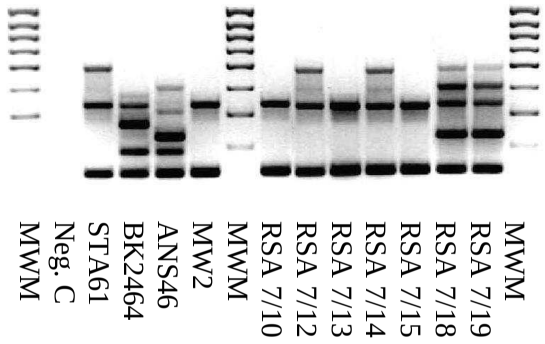

Figure E18: SCC*mec* multiplex PCR gel image #18.

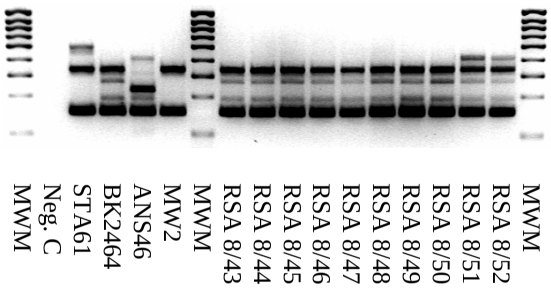

Figure E19: SCC*mec* multiplex PCR gel image #19.

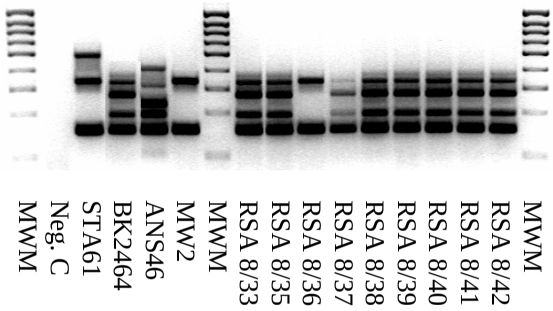

Figure E20: SCC*mec* multiplex PCR gel image #20.

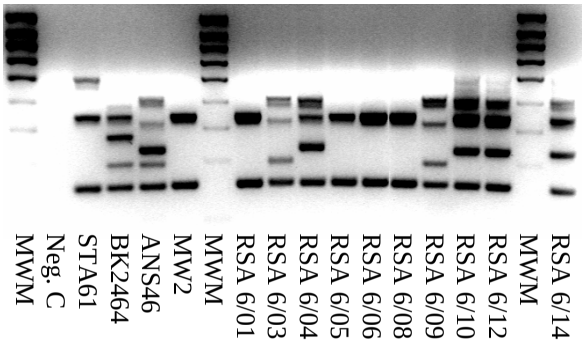

Figure E21: SCC*mec* multiplex PCR gel image #21.

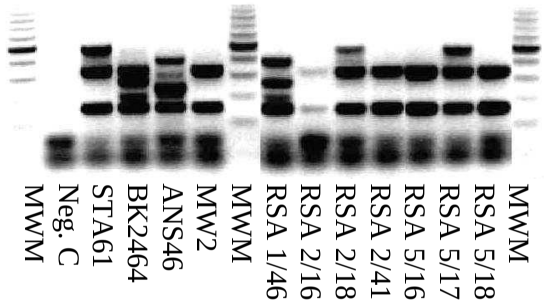

Figure E22: SCC*mec* multiplex PCR gel image #22.

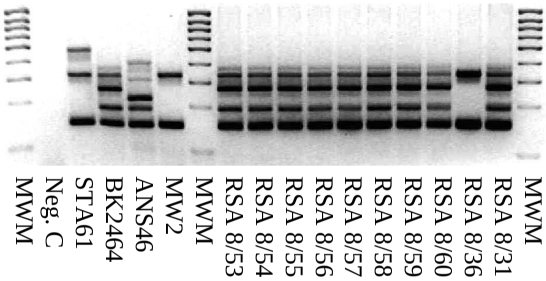

Figure E23: SCC*mec* multiplex PCR gel image #23.

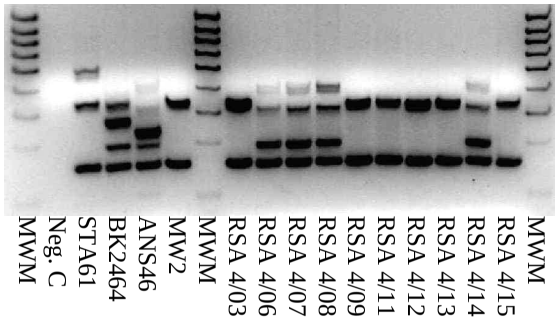

Figure E24: SCC*mec* multiplex PCR gel image #24.

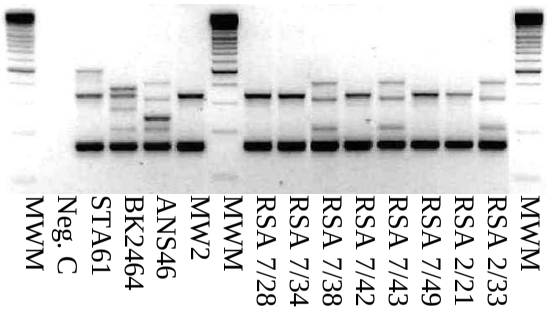

Figure E25: SCC*mec* multiplex PCR gel image #25.

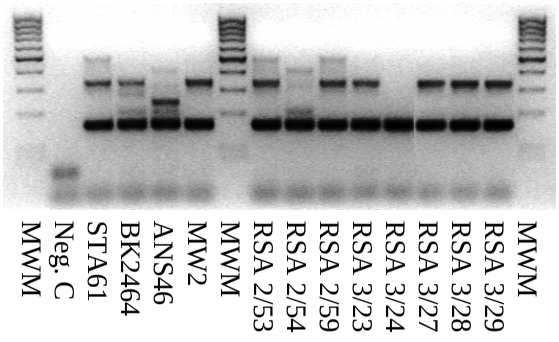

Figure E26: SCC*mec* multiplex PCR gel image #26.

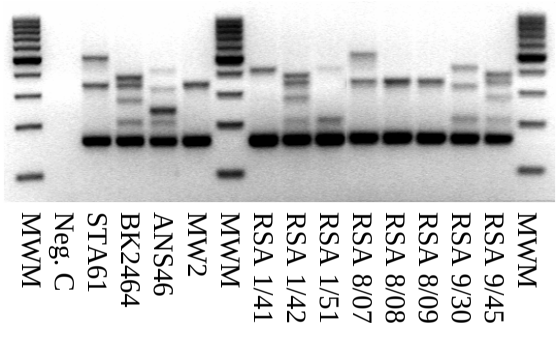

Figure E27: SCC*mec* multiplex PCR gel image #27.

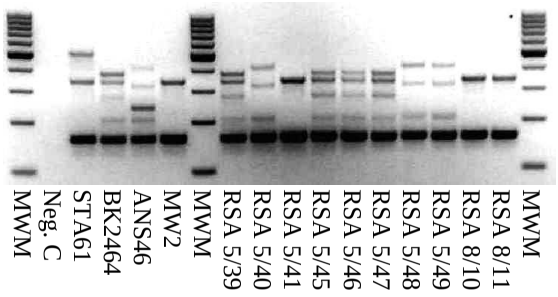

Figure E28: SCC*mec* multiplex PCR gel image #28.

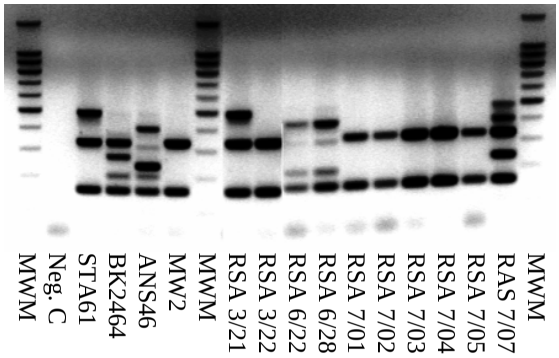

Figure E29: SCC*mec* multiplex PCR gel image #29.

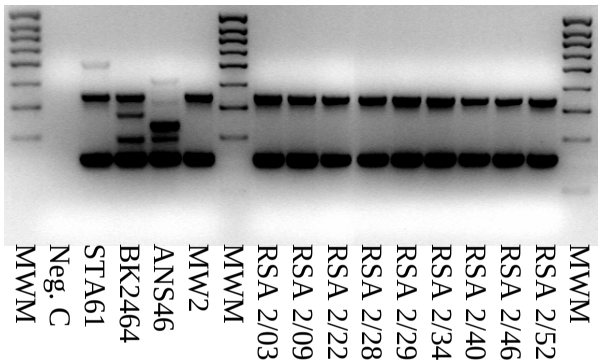

Figure E30: SCC*mec* multiplex PCR gel image #30.

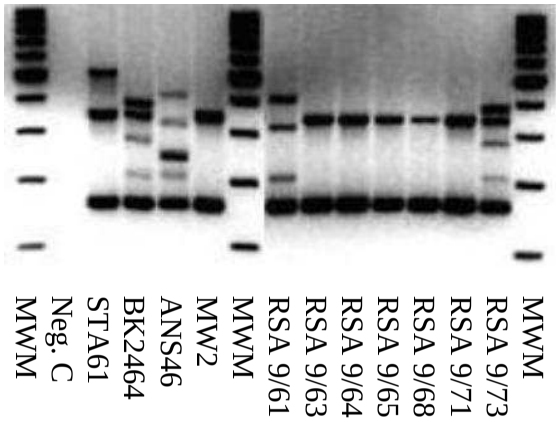

Figure E31: SCC*mec* multiplex PCR gel image #31.

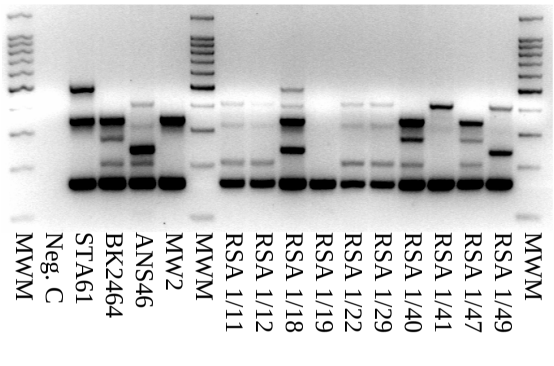

Figure E32: SCC*mec* multiplex PCR gel image #32.

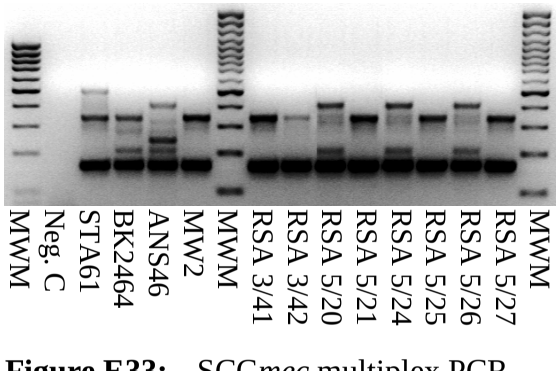

Figure E33: SCC*mec* multiplex PCR gel image #33.

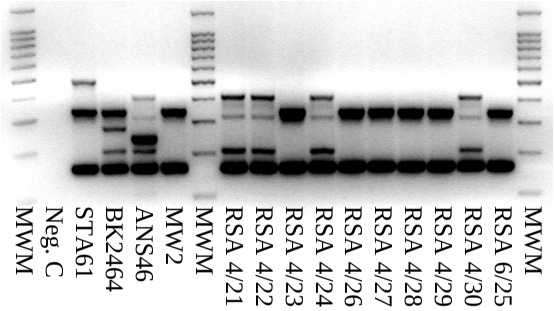

Figure E34: SCC*mec* multiplex PCR gel image #34.

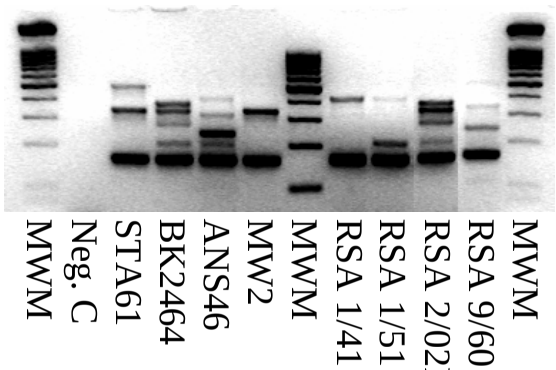

Figure E35: SCC*mec* multiplex PCR gel image #35.

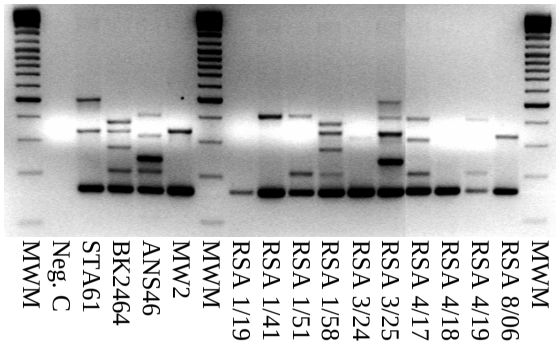

Figure E36: SCC*mec* multiplex PCR gel image #36.

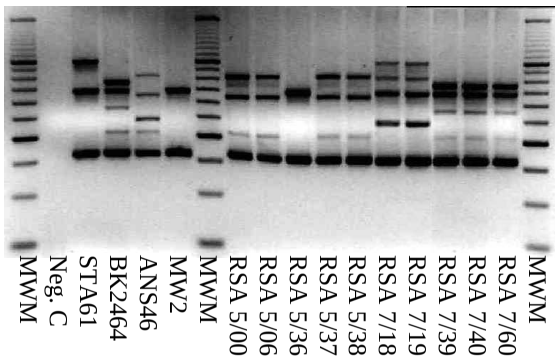

Figure E37: SCC*mec* multiplex PCR gel image #37.

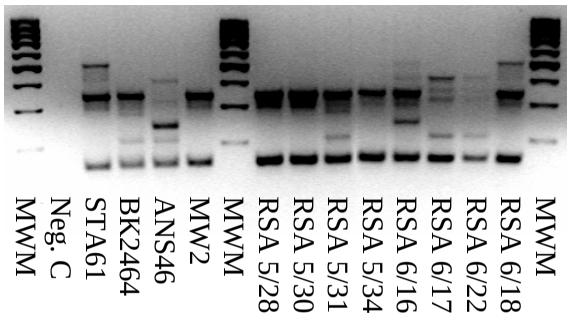

Figure E38: SCC*mec* multiplex PCR gel image #38.

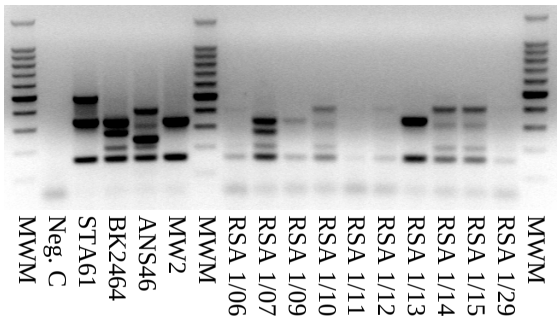

Figure E39: SCC*mec* multiplex PCR gel image #39.

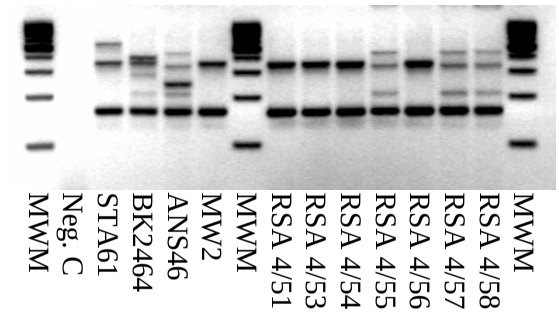

Figure E40: SCC*mec* multiplex PCR gel image #40.

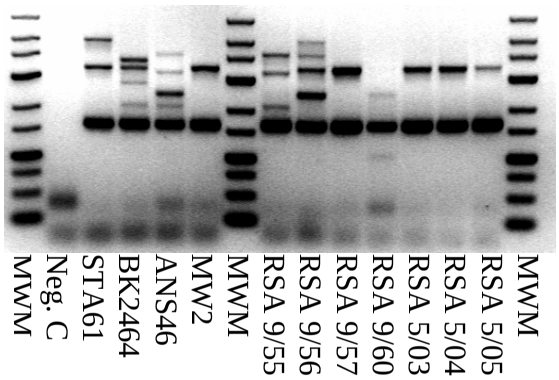

Figure E41: SCC*mec* multiplex PCR gel image #41.

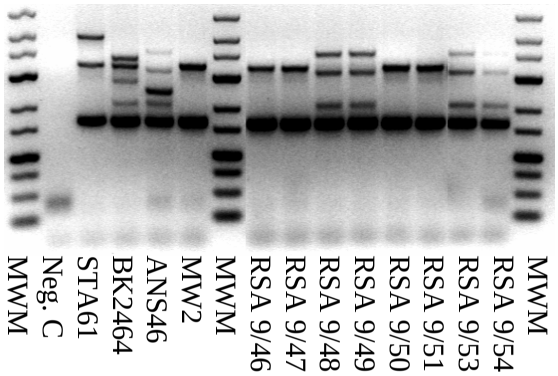

Figure E42: SCC*mec* multiplex PCR gel image #42.

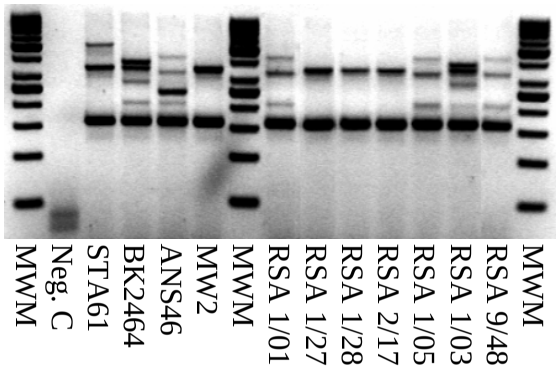

Figure E43: SCC*mec* multiplex PCR gel image #43.

8.6 APPENDIX F: Gel Images Obtained After Agarose Gel Electrophoresis of PVL PCR Experiments

PVL gel images obtained after agarose gel electrophoresis. Strain MW2 was used as the positive control. Lanes indicated with an asterisk were excluded from all analysis. Neg. C = Negative control. MWM = Molecular weight marker.

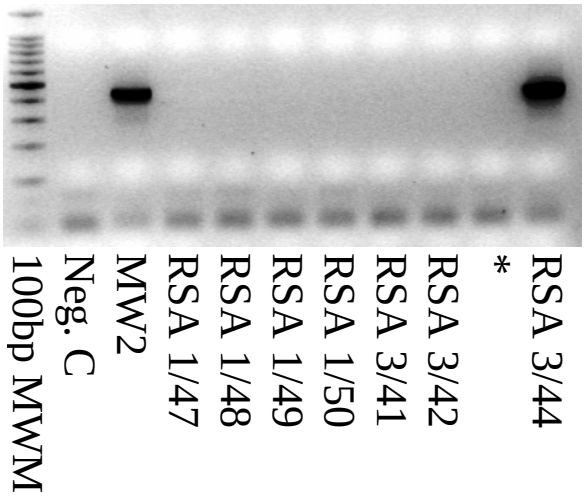

Figure F1: PVL PCR agarose gel electrophoresis image #1.

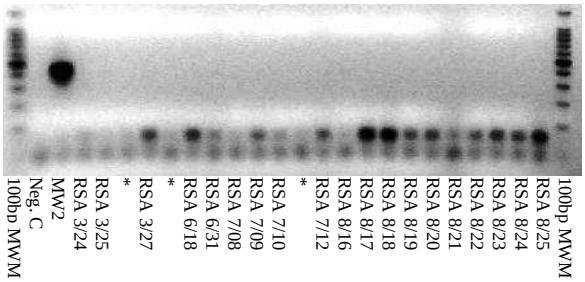

Figure F2: PVL PCR agarose gel electrophoresis image #2.

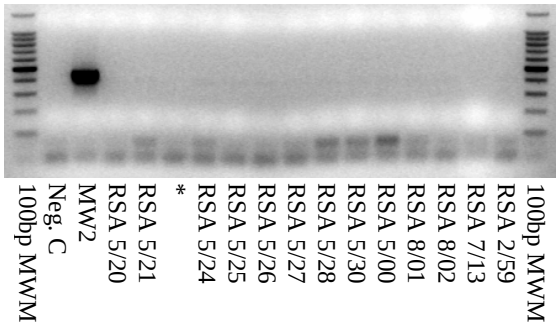

Figure F3: PVL PCR agarose gel electrophoresis image #3.

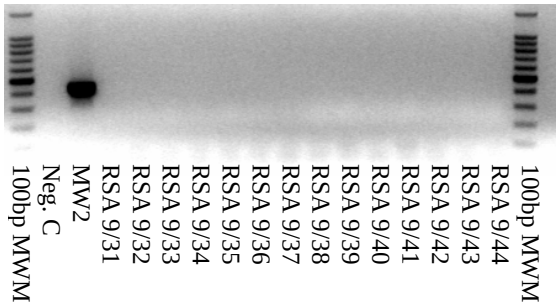

Figure F4: PVL PCR agarose gel electrophoresis image #4.

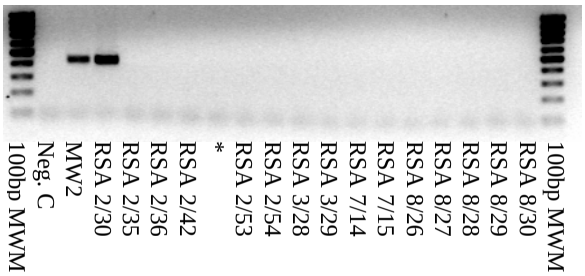

Figure F5: PVL PCR agarose gel electrophoresis image #5.

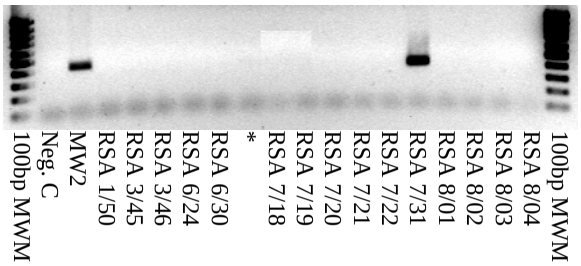

Figure F6: PVL PCR agarose gel electrophoresis image #6.

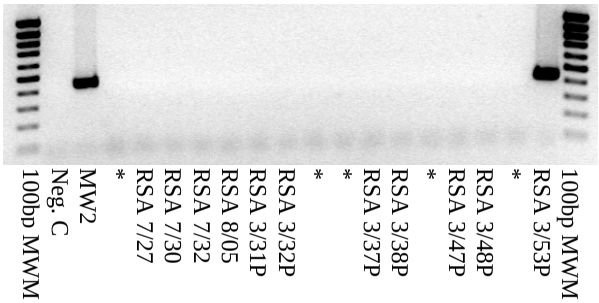

Figure F7: PVL PCR agarose gel electrophoresis image #7.

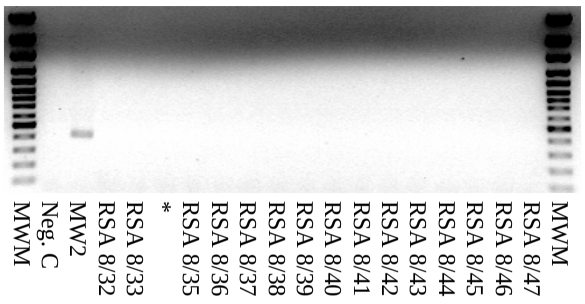

Figure F8: PVL PCR agarose gel electrophoresis image #8.

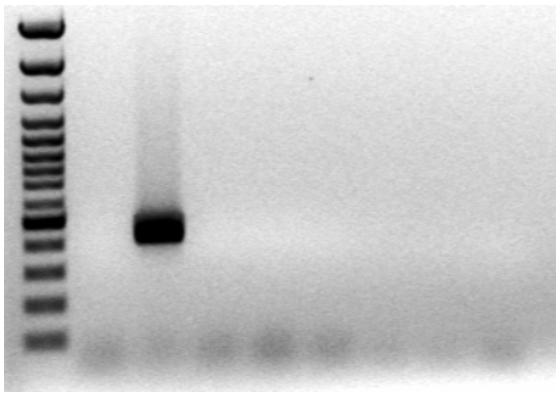

Figure F9: PVL PCR agarose gel electrophoresis image #9.

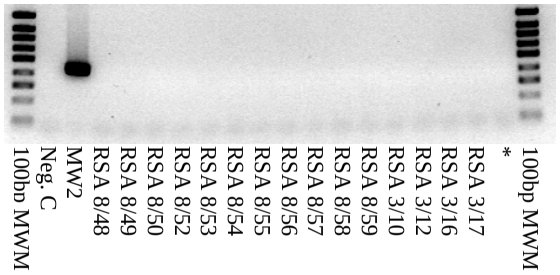

Figure F10: PVL PCR agarose gel electrophoresis image #10.

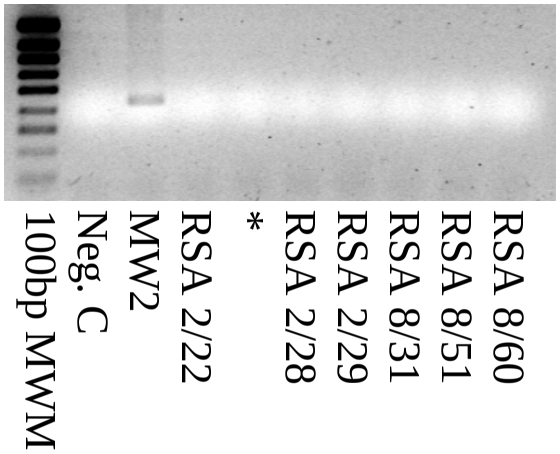

Figure F11: PVL PCR agarose gel electrophoresis image #11.

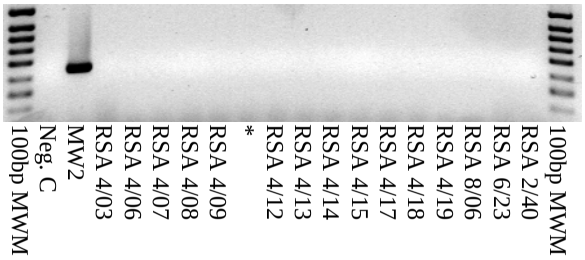

Figure F12: PVL PCR agarose gel electrophoresis image #12.

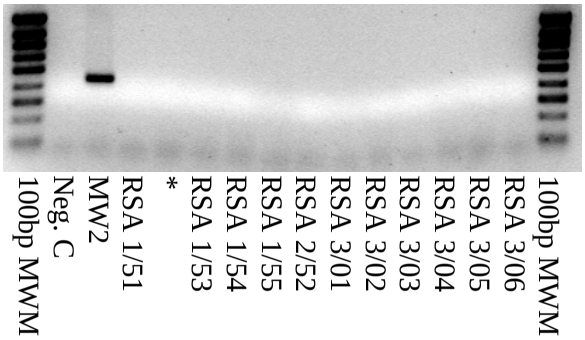

Figure F13: PVL PCR agarose gel electrophoresis image #13.

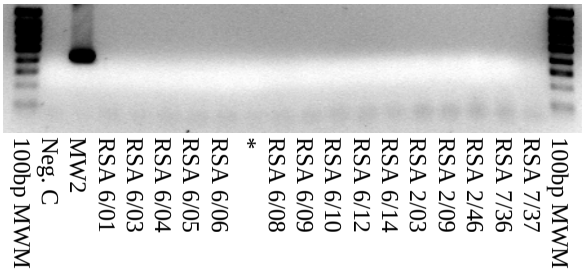

Figure F14: PVL PCR agarose gel electrophoresis image #14.

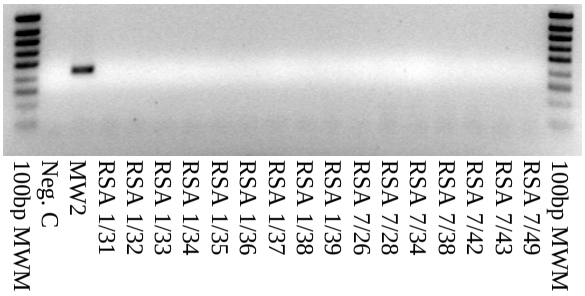

Figure F15: PVL PCR agarose gel electrophoresis image #15.

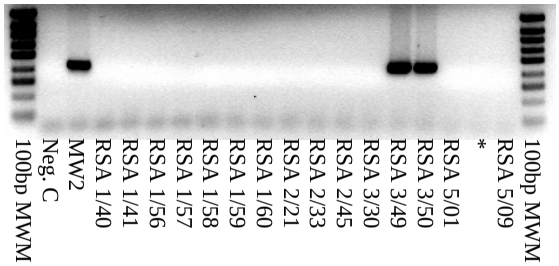

Figure F16: PVL PCR agarose gel electrophoresis image #16.

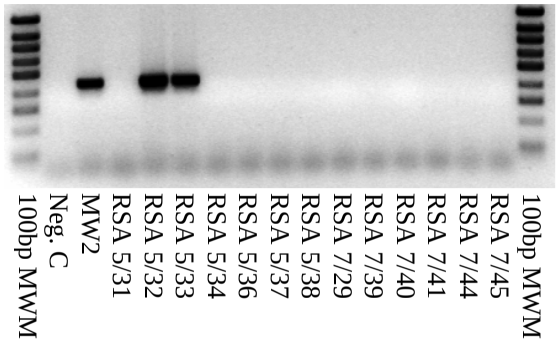

Figure F17: PVL PCR agarose gel electrophoresis image #17.

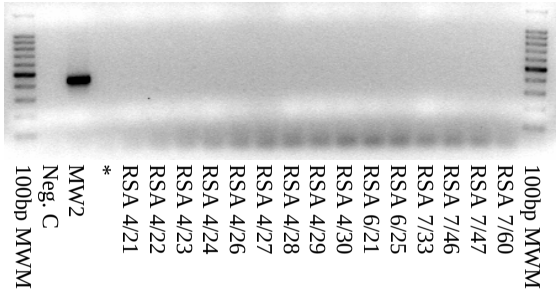

Figure F18: PVL PCR agarose gel electrophoresis image #18.

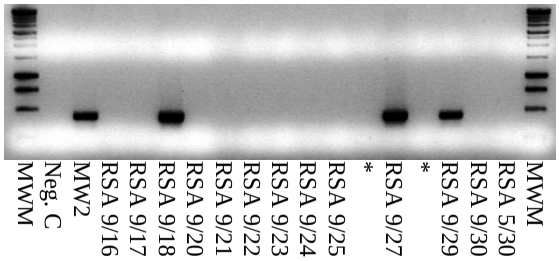

Figure F19: PVL PCR agarose gel electrophoresis image #19.

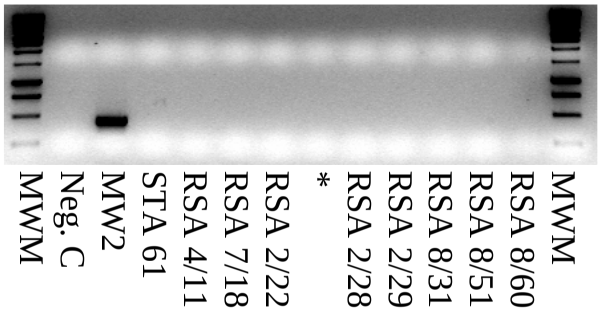

Figure F20: PVL PCR agarose gel electrophoresis image #20.

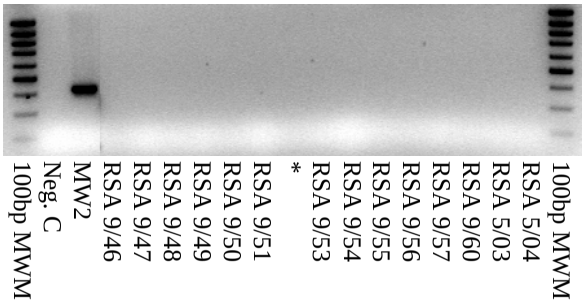

Figure F21: PVL PCR agarose gel electrophoresis image #21.

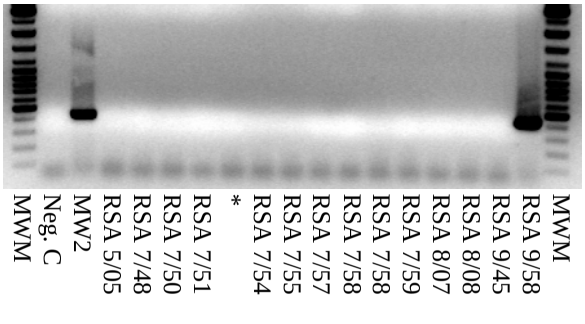

Figure F22: PVL PCR agarose gel electrophoresis image #22.

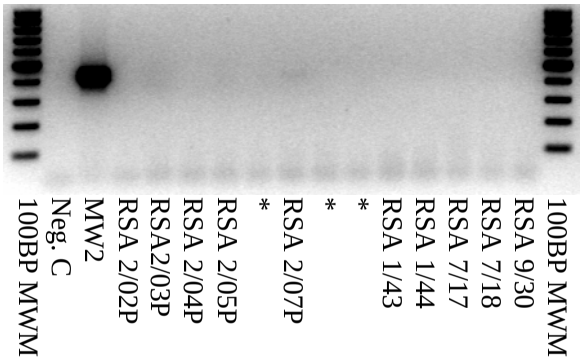

Figure F23: PVL PCR agarose gel electrophoresis image #23.

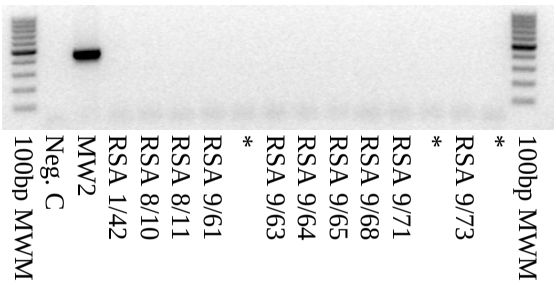

Figure F24: PVL PCR agarose gel electrophoresis image #24.

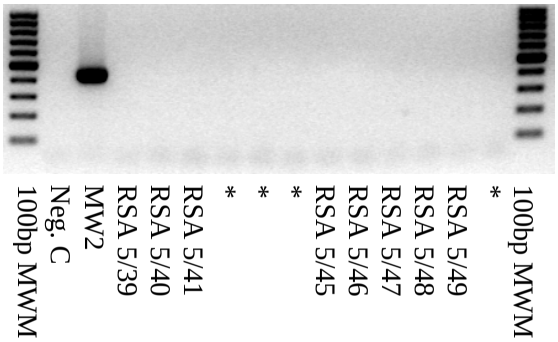

Figure F25: PVL PCR agarose gel electrophoresis image #25.

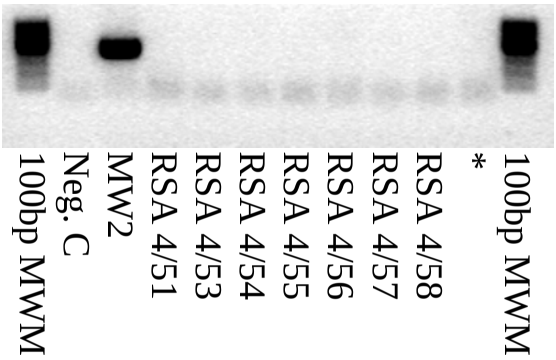

Figure F26: PVL PCR agarose gel electrophoresis image #26.

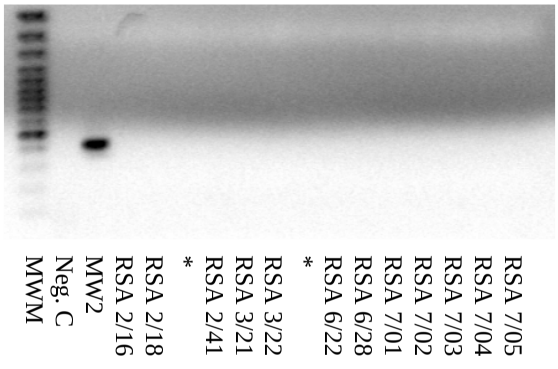

Figure F27: PVL PCR agarose gel electrophoresis image #27.

Figure F28: PVL PCR agarose gel electrophoresis image #28.

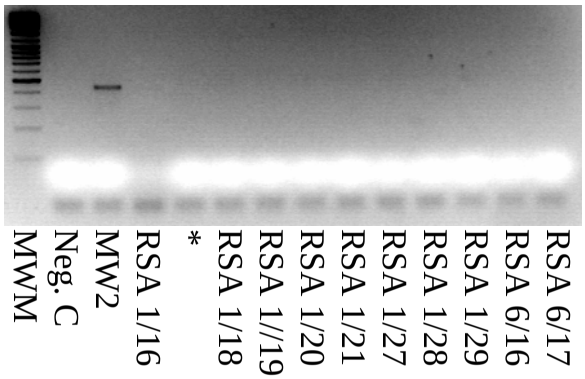

Figure F29: PVL PCR agarose gel electrophoresis image #29.

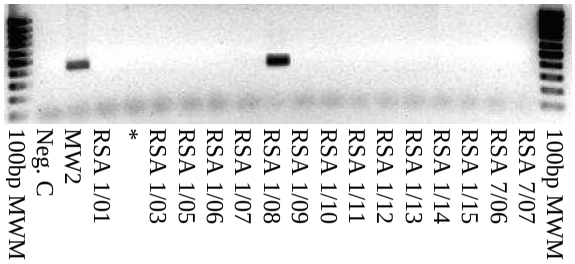

Figure F30: PVL PCR agarose gel electrophoresis image #30.

8.7 APPENDIX G: PCR Gel Images Obtained After Amplification of the SSR Region of the *spaA* Gene of Each Isolate

spaA gel images obtained after agarose gel electrophoresis. Lanes indicated with an asterisk were not included in analysis. Strain STA61 was used as the positive control. MWM = Molecular weight marker.

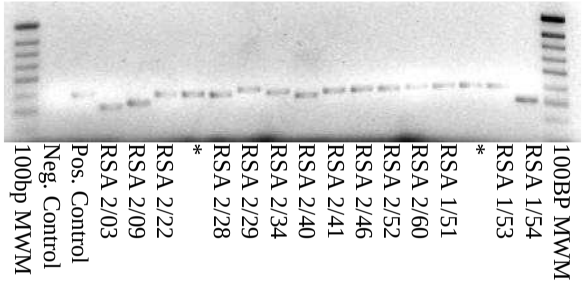

Figure G1: *spaA* PCR agarose gel electrophoresis image #1.

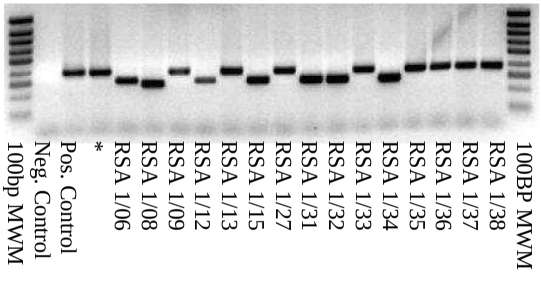

Figure G3: *spaA* PCR agarose gel electrophoresis image #3.

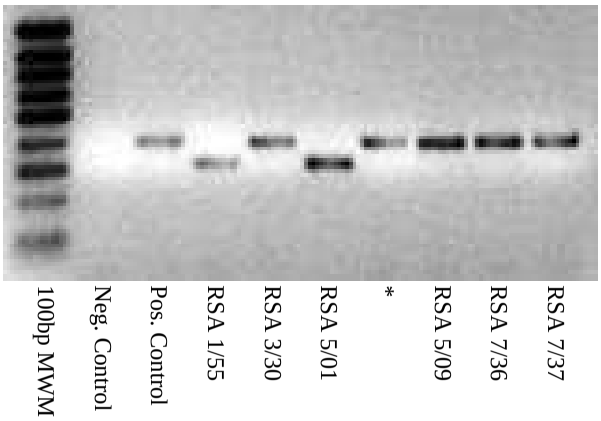

Figure G2: *spaA* PCR agarose gel electrophoresis image #2.

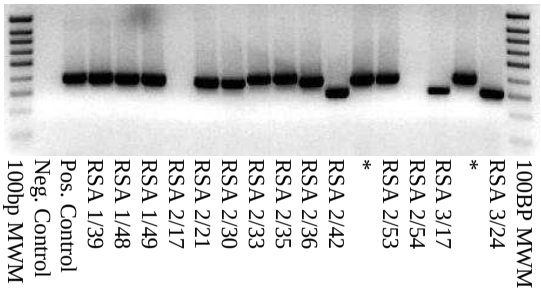

Figure G4: *spaA* PCR agarose gel electrophoresis image #4.

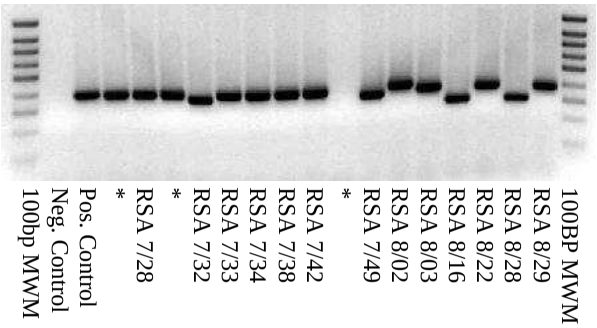

Figure G5: *spaA* PCR agarose gel electrophoresis image #5.

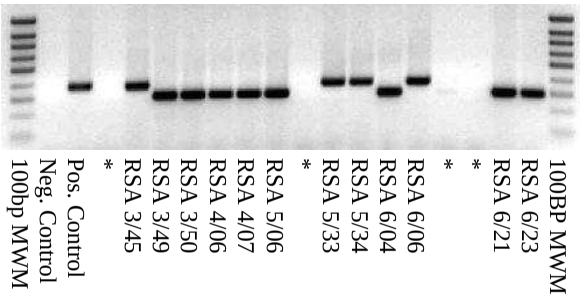

Figure G6: *spaA* PCR agarose gel electrophoresis image #6.

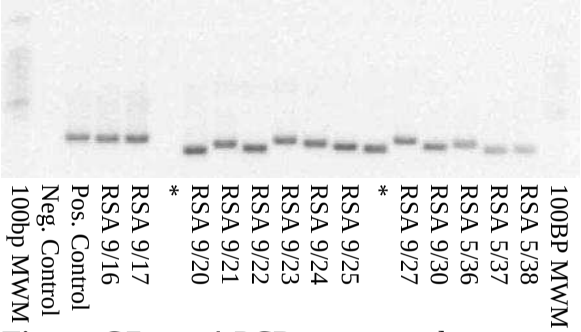

Figure G7: *spaA* PCR agarose gel electrophoresis image #7.

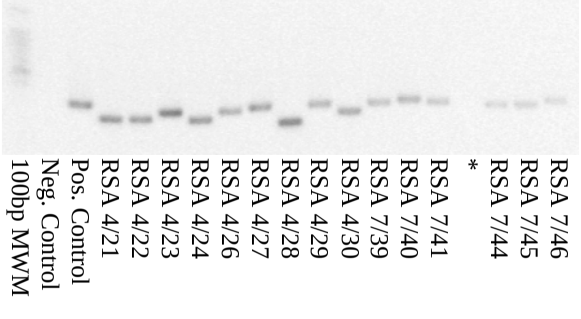

Figure G8: *spaA* PCR agarose gel electrophoresis image #8.

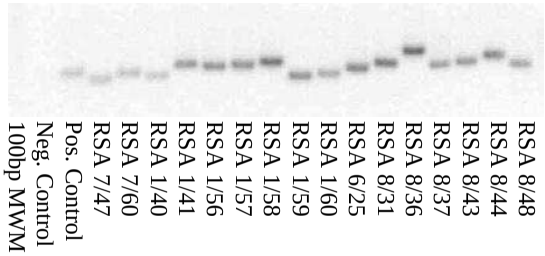

Figure G9: *spaA* PCR agarose gel electrophoresis image #9.

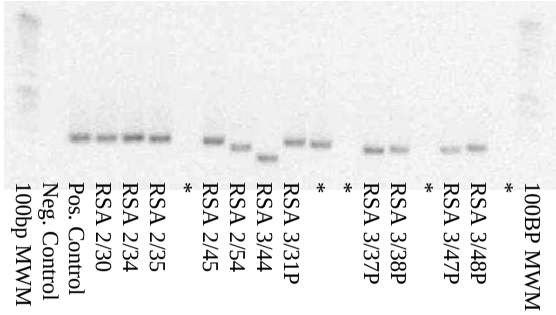

Figure G10: *spaA* PCR agarose gel electrophoresis image #10.

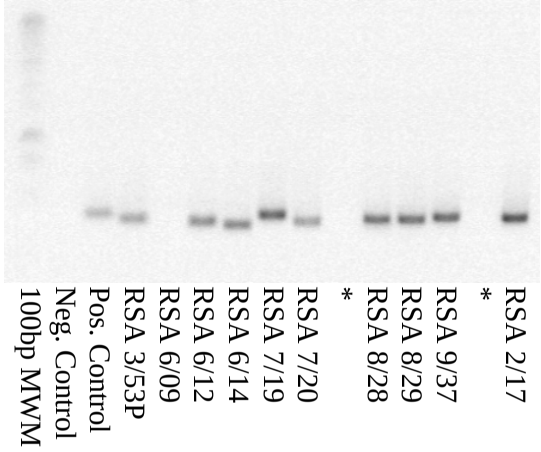

Figure G11: *spaA* PCR agarose gel electrophoresis image #11.

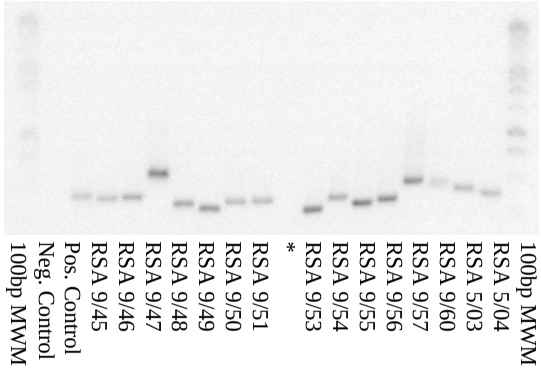

Figure G12: *spaA* PCR agarose gel electrophoresis image #12.

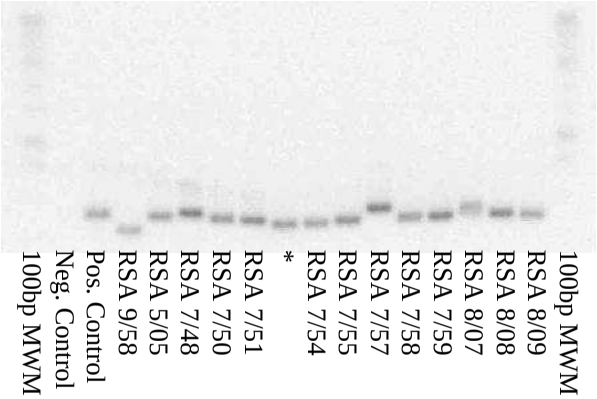

Figure G13: *spaA* PCR agarose gel electrophoresis image #13.

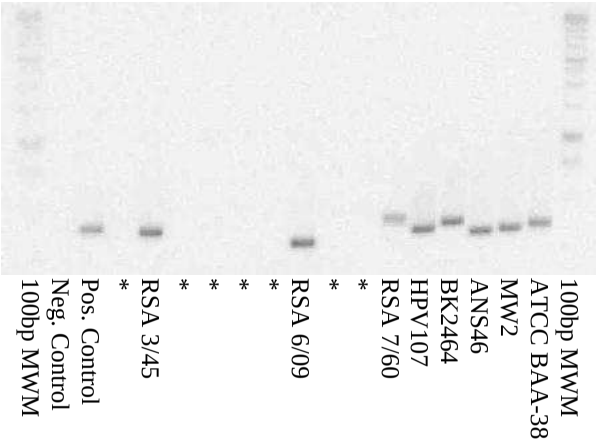

Figure G14: *spaA* PCR agarose gel electrophoresis image #14.

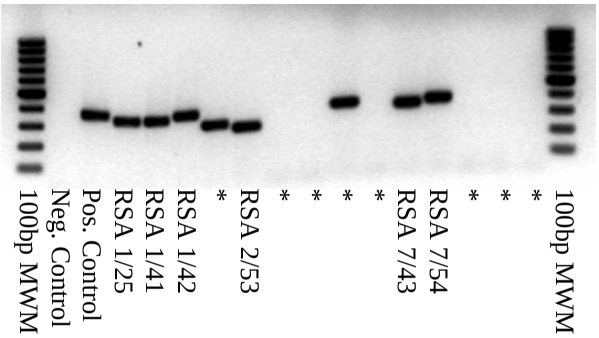

Figure G15: *spaA* PCR agarose gel electrophoresis image #15.

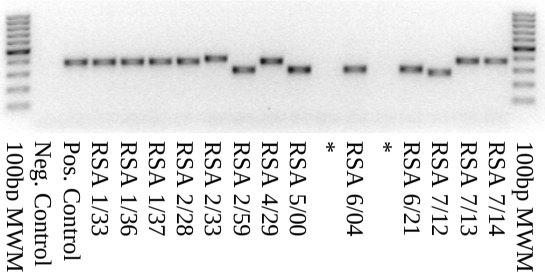

Figure G16: *spaA* PCR agarose gel electrophoresis image #16.

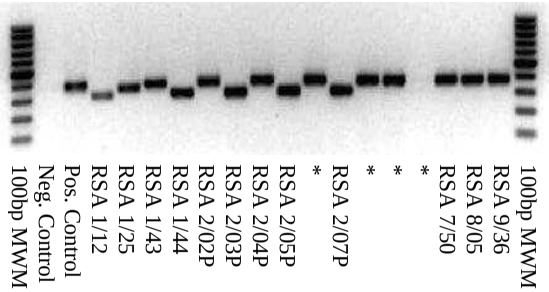

Figure G17: *spaA* PCR agarose gel electrophoresis image #17.

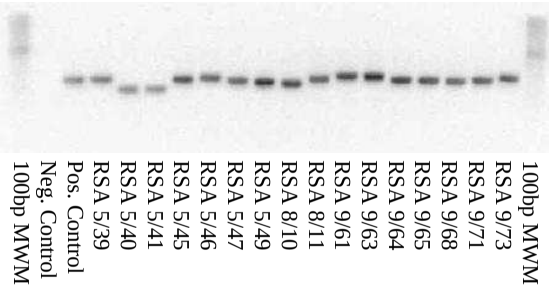

Figure G18: *spaA* PCR agarose gel electrophoresis image #18.

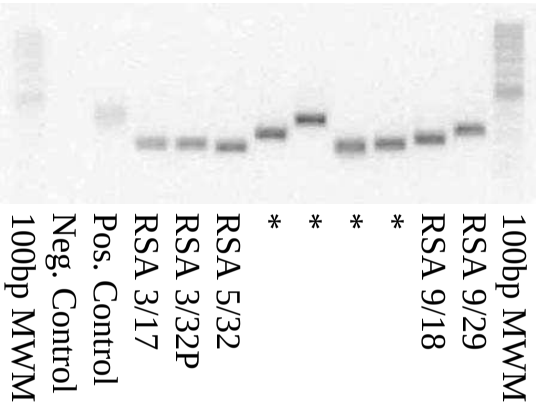

Figure G19: *spaA* PCR agarose gel electrophoresis image #19.

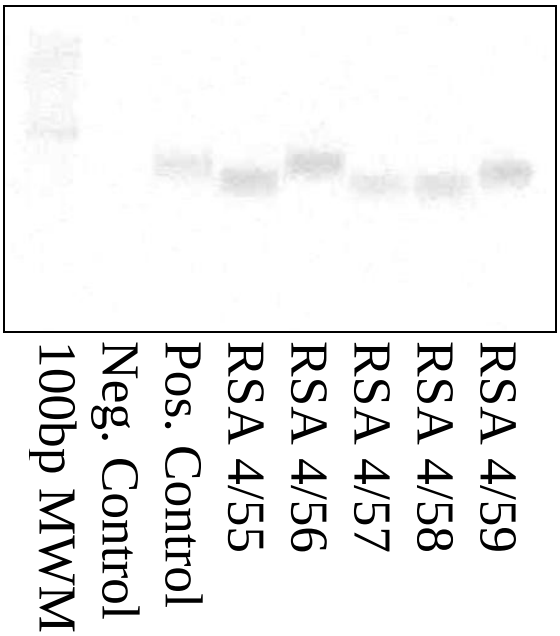

Figure G23: *spaA* PCR agarose gel electrophoresis image #23.

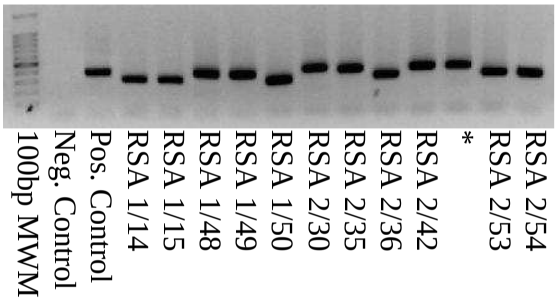

Figure G24: *spaA* PCR agarose gel electrophoresis image #24.

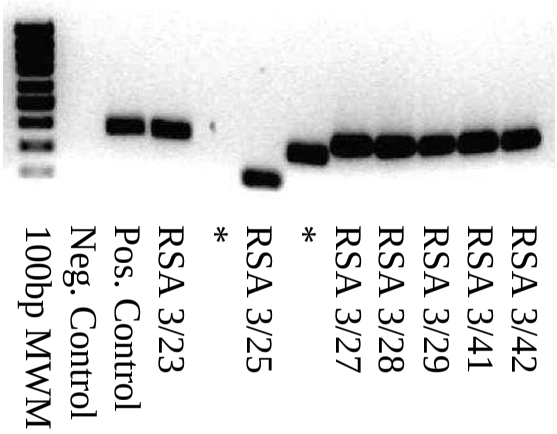

Figure G25: *spaA* PCR agarose gel electrophoresis image #25.

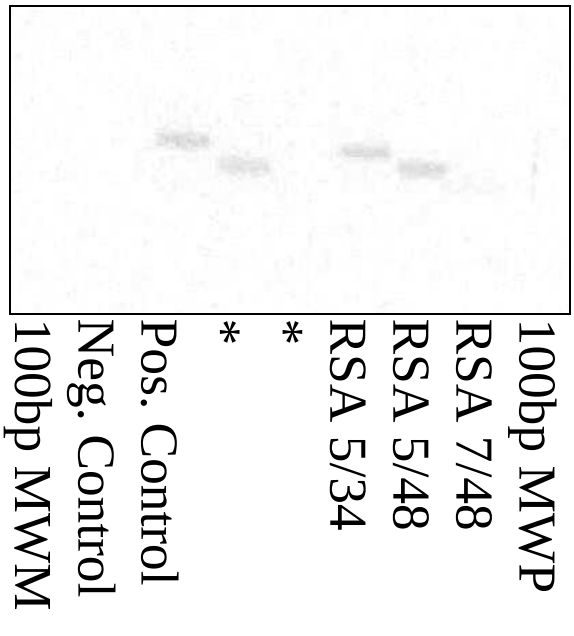

Figure G20: *spaA* PCR agarose gel electrophoresis image #20.

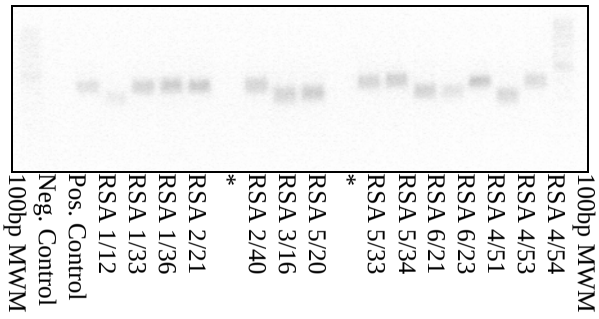

Figure G21: *spaA* PCR agarose gel electrophoresis image #21.

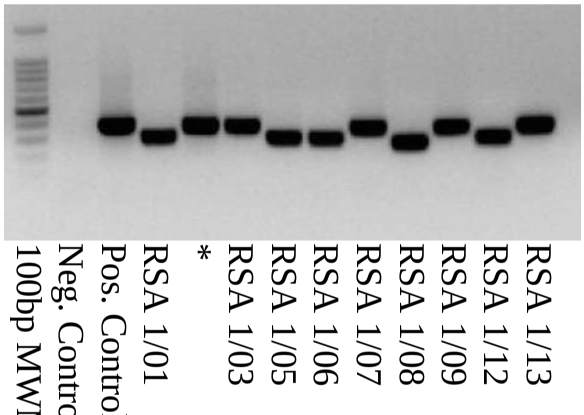

Figure G22: *spaA* PCR agarose gel electrophoresis image #22.

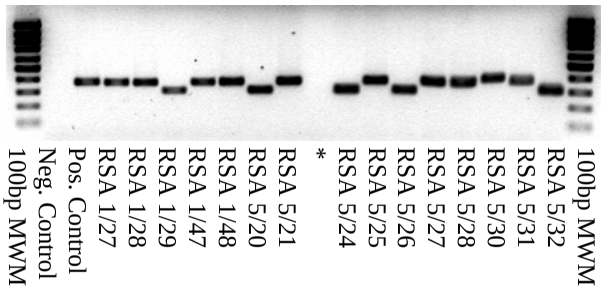

Figure G26: *spaA* PCR agarose gel electrophoresis image #26.

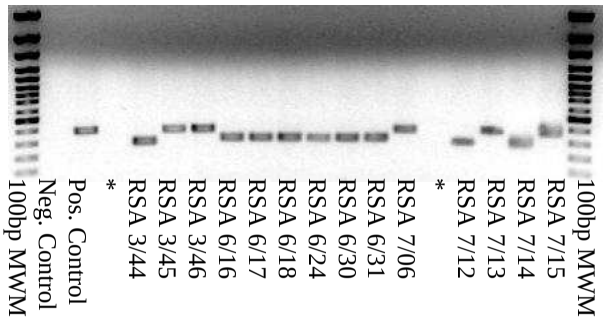

Figure G27: *spaA* PCR agarose gel electrophoresis image #27.

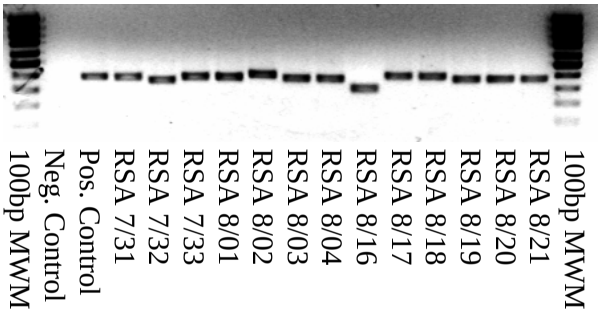

Figure G28: *spaA* PCR agarose gel electrophoresis image #28.

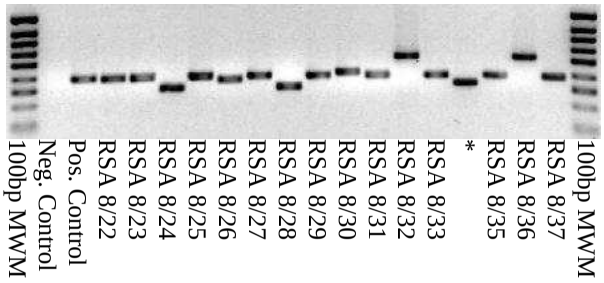

Figure G29: *spaA* PCR agarose gel electrophoresis image #29.

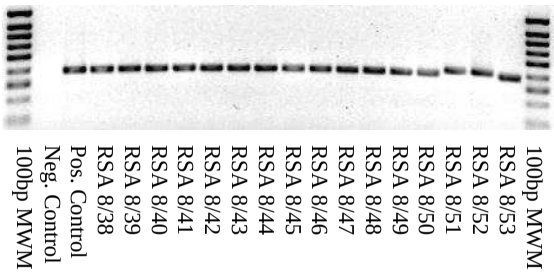

Figure G30: *spaA* PCR agarose gel electrophoresis image #30.

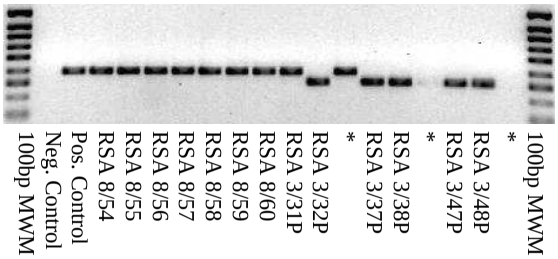

Figure G31: *spaA* PCR agarose gel electrophoresis image #31.

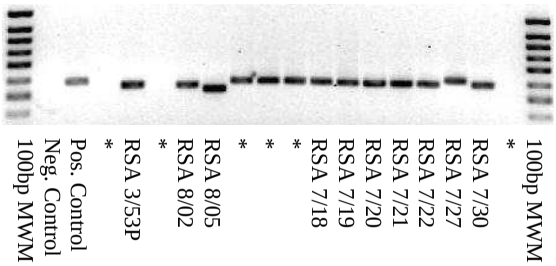

Figure G32: *spaA* PCR agarose gel electrophoresis image #32.

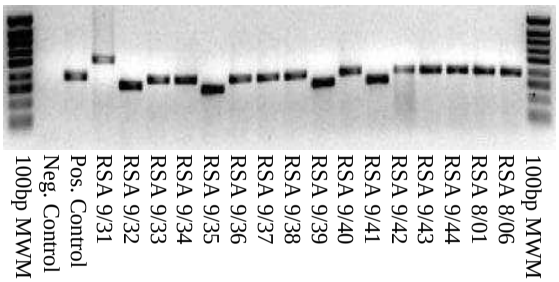

Figure G33: *spaA* PCR agarose gel electrophoresis image #33.

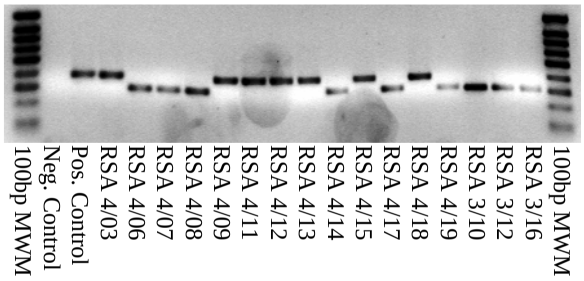

Figure G34: *spaA* PCR agarose gel electrophoresis image #34.

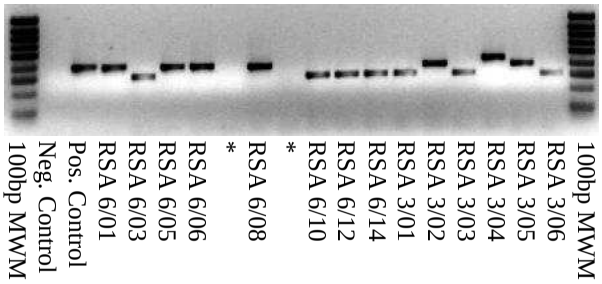

Figure G35: *spaA* PCR agarose gel electrophoresis image #35.

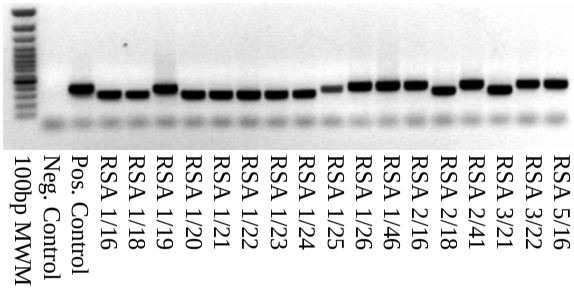

Figure G36: *spaA* PCR agarose gel electrophoresis image #36.

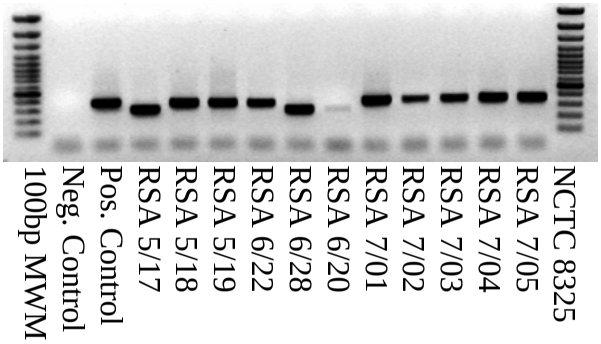

Figure G37: *spaA* PCR agarose gel electrophoresis image #37.

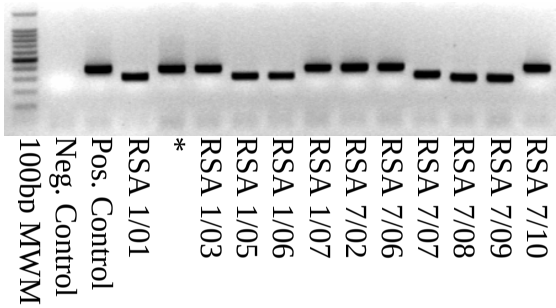

Figure G38: *spaA* PCR agarose gel electrophoresis image #38.

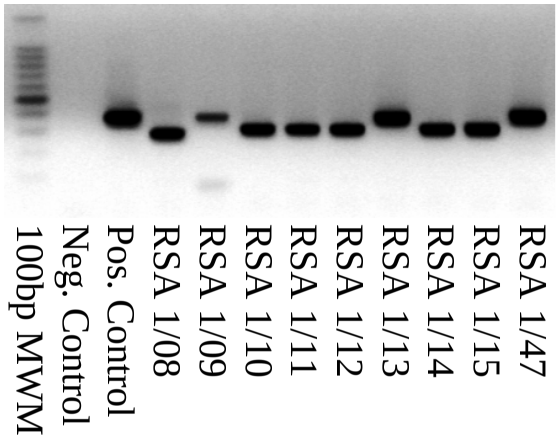

Figure G39: *spaA* PCR agarose gel electrophoresis image #39.

8.8 APPENDIX H: Nucleotide Sequences of the Various Repeat Units of the SSR Region of the *spaA* Gene

RIDOM repeat ID	Kreiswirth ID	Sequence
r01	-	GAGGAAGACAACAACAAGCCTAGC
r02	A1	AAAGAAGACAACAAAAAACCTGGC
r03	D2	GAGGAAGACAATAACAAACCTGGT
r04	Z1	GAGGAAGACAATAACAAGCCTGGT
r05	C1	AAAGAAGACAACAAAAAGCCTGGC
r06	G2	AAAGAAGACGGCAAAAAACCTGGC
r07	U1	GAGGAAGACAACAACAAACCTGGT
r08	X1	GAGGAAGACAACAACAAGCCTGGT
r09	A2	GAGGAAGACGGCAACAAACCTGGT
r10	C2	AAAGAAGACAATAACAAGCCTGGT
r11	Y1	GAGGAAGACAATAACAAGCCTGGC
r12	G1	AAAGAAGACAACAACAAGCCTGGT
r13	E1	AAAGAAGACAACAACAAACCTGGT
r14	I2	GAGGAAGACAACAACAAACCTGGC
r15	W1	GAGGAAGACAACAACAAGCCTGGC
r16	K1	AAAGAAGACGGCAACAAACCTGGT
r17	M1	AAAGAAGACGGCAACAAGCCTGGT
r18	H2	AAAGAAGATGGCAACAAGCCTAGT
r19	H1	AAAGAAGACAATAACAAGCCTGGC
r20	D1	AAAGAAGACAACAACAAACCTGGC
r21	F1	AAAGAAGACAACAACAAGCCTGGC
r22	L1	AAAGAAGACGGCAACAAGCCTGGC
r23	J1	AAAGAAGACGGCAACAAACCTGGC
r24	Q1	AAAGAAGATGGCAACAAGCCTGGT
r25	O1	AAAGAAGATGGCAACAAACCTGGT
r26	T1	GAGGAAGACAACAAAAAACCTGGT
r28	R1	AAAGAAGATGGTAACAAACCTGGC
r29	F2	AAAGAAGACAACAAAAAGCCTAGC
r30	O2	AAAGAAGACGGCAACAAAAAACCTGGT
r31	N1	AAAGAAGATGGCAACAAACCTGGC
r32	E2	AAAGAAGACAGCAACAAGCCTGGC
r33	P1	AAAGAAGATGGCAACAAGCCTGGC
r34	B1	AAAGAAGACAACAAAAAACCTGGT
r35	C3	GAGGAAGACAACAAAAAACCTGGC
r36	W2	AAAGAAGACAACAAAAAGCCTGGT
r37	D3	AAAGAAGACGGCAACAACAAACCTGGC
r38	F3	AAAGAAGACTACAAAAAACCTGGT
r39	E3	AAAGAAGACAGCAACAAACCTGGT
r41	U2	CAAGAAGACGGCAACAAGCCTGGT
r42	M2	AAAGAAGACAACATCAAACCTGGT
r43	X2	AAAGAAGACGGTAACAAACCTGGT
r44	Z2	AAAGAAGACAACAAGCCTGGT

r45	A3	AAAGAAGATGGCAACAGACCTGGC
r46	Y3	AAACAAGACAACAAAAAACCTGGT
r47	Z3	AAAGAAGACAACAACAAAAAACCTGGC
r48	V2	CAAGAAGACAACAACAAGCCTGGT
r49	Y2	GAGGAAGACAACAAACCTGGC
r50	T2	AAAGAAGACGGCAACAAGCCTAGT
r51	P2	AAAGAAGACAACAAACCTGGT
r52	R3	AAAGAAGACAACAAAAAACTTGGC
r53	G3	AAAGAAGACGTCAACAAACCTGGC
r54	H3	AAAGAAGACAACAAGCCTGGC
r55	A4	CAAGAAGACAACAACAAACCTGGC
r56	J2	AAGGAAGACAACAACAAGCCTGGT
r57	S1	AAAGAAGACAACAATAAGCCTGGC
r58	B4	AAAGAATACGGCAACAAGCCTGGT
r59	T3	AAAGAAGACAGTAACAAACCTGGC
r60	S2	GAGGAAGACAACAAACCTGGT
r61	W3	AAAGGAGATGGCAACAAGCCTGGC
r62	U3	AAAGAAGACGGCAATAAACCTGGC
r63	V3	GAGGAAAACAACAACAAGCCTGGT
r64	X3	AAAGAAGACAATAAAAAACCTGGT
r65	S3	AAAGAAGATGGCAAAAAACCTGGT
r66	F4	AAAGAAGACAGCAACAAGCCTGGT
r68	E4	AAAGAAGACGGTAACAAGCCTGGT
r69	C4	AAAGATGACGGCAACAAGCCTGGT
r70	D4	AAAGATGACGGCAACAAACCTGGT
r71	Q3	AAAGAAGATGGAAACAAGCCTGGT
r72	P3	AAAGAATACAACAACAAGCCTGGT
r73	G4	AAAGAAGATAACAACAAGCCTGGC
r74	H4	AATGAAGATGGCAACAAGCCTGGC
r75	I4	AAAGAAGATGGTAACAAACCTGGT
r76	K3	AAAGAAGACGACAACAAACCTGGT
r77	-	AAAGAAGACGGCAACAAGCCTGAT
r78	J4	AAAGAATACGGCAACAAACCTGGT
r80	K4	AAAGAAGACAACAATAAACCTGGT
r81	-	AAAGAAGACAGCAAAAAACCTGGT
r82	-	AAAGAAGACAGCAACAAACCTGGC
r83	-	AAAGAAGACGGCAAAAAACCTGGT
r84	-	AAAGAAGATGGCAACAAACCTGAC
r85	-	AAAGAAGACGGCAATAAACCTGGT
r86	-	AAAGAAGACGACAAAAAACCTGGC
r87	-	AAAGAAGATGGCAACAAGCCTGAT
r88	-	AAAGAAGACGGCAGCAAGCCTGGT
r89	-	GAAGAAGACGGCAACAAACCTGGT
r90	I1	AAAGAAGGCAACAAAAAACCTGGT
r91	-	AAATAAGACAACAAAAAACCTGGT
r92	-	AAAGAAGACAACAACAAACATGGC
r93	-	CAAGAAGACAACAACAAGCCTGGC
r94	-	CAAGAAGACAACAACAAACCTGGT

r95	-	GAGGAATACAATAACAAGCCTGGC
r96	-	AAAGAAGACGGCAACAAAACTGGT
r97	-	AAAGAAGACACCAACAAGCCTGGT
r98	-	AAAAAAGACGGCAACAAACCTGGT
r99	-	GAGGAAGACAATAACAAGCCTAGT
r100	-	AAAGAAGACAACAAAAAACCTGAC
r101	-	AAATAAGACAACAACAAGCCTGGT
r102	-	AAAGAAGACAACAACAAACCTGAT
r103	-	AAAGAAGACGGCAACAAACCTGGG
r104	-	AAAGAAGGCGGCAACAAACCTGGT
r105	-	AAAGAAGATAACAAAAAACCTGGT
r106	-	GAGCAAGACAACAACAAGCCTGGT
r107	-	AAAGAAGACGGCAACAAACCTGAC
r108	-	AAAAAAGACAACAACAAACCTGGT
r109	-	AAAGAAGACGGCAACAAACCTAGC
r110	-	AAAGAAGACGACAACAAGCCTGGT
r111	-	AAAGAAGATGGCAACAAACTTGGT
r112	-	AAAGAAGATGGAAACAAGCCTGGC
r113	-	AAAGAAGACAACAAAAAACCCGGT
r114	-	AAAGAAGACGGTAACAAACCTGGC
r115	-	AAAGAAGACAACAAAAAGCCTACC
r116	-	AAATAAGACAATAACAAGCCTGGC
r117	-	AAAGAAGATGGTAACAAGCCTGGT
r118	-	AAAGAAGACGTCAACAAGCCTGGT
r119	-	AAATAAGACGGCAACAAGCCTGGT
r120	-	AAAGAAGACAACAAAAAACCTGAT
r121	-	GAGGAAGACAACAAGCCTGGT
r122	-	GAGGAAGACAACAAAAAACCTGGA
r123	-	GAGGAAGACAACAACAATCCTGGC
r124	-	GAGGAAGGCAACAAAAAACCTGGT
r125	-	GAGGAAGACGGCAACAAACCTGGC
r126	-	GAGGAAGACAACAAAAAGCCTAGC
r127	-	AAAGAAGACAACAAAAAACCTGTC
r128	-	AAAGAAGACAACAACAAGCCTAGT
r129	-	AAAGAACACAACAAAAAACCTGGT
r130	-	AAAGAAGACGGCAACAAGTCTGGT
r131	-	AAAGAAGACAACAACAAGCTTGGC
r132	-	AAAGAAGACGGCAACAAGCCTAGC
r133	-	AAAAAAGACGGCAACAAGCCTGGT
r134	-	AAAGAAGGCAACAAGCCTGGC
r135	-	GAGGAAGACAACAAAAAGCCTGGT
r136	-	GAGGAAGACAACAACAAATCTGGT
r137	-	GAGGAAGGCAATAACAAGCCTGGC
r138	-	AAAGAAGACAACAAAAAACTTGGT
r139	-	AAAGAAGACAACAACAACCCTGGT
r140	-	GGAGAAGACAACAAAAAACCTGGT
r141	-	AAAGAAGACGGCAACCAGCCTGGT
r142	-	GAGAAAGACAACAACAAGCCTAGC

r143	-	AAAGAAGACAACAAAAAGCCTAGT
r144	-	AAAGAAGACACCAACAAACCTGGT
r145	-	AAAGAAGACGGCAACAAGCCTGGA
r146	-	AAAGAAGATGGTAACAAACCTGGA
r147	-	AAAGAATACAACAAAAAACCTGGT
r148	-	AAAGAAGACAACAACAAGACTGGT
r149	-	AAAGAAGACGGCAACAATCCTGGT
r150	-	AAAGAAGAAGGCAACAAACCTGGT
r151	-	AAAGAAGACGGCAAGAAGCCTGGT
r152	-	AAAGAAGACAACAACAACAAGCCTGGC
r153	-	AAAGAAGATGGCAATAAGCCTGGT
r154	-	AAAGAAGACAACAAAAAACCTGTA
r155	-	AAAGAAGACGGCAACAAGCCTTGT
r156	-	AAAGAAGACAACAACAAGCCTAGC
r157	-	AAAAAAGACAACAAAAAACCTGGC
r158	-	AAAGAAGACGGAAACAAACCTGGT
r159	-	AAAGAAGACAACAAAAAACCTAGC
r160	-	GAGGAAGACAACAAAAAGCCTGGC
r161	-	AAAGAAGAGGGCAACAAGCCTGGT
r162	-	AAAGAAGATAGCAACAAACCTGGT
r163	-	GAGGAAGACAACTACAAGCCTGGT
r164	-	GAGGAAGACGGCAACAAGCCTGGT
r165	-	AAAGAAGACGGCAACAAAGCTGGC

- = Not available

8.9 APPENDIX I: Pulsed-Field Gel Electrophoresis Gel Images Obtained After Macro-Restriction Analysis of Each Isolate

Pulsed-field gel electrophoresis (PFGE) gel images obtained after MRA. All lanes marked with an asterisk were excluded from analysis and UPGMA tree construction, mainly due to uninterpretable banding patterns. The agarose plugs of these isolates were subjected to PFGE again. Strain NCTC 8325 was used as the molecular size standard.

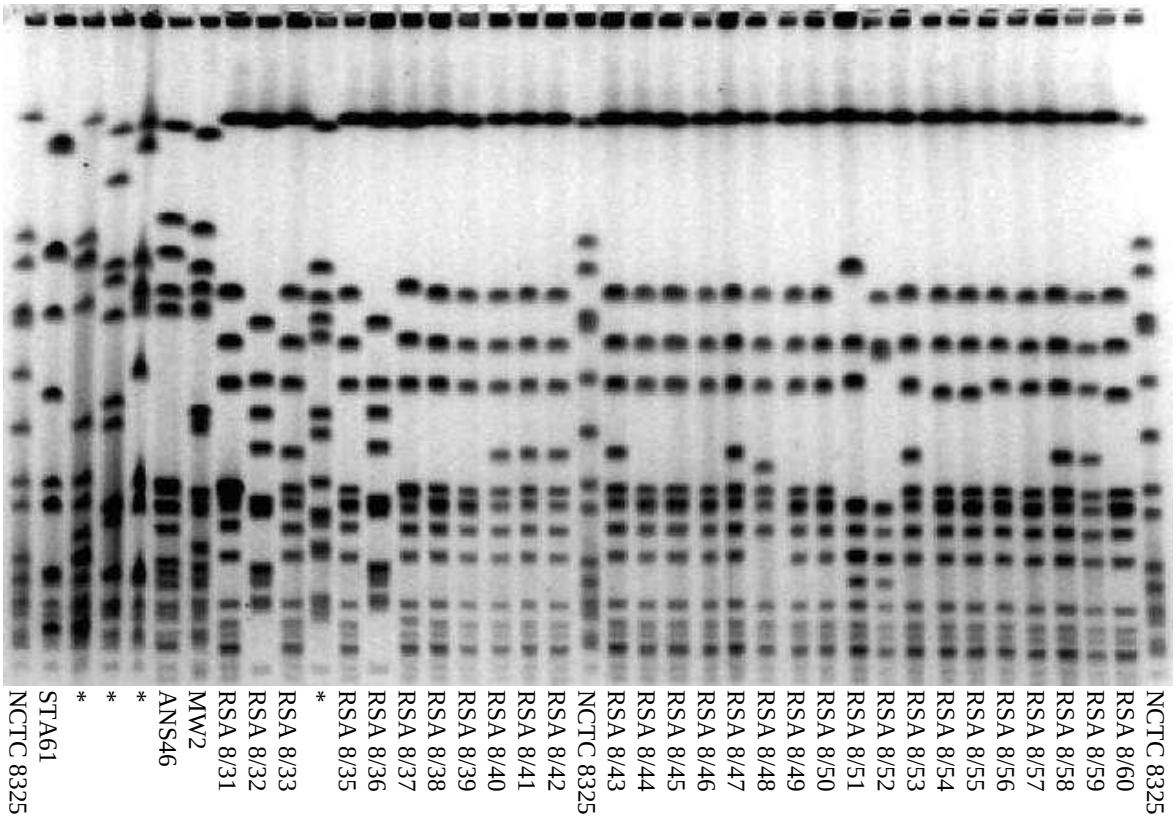

Figure I1: Pulsed-field gel electrophoresis image #1.

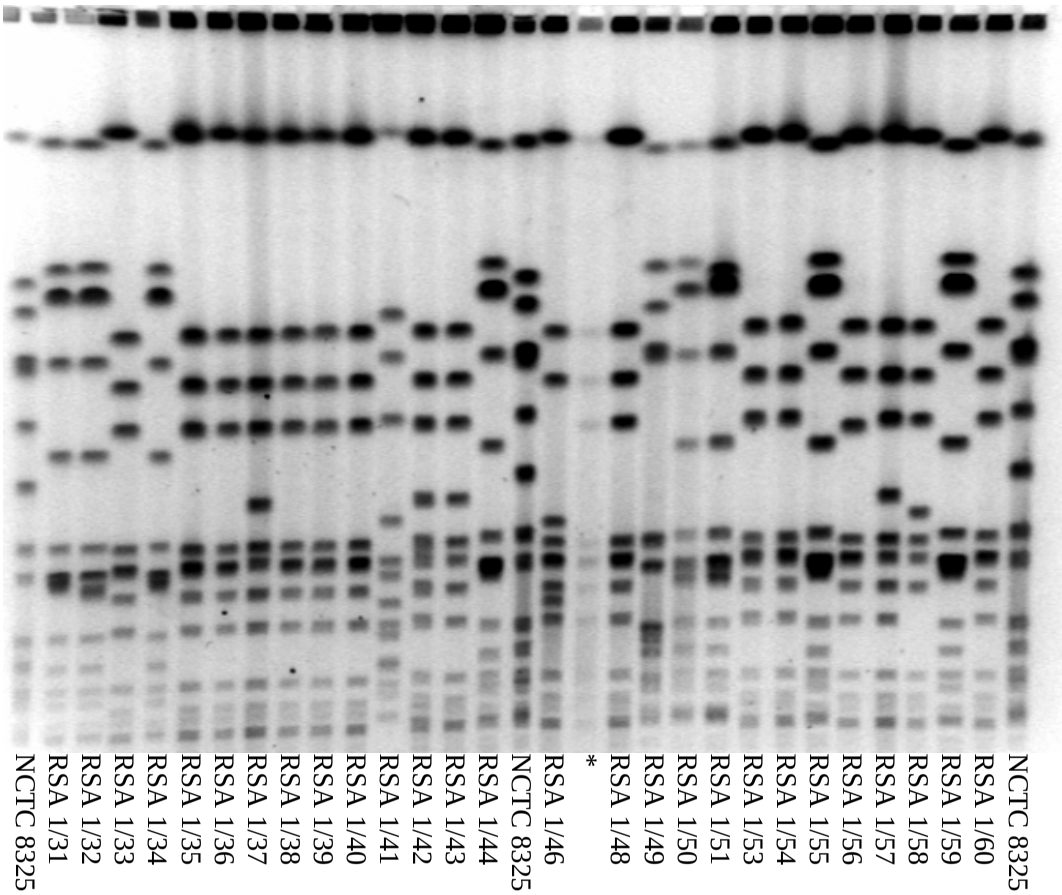

Figure I2: Pulsed-field gel electrophoresis image #2.

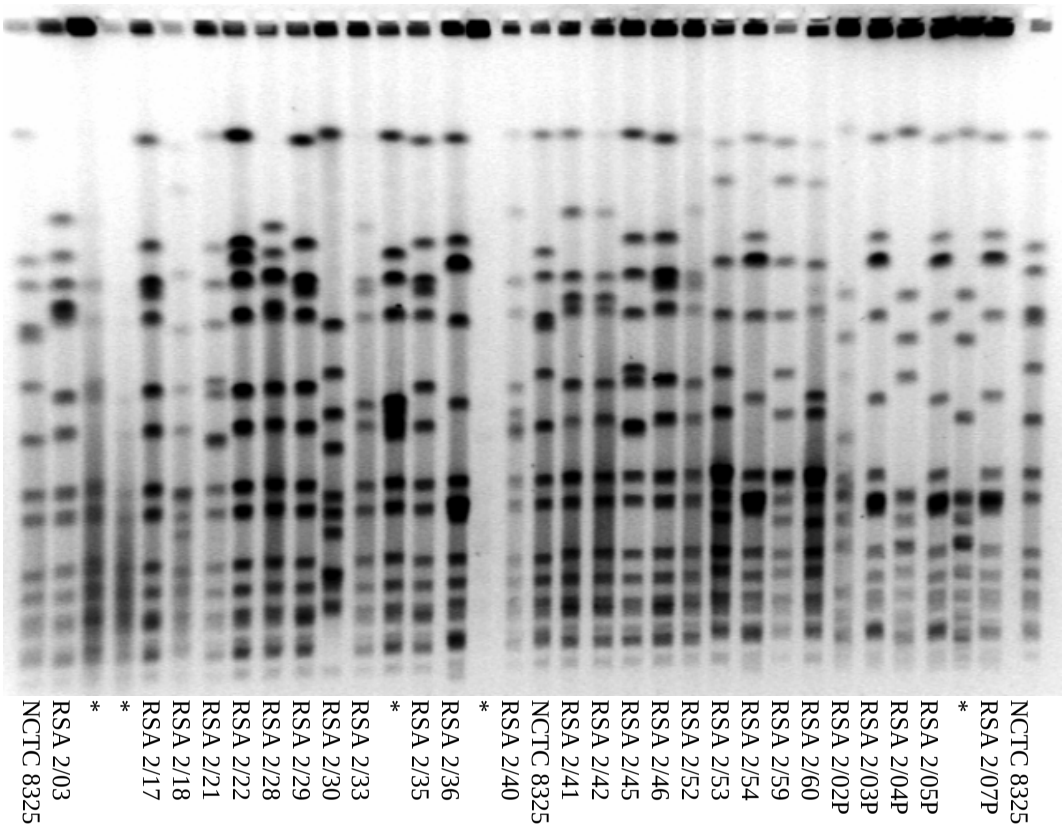

Figure I3: Pulsed-field gel electrophoresis image #3.

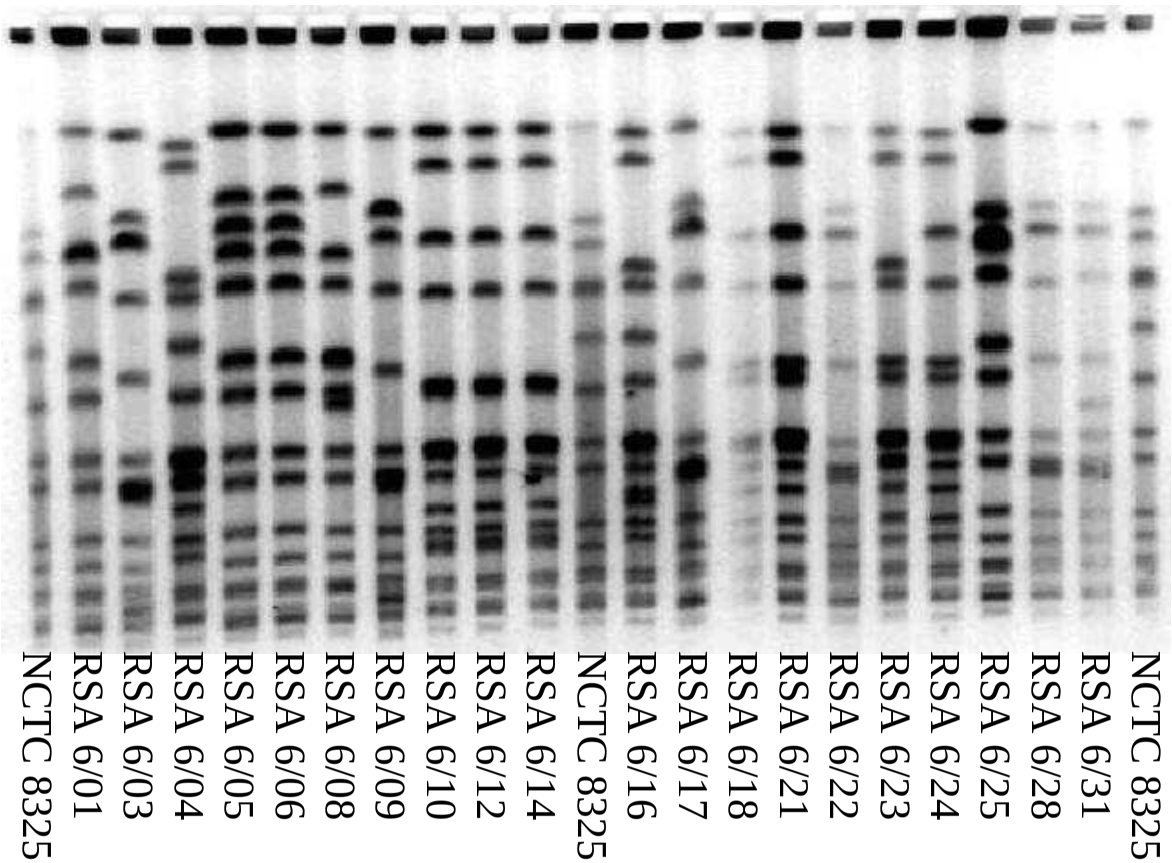

Figure I4: Pulsed-field gel electrophoresis image #4.

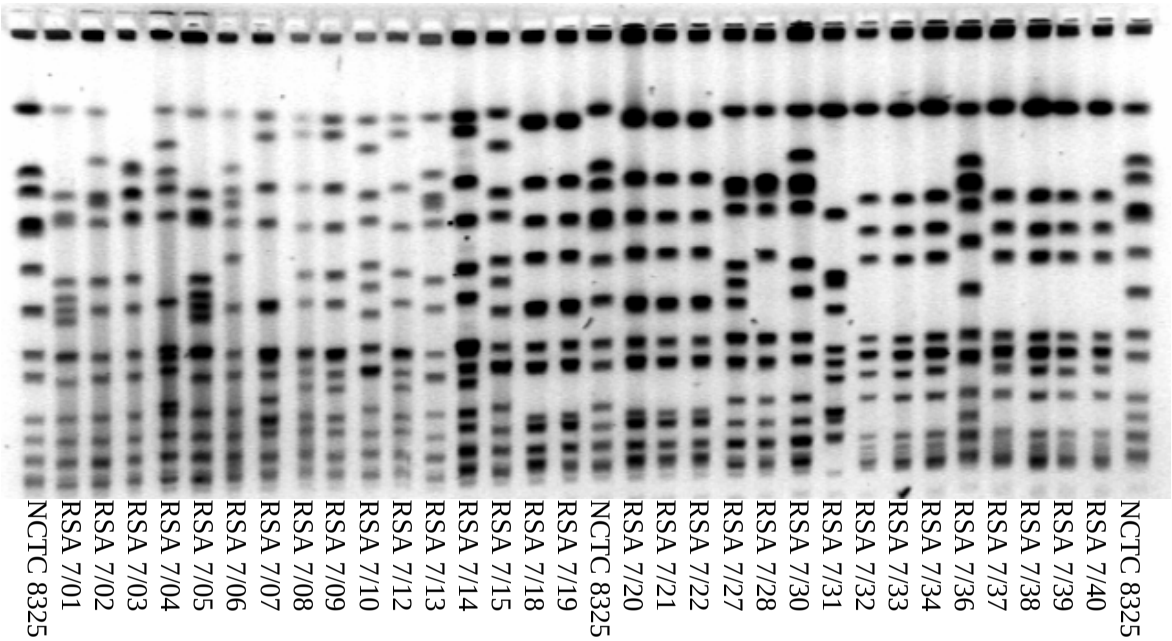

Figure I5: Pulsed-field gel electrophoresis image #5.

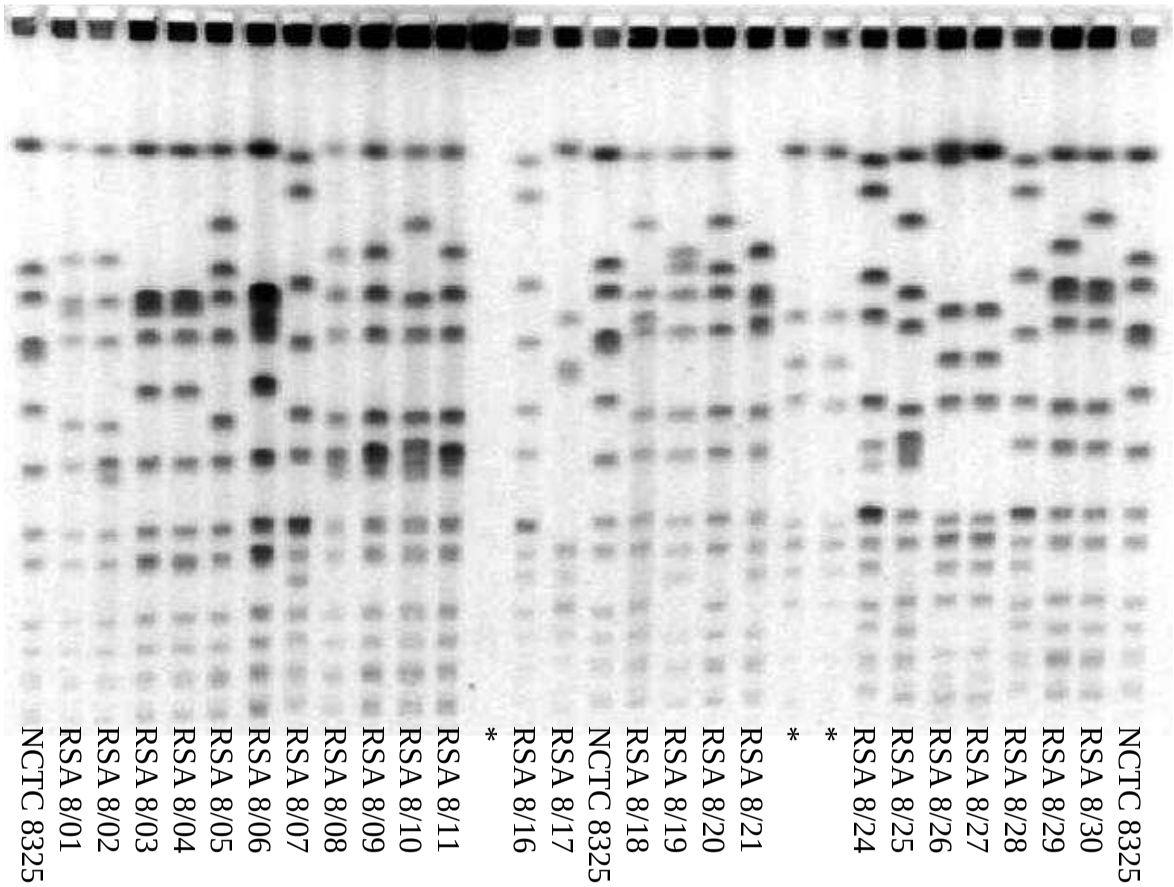

Figure I6: Pulsed-field gel electrophoresis image #6.

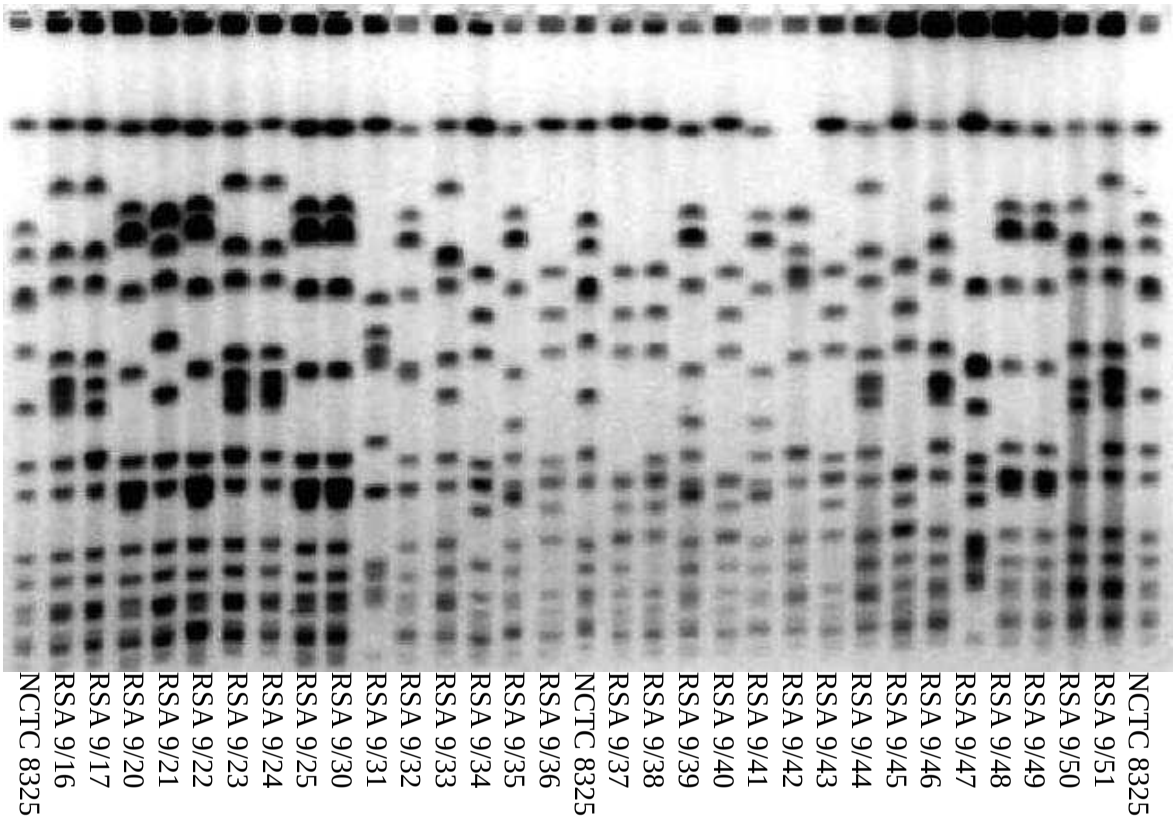

Figure I7: Pulsed-field gel electrophoresis image #7.

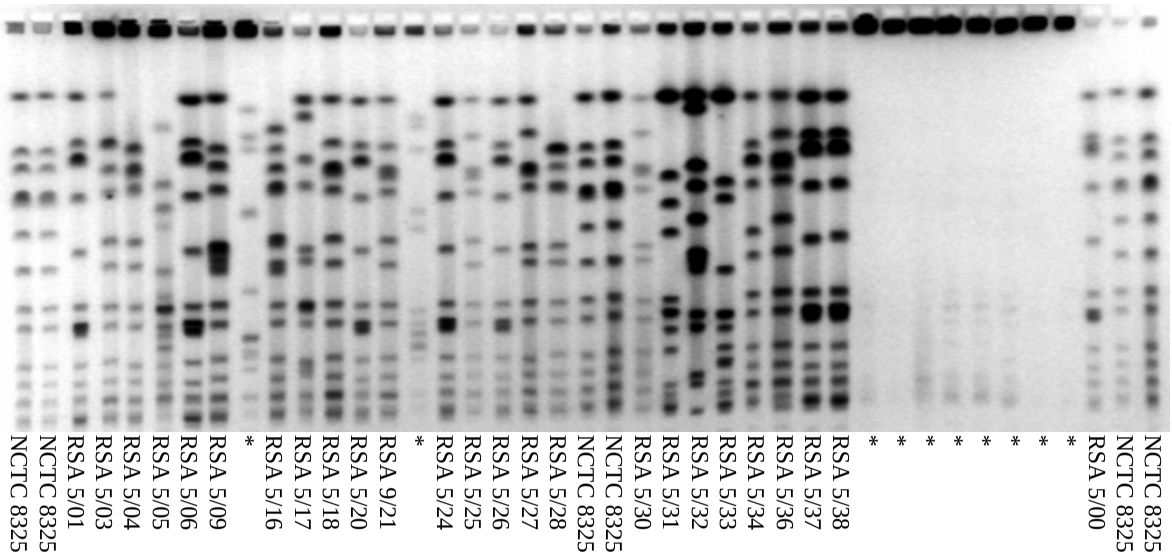

Figure I8: Pulsed-field gel electrophoresis image #8.

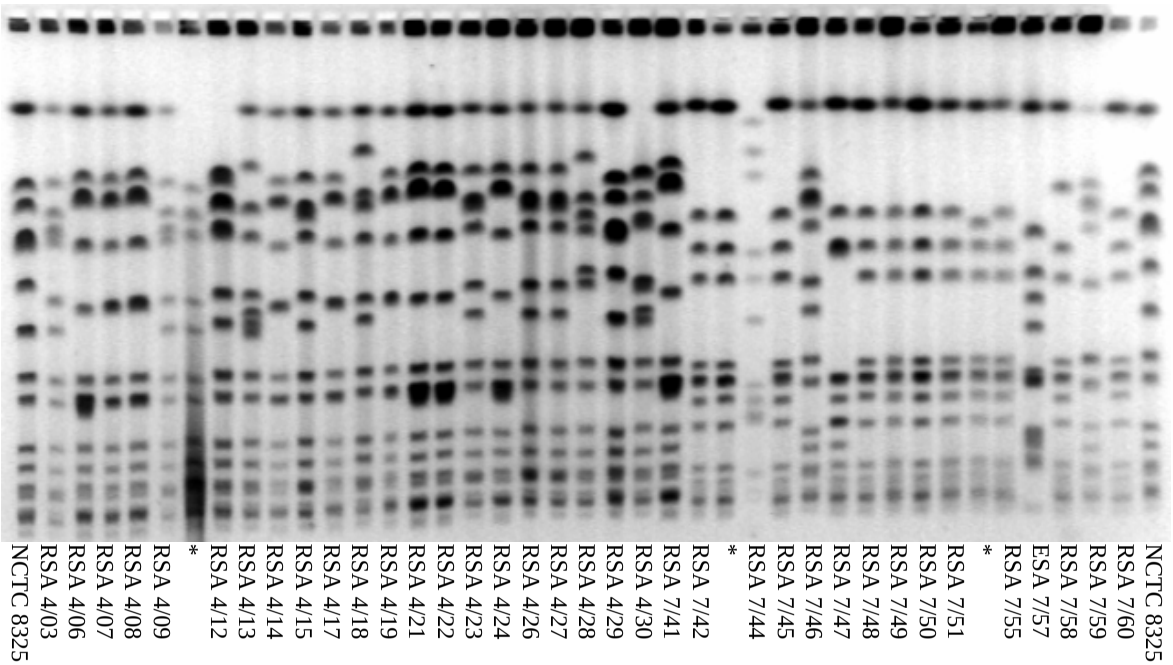

Figure I9: Pulsed-field gel electrophoresis image #9.

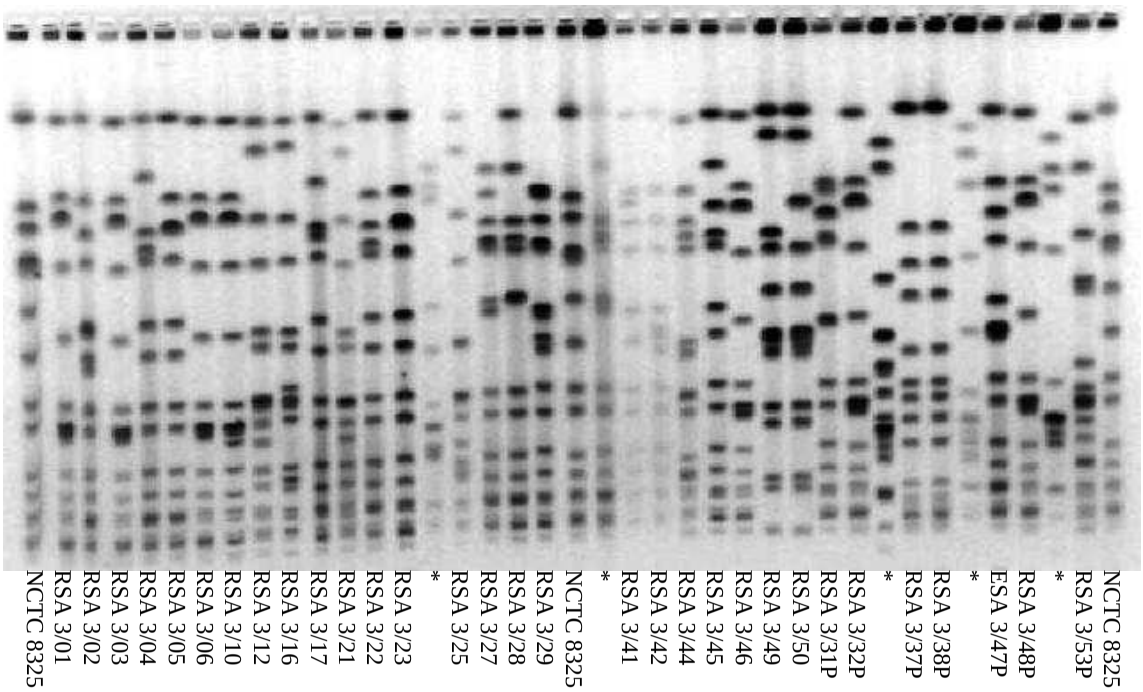

Figure I10: Pulsed-field gel electrophoresis image #10.

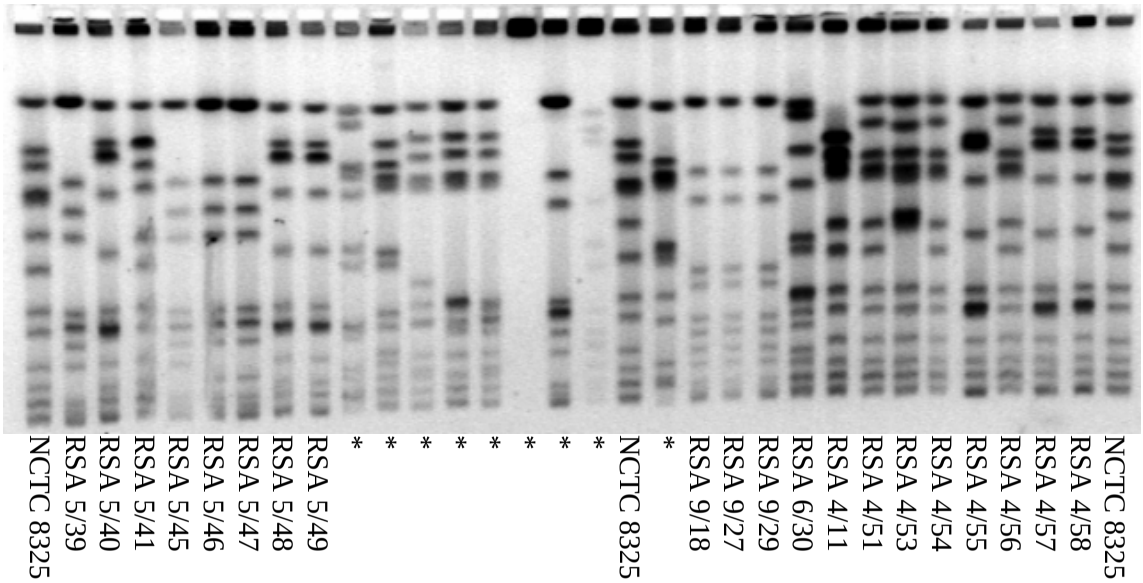

Figure I11: Pulsed-field gel electrophoresis image #11.

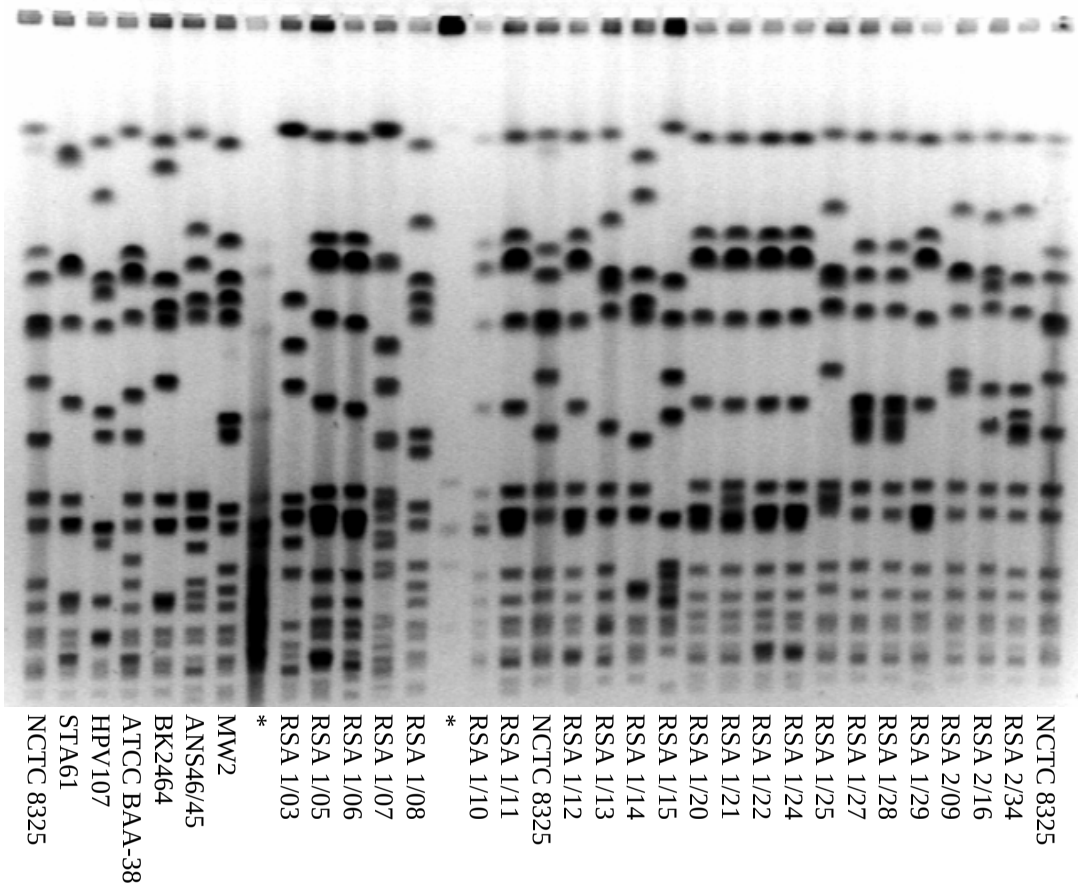

Figure I12: Pulsed-field gel electrophoresis image #12.

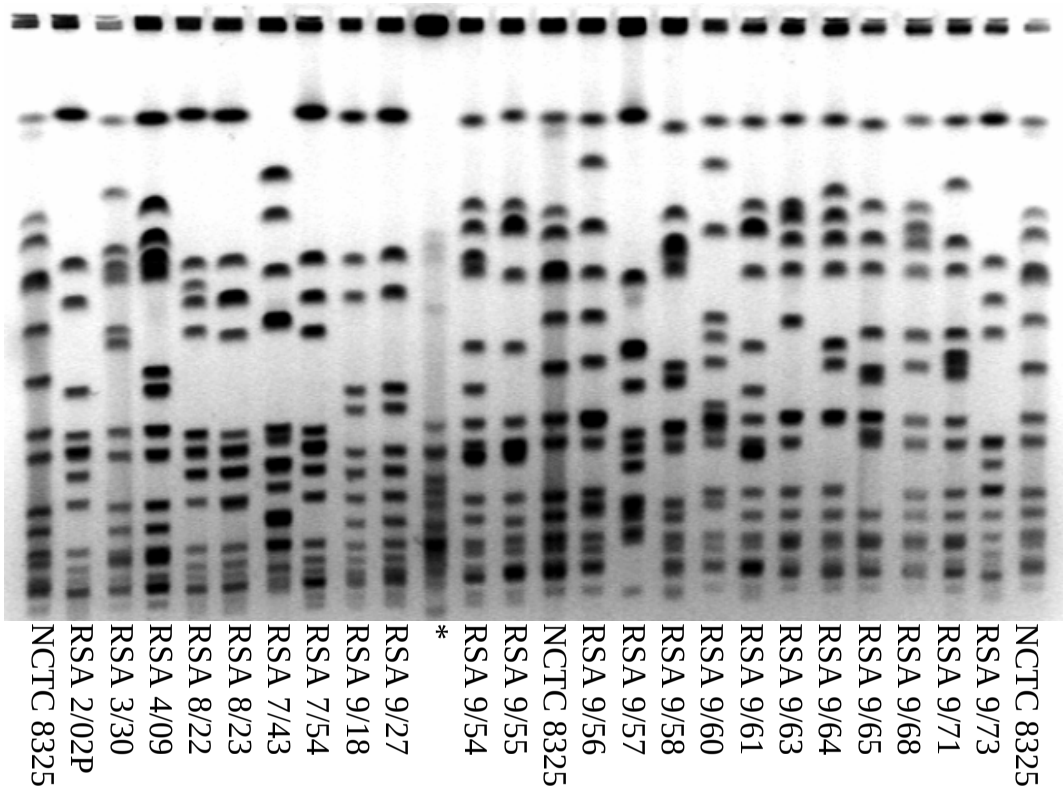

Figure I13: Pulsed-field gel electrophoresis image #13.

8.10 APPENDIX J: *spa* Type Repeat Motif Alignments

Table J1: Alignment of the repeat patterns of *spa* types associated with *spa*-CC 064.

***spa* type**	**Repeat motif**
t008	11-19- -12-21-17-34- -24-34-22- -25
t051	11-19-21-12-21-17-34- -24-34-22 -25
t064	11-19- -12-05-17-34- -24-34-22- -25
t104	11-10- -34-22- -25
t951	11-10- -05-17-34- -24-34-22-- 25
t1256	11-19- -12-05-17-34- -24-34-22- -65
t1257	11-19- -34-05-17-34- -24-34-22- -25
t1443	11-19- -12-05-17-34-24- -24-34-22- -25
t1555	11-19- -34-05-17-34-24-34-24-34-22- -25
t1774	11-19-19-12-05-17-34- -24-34-22- -25
t1779	11-19-12-12-05-17-34- -24-34-22- -25
t1930	11-19- -12-05-17-34- -24-34-22-33-25
t1952	11-19- -12-05-17-34- -24-34- -16
t1971	11-34- -05-17-34- -24-34-22- -25

Table J2: Alignment of the repeat patterns of *spa* types associated with *spa*-CC 032.

***spa* type**	**Repeat motif**
t020	26-23- -31-29-17-31-29-17-25-17-28-16-28
t032	26-23-23-13-23-31-29-17-31-29-17-25-17-25-16-28
t891	26-23- -13-23-31-05- -17-25-17-25-28
t1468	26-23-13-23-31-31-29-17-31-29-17-25-17-25-16-28
t1972	26-16-23- -31-05- -17-25-17-25-28
EMRSA-15	26-23-23-13-23-31-29-17-31-29-17-25-17-25-16-28
Barmin	26-23-23-13-23-31-29-17-31-29-17-25-17-25-16-28

Table J3: Alignment of the repeat patterns of *spa* types associated with *spa*-CC 045.

***spa* type**	**Repeat motif**
t001	26-30-17-34-17-20-17-12- -17-16
t045	26- -17-20-17-12- -17-16
t1154	26- -20-17-12- -17-16
t1880	26- -13-20-17-12-12-17-16
EMRSA-3	26-30-17-34-17-20-17-12- -17-16

Table J4: Alignment of the repeat patterns of *spa* types associated with *spa*-CC #5:

NF.

***spa* type**	**Repeat motif**
t015	08-16-02-16-34-13-17-34-16-34
t465	08-23- -16-34-13-17-34-16-34

Table J5: Alignment of the repeat patterns of *spa* types associated with *spa*-CC #6:

NF.

***spa* type**	**Repeat Motif**
t128	07-23-23-21-16-34-33-13
t172	14- -21-16-34-33-13

Table J6: Alignment of the repeat patterns of *spa* types associated with *spa*-CC 012.

***spa* type**	**Repeat motif**
t012	15-12-16- -02-16-02-25-17-24-24
t018	15-12-16- -02-16-02-25-17-24-24-24
t021	15-12-16- -02-16-02-25-17-24
t037	15-12-16- -02- -25-17-24
t046	08- -16- -02-16-02-25-17-24-24-24
t318	15-12-16-16-02-16-02-25-17-24
t399	15-12-16- -02- -17-24-24
t840	15-12- -02-16-02-25-17-24-24
t1504	15-12-16-16-02- -25-17-24

Table J7: Alignment of the repeat patterns of the singleton *spa* types t174 and t1951.

***spa* type**	**Repeat motif**
t174	14-21-16- -34-33-136
t1951	07-21-17-13-13-34-34-33-34

8.11 APPENDIX K: *spa* Repeat Unit Alignments

Table K1: Alignment of the nucleotide sequences of repeats r21 and r05.

Repeat	Nucleotide sequence
r21	AAAGAAGACAACAA**C**AAGCCTGGC
r05	AAAGAAGACAACAA**A**AAGCCTGGC

Table K2: Alignment of the nucleotide sequences of repeats r19, r10 and r34.

Repeat	Nucleotide sequence
r10	AAAGAAGACAA**T**AA**C**AA**G**CCTGG**T**
r19	AAAGAAGACAA**T**AA**C**AA**G**CCTGG**C**
r34	AAAGAAGACAA**C**AA**A**AA**A**CCTGG**T**

Table K3: Alignment of the nucleotide sequences of repeats r25, r65 and r16.

Repeat	Nucleotide sequence
r65	AAAGAAGA**T**GGCAA**A**AAACCTGGT
r25	AAAGAAGA**T**GGCAA**C**AAACCTGGT
r16	AAAGAAGA**C**GGCAA**C**AAACCTGGT

Table K4: Alignment of the nucleotide sequences of repeats r23 and r16.

Repeat	Nucleotide sequence
r23	AAAGAAGACGGCAACAAACCTGG**C**
r16	AAAGAAGACGGCAACAAACCTGG**T**

Table K5: Alignment of the nucleotide sequences of repeats r29 and r05.

Repeat	Nucleotide sequence
r29	AAAGAAGACAACAAAAAGCCT**A**GC
r05	AAAGAAGACAACAAAAAGCCT**G**GC

Table K6: Alignment of the nucleotide sequences of repeats r17 and r13.

Repeat	Nucleotide sequence
r17	AAAGAAGAC**GG**CAACAA**G**CCTGGT
r13	AAAGAAGAC**AA**CAACAA**A**CCTGGT

Table K7: Alignment of the nucleotide sequences of repeats r16 and r13.

Repeat	Nucleotide sequence
r16	AAAGAAGACGGCAACAAACCTGG**T**
r23	AAAGAAGACGGCAACAAACCTGG**C**

TableK8: Alignment of the nucleotide sequences of repeats r14 and r07.

Repeat	Nucleotide sequence
r14	GAGGAAGACAACAACAAACCTGG**C**
r07	GAGGAAGACAACAACAAACCTGG**T**

Table K9: Alignment of the nucleotide sequences of repeats r08 and r15.

Repeat	Nucleotide sequence
r08	GAGGAAGACAACAACAAGCCTGG**T**
r15	GAGGAAGACAACAACAAGCCTGG**C**

8.12 APPENDIX L: Provincial Map of South Africa