MOLECULAR TYPING OF HOSPITAL-ACQUIRED, COMMUNITY-ACQUIRED AND MULTIDRUG-RESISTANT METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

Jonathan Martin Caddick

Doctor of Philosophy

Aston University

July 2005

THE UNIVERSITY OF ASTON IN BIRMINGHAM

Molecular typing of hospital-acquired, community-acquired and multidrug-resistant methicillin-resistant *Staphylococcus aureus*

A thesis submitted by Jonathan Martin Caddick BSc (Hons)

For the degree of Doctor of Philosophy

2005

SUMMARY

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a significant cause of nosocomial morbidity and mortality which has become a worldwide problem. The emergence of multidrug-resistant MRSA (MR-MRSA), vancomycin-intermediate *S. aureus* (VISA), vancomycin-resistant *S. aureus* (VRSA) strains and highly virulent community-acquired MRSA (CA-MRSA) strains, which produce the Panton-Valentine leucocidin toxin (PVL), have also been reported in recent years. This thesis has sought to investigate the phenotypic and genotypic characteristics of MRSA and MR-MRSA strains isolated from bacteraemic patients in the hospital environment and patients with skin and soft tissue infections in the community. Isolates were phenotypically tested using antibiotic sensitivity testing and vancomycin E-tests. Isolates were genotypically characterised using a number of molecular typing techniques. A reproducible and optimised random amplification of polymorphic DNA (RAPD) protocol was compared with pulsed-field gel electrophoresis (PFGE) of *Sma*I chromosomal macrorestriction digests. Further characterisation was performed using staphylococcal cassette chromosome *mec* (SCC*mec*) type assignment by multiplex PCR and restriction enzyme analysis of plasmid DNA (REAP). Isolates were also screened for the PVL gene using PCR. Principal components analysis (PCA) and cluster analysis were performed to compare strain clustering and Spearman's correlation coefficient (r_s) was employed to identify discriminatory bands from PFGE profiles. A reverse hybridisation, binary typing method that employs discriminatory DNA probes was evaluated and preliminary optimisation of a new system was initiated. Antibiotic sensitivity testing and vancomycin E-tests identified that a large proportion of hospital strains are MR-MRSA and that all strains remain highly sensitive to vancomycin. Both PFGE and RAPD were able to differentiate MRSA strains into three main clusters; group 1 was associated with closely related HA-MRSA and CA-MRSA; group 2 was associated with closely related MR-MRSA; and group 3 was associated with a diverse group of HA-MRSA and CA-MRSA. Further molecular typing revealed that all MR-MRSA strains were SCC*mec* type II, did not carry plasmid DNA, were clonal and may be well adapted for survival in the hospital environment. The majority of HA-MRSA and CA-MRSA were SCC*mec* type IV, carried plasmid DNA and may represent a group of strains adapted for survival in both the hospital and community environments. The results of cluster analysis and PCA of PFGE profiles indicated similar strain clustering, however, PCA identified a fourth cluster of strains that was not identified by cluster analysis. In addition, novel analysis of the PCA data using r_s identified the most discriminatory bands from PFGE profiles. Four bands were identified that were suitable for differentiating MR-MRSA from MRSA strains. Optimisation of a binary typing system that uses discriminatory DNA probes from multiple molecular typing techniques, identified using PCA and r_s, has been initiated and has had limited success, however, further optimisation is required. The phenotypic and genotypic properties of this pathogen may provide a basis for future studies regarding the survival and dissemination of MRSA strains from distinct genetic backgrounds in different environments.

Keywords: Methicillin-resistant *Staphylococcus aureus*, multidrug-resistant, molecular typing, PFGE, RAPD, REAP, SCC*mec*, principal components analysis (PCA), binary typing.

For mom and dad,
nan and grandpa,
and Jude.

ACKNOWLEDGEMENTS

For giving me the opportunity to undertake the studies presented within this thesis I would sincerely like to thank Dr. A. C. Hilton, Dr. P. A. Lambert, Dr. T. Worthington and Prof. T. S. J. Elliott. Thank you also to Dr. M. Allen of Wyeth, for supporting our work and for providing opportunities to present our findings at international conferences.

For continual guidance and support with every aspect of my work I would especially like to thank Dr. A. C. Hilton and Dr. P. A. Lambert. I would like to thank Dr. A. Casey, Claire Powell and Dr. T. Worthington for helping me with all my queries while at the University Hospital Birmingham. For all the answers when it came to statistics I would like to thank Dr. R. A. Armstrong. For their guidance with GelCompar II and with binary typing I would like to thank Dr. T. M. Weller and Kulvir Chana from the City Hospital in Birmingham. I would like to thank Jess Rollason for assistance in my final year with SCC*mec* type assignment and PVL detection. For all their help behind the scenes I would also like to thank all the technical staff at Aston University and the University Hospital Birmingham.

For Maria Braoudaki, Lisbeth Brevik Aamaas, Anna Casey, Emily Darwin, Alex Foster, Lucy Harper, Karen Jones, Sue Lang, Roy McKenzie, Alex Perry, Jess Rollason, Andrew Traube, Laura Wheeldon, my young Padawan's Lisa Baldwin, Michelle Hill, Vicky James, and Helen Spear, a special thank you. Thank you!

Nunc est bibendum

LIST OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

A	Absorbance
AP-PCR	Arbitrarily primed polymerase chain reaction
AST	Antibiotic sensitivity testing
ATCC	American Type Culture Collection
BHI	Brain heart infusion
BSAC	British Society for Antimicrobial Chemotherapy
bp	Base pairs
CA	Community-acquired
CFU	Colony forming unit
CHIP	Chemotaxis inhibitory protein
CSPD	Disodium3-(4-meth-oxy-spiro(1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan)-4-yl)phenyl phosphate
Da	Daltons
DHFR	Dihydrofolate reductase
DHPS	Dihydropterate synthetase
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EARSS	European Antimicrobial Resistance Surveillance System
EDTA	Ethylenediamine-tetraacetic acid
EMRSA	Epidemic methicillin-resistant *S. aureus*
FAME	Fatty acid esterifying enzyme
HA	Hospital-acquired
HCl	Hydrochloric acid
hVISA	Hetero-vancomycin-intermediate *S. aureus*
IleS	Isoleucyl tRNA synthetase
IS	Insertion sequence
kbp	Kilo-base pairs
KCl	Potassium chloride
kDa	Kilo Daltons
Mg^{2+}	Magnesium ion
$MgCl_2$	Magnesium chloride

MH	Mueller-Hinton
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
mbp	Mega-base pairs
MLS	Macrolide, lincosamide and streptogramin B
MLEE	Multi-locus enzyme electrophoresis
MLST	Multi-locus sequence typing
MSCRAMMS	Microbial surface components recognising adhesive matrix molecules
MR	Multidrug-resistant
MRSA	Methicillin-resistant *S. aureus*
MSSA	Methicillin-sensitive *S. aureus*
MW	Molecular weight
NaOH	Sodium hydroxide
NCCLS	National Committee for Clinical Laboratory Standard
NCTC	National collection of type cultures
NMR	Non-multidrug-resistant
OD	Optical density
ORF	Open reading frame
PABA	P-aminobenzoic acid
PBS	Phosphate buffered saline
PBP	Penicillin binding protein
PCA	Principle components analysis
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PVL	Panton-Valentine leucocidin
RAPD	Random amplification of polymorphic DNA
REANOVA	Repeated measures analysis of variance
REAP	Restriction enzyme analysis of plasmid DNA
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
SCC*mec*	Staphylococcal cassette chromosome *mec*
SDS	Sodium dodecyl sulphate
SDW	Sterile double distilled water

TAE	Tris acetate, EDTA
TBE	Tris, boric acid, EDTA
Taq	DNA polymerase from *Thermus aquaticus*
TBS	Tris buffered saline
Tn	Transposon
Tris	Tris [hydroxymethyl] aminomethane
tRNA	transfer ribonucleic acid
UHB	University Hospital Birmingham NHS Foundation Trust, UK
UPGMA	Unweighted pair group method of arithmetic averages
UV	Ultra violet
VISA	Vancomycin-intermediate *S. aureus*
VRSA	Vancomycin-resistant *S. aureus*

CHAPTER 1 INTRODUCTION

1.1 *Staphylococcus aureus*

The genus *Staphylococcus* was named by Ogsten in 1881 when he observed grape-like clusters of berry shaped bacteria during a microscopical examination of a pus sample, removed from the leg of a young male patient (Ogsten, 1881). The name was derived from the Greek [*staphulē*] meaning bunch of grapes and [*kokkos*] meaning berry or grain. Three years later, Rosenbach successfully isolated and grew these microorganisms in pure culture and gave the bacteria the specific epithet *Staphylococcus aureus* (Rosenbach, 1884). The species name was derived from the Latin [*aureus*] meaning golden and related to the yellow-to-orange pigmented appearance of the microorganism's colonies.

Staphylococcus aureus is a member of the Micrococcaceae family and is identifiable as a non-spore forming, Gram-positive, catalase-positive, oxidase-negative, facultative anaerobic coccus approximately 1μm in diameter that divides in more than one plane to form grape-like clusters of cells (Wilkinson, 1997). The microorganism can grow at temperatures ranging between 15-45°C, optimally at 35-37°C and is notably halotolerant, typically surviving in salt concentrations of up to 15%. The characteristics which distinguish *S. aureus* from other staphylococcal species are the golden pigment of its colonies and positive results for coagulase, mannitol-fermentation and deoxyribonuclease tests (Wilkinson, 1997).

Staphylococcus aureus is a human skin commensal and is commonly isolated from the nares, axillae, vagina, pharynx and damaged skin surfaces. Humans are a natural reservoir for *S. aureus* and it is estimated that 30-70% of the population are colonised and 10-20% persistently colonised by the microorganism (Noble *et al.*, 1967; Casewell and Hill, 1986; Peacock *et al.*, 2001).

Staphylococcus aureus has been identified as a significant cause of nosocomial infection, which before the introduction of antibiotic chemotherapies was considered a major life-threatening pathogen (Cookson *et al.*, 2003). Since first being identified as

a disease causing microorganism, *S. aureus* has demonstrated a proven ability to adapt to the selective pressure of antibiotics, which has led to the emergence of methicillin-resistant *S. aureus* (MRSA). Initially, MRSA was confined to hospitals but in recent years has disseminated and emerged as a serious community acquired infection. Presently, MRSA is commonly associated with multi-drug resistance, which has led to mounting concern regarding the situation of increasing resistance to vancomycin, the antibiotic of last resort.

1.2 *S. aureus* and the emergence of methicillin-resistant *S. aureus*

1.2.1 Resistance to β-lactam antibiotics

Penicillin, the first of the β-lactam antibiotics, was discovered by Fleming in 1929. Subsequent work in the late 1930s and early 1940s was performed by Florey and Chain and led to penicillin being used therapeutically for the first time in 1941 (Dyke and Gregory, 1997). The first report that bacterial extracts could render penicillin innocuous was published in 1940 demonstrating that certain bacterial species could be resistant to such therapy (Lyon and Skurray, 1987). By 1948 the first report of penicillin-resistant *S. aureus* was published and an estimated 60% of *S. aureus* isolates in the UK were found to be resistant to penicillin (Barber and Rozwadowska-Dowzenko, 1948; Cookson *et al.*, 2003). Presently, it is estimated that as many as 93% of *S. aureus* isolates are resistant to β-lactamase labile penicillins (Thornsberry, 1995).

Resistance to penicillin is attributed to an enzyme called penicillinase which since the 1960s has been known as β-lactamase. Collectively β-lactamases are the commonest cause of bacterial resistance to β-lactam antibiotics (Livermore, 1995). The staphylococcal enzymes, of which there are four types designated A-D, based on the two main systems of classification are molecular class A type and have catalytic properties of group 2a enzymes (Bush *et al.*, 1995; Livermore, 1995). In addition they typically spread through plasmid transfer and the functional enzyme is largely cell surface attached and not found extracelluarly (Livermore, 1995). The β-lactamases of molecular class A and group 2a are serine enzymes that are inhibited by clavulanate

and are able to hydrolyse the β-lactam ring of penicillin and carbenicillin, rendering them inactive (Bush *et al.*, 1995; Livermore, 1995; Dyke and Gregory, 1997). The *S. aureus* β-lactamase is encoded by the *blaZ* gene, which is under the control of two adjacent regulatory genes, the antirepressor *blaR1* and the repressor *blaI* (Kernodle, 2000) (figure 1.1).

To overcome the failure of β-lactam therapy an intensive search was undertaken to identify β-lactams that were not susceptible to β-lactamase activity. Investigation led to the discovery of 6-aminopenicillanic acid (6-APA) in 1959 (Cookson *et al.*, 2003), the basic building block of all semisynthetic penicillins. Treating 6-APA with various acyl halides allowed bulky side chains to be added to the original lead compound protecting the essential β-lactam ring from β-lactamase hydrolysis.

1.2.2 Methicillin-resistant *S. aureus*

In 1960 methicillin, the first semisynthetic penicillinase-resistant penicillin was developed as a therapeutic agent for the treatment of penicillin-resistant *S. aureus* strains. In the same year Jevons identified the first methicillin-resistant *S. aureus* (MRSA) (Jevons, 1961). The mechanism of resistance could not be due to β-lactamase activity as methicillin is intrinsically protected against such degradation. Therefore resistance was attributable to another mechanism.

It is widely accepted that MRSA arose when *S. aureus* acquired *mecA*, which is carried on a large mobile genetic element known as the staphylococcal cassette chromosome *mec* (SCC*mec*) (Ito *et al.*, 1999; Katayama *et al.*, 2000; Ito *et al.*, 2001). The SCC*mec* is a 21-67 kb fragment of DNA that inserts precisely into the *S. aureus* chromosome at the 3' end of *orf*X, a unique site located near the origin of replication (Kuroda *et al.*, 2001; Baba *et al.*, 2002). The *mecA* gene encodes a 78 kDa PBP, designated 2' (PBP2') or 2a (PBP2a), which has reduced affinity for β-lactam antibiotics (Brown and Reynolds, 1980; Hartman and Tomasz, 1984) (figure 1.1). In addition to *mecA* there are two regulatory elements *mecR1*, encoding a signal-transduction protein, and *mecI*, encoding a transcriptional repressor, plus additional DNA that contains transposons, insertion sequences, resistance determinants, and

integrated plasmids (Ito *et al.*, 1999; Ito *et al.*, 2001). This additional DNA may carry genes that confer resistance to groups of antibiotics other than β-lactams (Rohrer *et al.*, 2003).

Figure 1.1 Induction and regulation of penicillin and methicillin resistance mechanisms. Adapted from (Lowy, 2003).

(a) Induction of staphylococcal β-lactamase synthesis in the presence of the β-lactam antibiotic penicillin. **I.** The DNA-binding protein BlaI binds to the operator region, repressing RNA transcription from both *blaZ* and *blaR1-blaI*. In the absence of penicillin, β-lactamase is expressed at low levels. **II.** Binding of penicillin to the transmembrane sensor-transducer BlaR1 stimulates its autocatalytic activation. **III-IV.** Active BlaR1 either directly or indirectly (via a second protein, BlaR2) cleaves BlaI into inactive fragments, allowing transcription of both *blaZ* and *blaR1-blaI* to commence. **V-VII.** β-lactamase, the extracellular enzyme encoded by *blaZ* **(V)**, hydrolyses the β-lactam ring of penicillin **(VI)**, thereby rendering it inactive **(VII)**. **(b)**. Mechanism of *S. aureus* resistance to methicillin. Synthesis of PBP2a proceeds in a fashion similar to that described for β-lactamase. Exposure of MecR1 to a β-lactam antibiotic induces MecR1 synthesis. MecR1 inactivates MecI, allowing synthesis of PBP2a. MecI and BlaI have coregulatory effects on the expression of PBP2a and β-lactamase.

It has been speculated that a PBP which shares 87.8% amino acid homology with PBP2a is an evolutionary precursor of the *mecA* gene acquired by *S. aureus* from the related coagulase negative species *Staphylococcus sciuri* (Wu *et al.*, 2001). There is also evidence that a *S. aureus* has acquired, via *in vivo* horizontal transfer, *mecA* DNA from *Staphylococcus epidermidis* to subsequently become a MRSA (Wielders *et al.*, 2001), thus demonstrating the role of coagulase negative staphylococci in *mecA* acquisition by *S. aureus*.

Five distinct types of SCC*mec* designated I-V and a few variants have been described to date; three (I-III) are associated with hospital-acquired MRSA (HA-MRSA) (Ito *et al.*, 2001) and two (IV and V) are associated with community-acquired MRSA (CA-MRSA) (Daum *et al.*, 2002; Ma *et al.*, 2002; Ito *et al.*, 2004). Each type of SCC*mec* element contains a *mec* gene complex that consists of *mecA* and its regulatory genes and a *ccr* gene complex that encodes cassette chromosome recombinases, which are responsible for the excision and integration of SCC*mec* into the *S. aureus* chromosome (Katayama *et al.*, 2000). The structure of the known classes of *mec* gene complexes are as follows: class A, insertion sequence 431 (IS*431*)-*mecA*-*mecR1*-*mecI*; class B, IS*431*-*mecA*-Δ*mecR1*-IS*1272*; class C, IS*431*-*mecA*-Δ*mecR1*-IS*431*; and class

D, IS*431-mecA*-Δ*mecR1* (Katayama *et al.*, 2000). The allotypes for the *ccrA* and *ccrB* genes are as follows: *ccrA1*, *ccrA2*, *ccrA3*, *ccrA4* for the *ccrA* gene and *ccrB1*, *ccrB2*, *ccrB3*, and *ccrB4* for the *ccrB* gene. The SCC*mec* element can be classified according to the combination of the *mec* gene complex class and the *ccr* gene complex type (Ito *et al.*, 2001; Ma *et al.*, 2002) as follows: type I SCC*mec*, class B *mec* gene complex and type 1 *ccr* gene complex; type II SCC*mec*, class A *mec* gene complex and type 2 *ccr* gene complex; type III SCC*mec*, class A *mec* gene complex and type 3 *ccr* gene complex; and type IV SCC*mec*, class B *mec* gene complex and type 2 *ccr* gene complex. The novel type V SCC*mec* has been designated as a class C2 *mec* gene complex and was identified with a single copy of a gene homologue that encoded a cassette chromosome recombinase, which was designated *ccrC* (Ito *et al.*, 2004). The region of the SCC*mec* element other than the *mec* and *ccr* gene complexes that contain DNA of cryptic function have been designated the J ("junkyard") region. Each SCC*mec* type is further classified into subtypes on the basis of the J-region sequence (figure 1.2) (Hiramatsu *et al.*, 2002).

Figure 1.2 Simplified illustrative representations of SCC*mec* types. Adapted from (Ito *et al.*, 2003). J1-J3 indicates J (junkyard) regions that may encode insertion sequences and transposons, encoding additional antimicrobial resistance genes.

Staphylococcus aureus strains containing a class A *mec* complex are not resistant to methicillin due to the repressive function of the *mecI* gene and are termed pre-MRSA (Kuwahara-Arai *et al.*, 1996). In MRSA strains that contain the class A *mec* complex, the repressive function of *mecI* has been inactivated by mutations in *mecI* or the *mecA* promoter region (Kuwahara-Arai *et al.*, 1996; Katayama *et al.*, 2000). The insertion element IS*431* (synonym IS*257*) can act as a receptor site for IS*431*-associated plasmids and transposons for integration into the SCC*mec* element by homologous recombination, which may facilitate the acquisition of additional resistance genes near to *mecA* (Cookson *et al.*, 2003). The type II and type III SCC*mec* elements also carry copies of the transposon Tn*554*, which encodes inducible resistance to erythromycin (Cookson *et al.*, 2003).

All worldwide MRSA strains appear to be descended from a limited number of clones that have acquired the *mecA* gene (Kreiswirth *et al.*, 1993; Hiramatsu, 1995; Enright *et al.*, 2002) and may have evolved, since the introduction of methicillin, from five major lineages (Robinson and Enright, 2003).

1.2.2.1 Staphylococcal cell wall structure and methicillin resistance

In common with all staphylococci, the cell wall of *S. aureus* is 50% peptidoglycan by weight (Lowy, 1998). The murein monomer of *S. aureus* peptidoglycan is composed of alternating polysaccharide subunits of repeating β-1-4-linked N-acetylglucosamine and N-acetylmuramic acid residues. Attached to the carboxyl residue of each murein monomer precursor there is usually a pentapeptide chain composed of L-alanyl-D-isoglutaminyl-L-lysyl-D-alanyl-D-alanine. The individual glycan chains are typically cross-linked by a pentaglycine bridge that links the ε-amino group of L-lysine in one tetrapeptide to the D-alanine residue in position four of an adjacent tetrapeptide (Wilkinson, 1997; Lowy, 1998). During this process the terminal D-alanine is removed. Cross-linkage of *S. aureus* peptidoglycan is typically high, generally 85-90% (Wilkinson, 1997). The multiple glycine residues that form the interpeptide bridge render *S. aureus* susceptible to the enzyme lysostaphin (Wilkinson, 1997).

There are four penicillin binding proteins (PBPs), PBP1-4, produced by *S. aureus* that are involved in peptidoglycan assembly and resultant cell wall synthesis (Labischinski, 1992). These PBPs have a biological activity similar to that of serineproteases and act as transpeptidases, catalysing the cross-linking of adjacent glycan chains (Waxman and Strominger, 1983; Murakami *et al.*, 1991). The process of which, is essential for shape, strength and mechanical integrity of all staphylococcal cell walls. Evidence suggests that *S. aureus* can survive and grow with only a single PBP left functioning (Labischinski, 1992).

All *β*-lactam antibiotics are structural analogues of the D-alanyl-D-alanine (D-ala-D-ala) sequence, to which PBPs bind to when cross-linking of peptidoglycan is taking place. A β-lactam antibiotic can form a stable covalent bond with the serine residue present in the active site of a PBP forming a penicilloyl-enzyme complex with a half

life of approximately ten minutes (Nicholas and Strominger, 1988). By inhibiting PBP function, peptidoglycan synthesis is impaired and cell wall integrity is affected which may lead to cell lysis. It is by this process that all β-lactams are widely considered to have a bacteriostatic or bactericidal effect against *S. aureus*, dependant on antibiotic concentration. All β-lactam antibiotics have a low binding affinity for PBP2a, the product of *mecA*, and are unable to inhibit the transpeptidase function of the enzyme. It is estimated that PBP2a binds β-lactam antibiotics with 100-1000-fold less affinity than the other staphylococcal PBPs (Chambers, 2004). In this way PBP2a can substitiute for the lost function of the other staphylococcal PBPs when antibiotic concentrations would otherwise be lethal. The substituted, transpeptidase activity from PBP2a assisted by the transglycosidase domain of the native PBP2 of *S. aureus*, maintain cell wall biosynthesis and cell wall integrity in the presence of β-lactam antibiotics (Pinho *et al.*, 2001). Any *S. aureus* strain that carries and expresses *mecA* in the presence of β-lactam antibiotics is capable of producing PBP2a and thus intrinsically resistant to all β-lactam antibiotics, including cephalosporins and carbapenems. Strains of *S. aureus* which are resistant to penicillinase-resistant β-lactam antibiotics and which carry *mecA* are considered MRSA. Expression of *mecA* and synthesis of PBP2a is similar to the process described for the expression of *BlaZ* (figure 1.1).

1.2.3 Multidrug-resistant MRSA

Methicillin-resistant *Staphylococcus aureus* strains have become a worldwide problem exacerbated by the emergence of strains that carry, in addition to *mecA*, resistance genes to other antimicrobial agents. Strains of MRSA that carry genes which confer resistance to other antimicrobial groups are commonly given the prefix, multidrug-resistant, multiresistant, multiple-resistant MRSA (MR-MRSA). Such strains demonstrate a reduced susceptibility to almost all clinically available antibiotics and are often only susceptible to glycopeptides and investigational drugs (Wenzel *et al.*, 1991; Aires de Sousa *et al.*, 1998). The emergence and evolution of MR-MRSA strains has stemmed from multiple factors that include, the widespread and often inappropriate use of antimicrobial drugs, the extensive use of antimicrobial agents as growth enhancers in animal feed, and an increase in regional and international travel,

which has allowed MR-MRSA to cross geographic barriers (Cohen, 1992; Tomasz, 1994; Swartz, 1997).

The SENTRY Antimicrobial Surveillance Program, a global network of sentinel hospitals in the USA, Canada, Latin America, Europe and the Western Pacific region, reported high rates of co-resistance in MRSA isolates between 1997 and 1999 (Diekema *et al.*, 2001). The rates and types of antibiotics, in addition to β-lactams, cephalosporins and carbapenems, to which MRSA demonstrate resistance varies significantly between different regions. In Latin America MRSA isolates were resistant to a median of 6 antimicrobial classes, in comparison to resistance to a median of 3 antimicrobial classes in the USA and Canada. Levels of erythromycin, clindamycin and ciprofloxacin resistance were found to be high among MRSA in all regions (up to 94.7%; 88%; 89.6%, respectively). Antimicrobial agents with significant regional differences included rifampin (4.9% in Canada compared to 44.4% in Europe), gentamicin (25.9% in Canada compared to 91.2% in Latin America) and tetracycline (14.8% in Canada compared to 82% in the Western Pacific region).

Presently, MR-MRSA strains are a major worldwide problem; mainly due to there intrinsic virulence, ability to cause a diverse array of life-threatening infections and, as more patients are treated outside the hospital setting, an increasing concern in the community (Aires de Sousa *et al.*, 1998; Diekema *et al.*, 2001).

1.2.4 Vancomycin-intermediate and vancomycin-resistant MRSA

Multidrug-resistant MRSA strains have limited the use of most antibimicrobial agents, with the exception of the glycopeptides (vancomycin and teicoplanin), however, evidence suggests that the increased use of vancomycin to treat infections caused by methicillin-resistant staphylococci (both coagulase-positive and negative), *Clostridium difficile*, and enterococcal infections has led to the emergence of *S. aureus* and MRSA isolates with reduced susceptibility to vancomycin (Kirst *et al.*, 1998).

1.2.4.1 Defining vancomycin resistance

Criteria set by different countries to define *S. aureus* strains with reduced susceptibility to vancomycin, has caused a great deal of confusion. Typically, a minimum inhibitory concentration (MIC) of 8μg/ml of vancomycin is what defines a *S. aureus* or MRSA strain with a reduced susceptibility to vancomycin (Cui and Hiramatsu, 2003). Criteria set by the National Committee for Clinical Laboratory Standard (NCCLS) define *S. aureus*/MRSA strains with a MIC of 4μg/ml as vancomycin-sensitive *S. aureus* (VSSA), those with a MIC of 8-16μg/ml of vancomycin as vancomycin-intermediate *S. aureus* (VISA), and those with a MIC of 32μg/ml as vancomycin-resistant *S. aureus* (VRSA) (Anonymous, 2000). The British Society for Antimicrobial Chemotherapy (BSAC) and the Swedish Reference Group for Antibiotics (SRGA-M) define *S. aureus*/MRSA strains with a MIC of 4μg/ml as VSSA and those with a MIC of 8μg/ml as VRSA (Olsson-Liljequist *et al.*, 1997; Anonymous, 1998). Table 1.1 summarises the main criteria for different countries.

Strains of *S. aureus* and MRSA with a MIC of 8μg/ml are often reported to resist prolonged vancomycin therapy (Hiramatsu *et al.*, 1997a; Ploy *et al.*, 1998; Smith *et al.*, 1999; Sieradzki *et al.*, 1999a; Chesneau *et al.*, 2000; Ferraz *et al.*, 2000; Hood *et al.*, 2000; Kim *et al.*, 2000; Wong *et al.*, 2000; Boyle-Vavra *et al.*, 2001; Hageman *et al.*, 2001; Oliveira *et al.*, 2001; Paton *et al.*, 2001) and are thus considered to be clinically resistant (Cui and Hiramatsu, 2003). For this reason the term VISA appears obsolete, however, the term VRSA is also used to describe isolates that possess the *vanA* gene, which confers a greater level of resistance to vancomycin. Therefore, it is proposed that *S. aureus* isolates that are "clinically resistant" to vancomycin and which have an MIC of 8 μg/ml, be designated VISA, contrary to (Cui and Hiramatsu, 2003), and only isolates of *S. aureus* that carry *vanA* be designated VRSA. The term glycopeptide-intermediate *S. aureus* (GISA), and glycopeptide-resistant *S. aureus* (GRSA) will not be used during this thesis as sensitivity of *S. aureus* and MRSA strains to teicoplanin is not routinely tested in most UK laboratories

Table 1.1 Glycopeptide breakpoint values for Staphylococcus aureus. Adapted from (Cui and Hiramatsu, 2003).

1.2.4.2 Emergence of vancomycin resistance

The first report of reduced susceptibility to vancomycin in a staphylococcal species was observed in a clinical isolate of *Staphylococcus haemolyticus* (Schwalbe *et al.*, 1987). It was ten years after this initial observation that the first VISA strain, designated Mu50, was reported in Japan in 1997 (Hiramatsu *et al.*, 1997a), followed by subsequent, unrelated cases of VISA strains reported from other countries including the United States of America (Smith *et al.*, 1999; Sieradzki *et al.*, 1999a; Boyle-Vavra *et al.*, 2001; Hageman *et al.*, 2001), United Kingdom (Hood *et al.*, 2000; Paton *et al.*, 2001), France (Ploy *et al.*, 1998; Chesneau *et al.*, 2000), Belgium (Denis *et al.*, 2002), Netherlands (Van Griethuysen *et al.*, 2003), South Korea (Kim *et al.*, 2000), South Africa (Ferraz *et al.*, 2000) and Brazil (Oliveira *et al.*, 2001). The majority of these VISA isolates were also methicillin-resistant, non clonal, and came from patients that had received prolonged vancomycin therapy and had MRSA infections in preceding months (Fridkin *et al.*, 2003). In addition, the majority of patients were reported as sharing similar factors such as, having an underlying illness, long term exposure to vancomycin, and unsuccessful vancomycin treatment.

In the same year that the first VISA strain was reported another category of *S. aureus* isolate with reduced susceptibility to vancomycin was isolated in Japan and designated hetero-VISA (hVISA) (Hiramatsu *et al.*, 1997b). The first hVISA strain, Mu3 demonstrated a low MIC of 2μg/ml of vancomycin, indicating sensitivity, however, sub-populations of cells that were capable of growth in the presence of 5-

9μg/ml of vancomycin, indicating resistance, were also observed. During MIC testing, using any known breakpoint system, hVISA strains may be mistakenly judged to be sensitive yet may spontaneously generate sub-populations of VISA cells at high frequency which are implicated in clinical vancomycin treatment failure (Cui and Hiramatsu, 2003).

Currently, three VRSA isolates carrying *vanA* have been reported. All isolates had a MIC of ≥128μg/ml of vancomycin and were from locations within the mainland of the USA. The first case occurred in Michigan (Anonymous, 2002a) the second in Pennsylvania (Anonymous, 2002) and the most recent in New York (Anonymous, 2004b). The first two cases of VRSA have been confirmed to be the result of independent genetic events (Clark *et al.*, 2005) but all three cases are thought to be unrelated.

1.2.4.3 Mechanisms of vancomycin resistance

Two mechanisms of *S. aureus* resistance to vancomycin have been identified (Walsh and Howe, 2002). One form is associated with VISA strains, which have a MIC to vancomycin of 8-16μg/ml (Hiramatsu *et al.*, 1997a). This form of resistance is also associated with the clinically vancomycin-resistant sub-populations of cells associated with hVISA strains (Hiramatsu *et al.*, 1997b). The reduced susceptibility in both VISA and hVISA subpopulations appears to result from changes in peptidoglycan biosynthesis (figure 1.3). The exact mechanisms for these alterations in peptidoglycan biosynthesis are currently unknown.

Figure 1.3 Proposed mechanism of clinical resistance to vancomycin observed in VISA and subpopulations of hVISA strains. Adapted from (Sieradzki *et al.*, 1999).

It is proposed that glycopeptide antibiotics initiate their antibacterial activity by binding to the D-ala-D-ala terminus of the N-acetyl-muramyl-pentapeptide subunit (murein monomer) of cell wall precursors while they emerge at biosynthetic sites on the bacterial plasma membrane (Reynolds, 1989; Groves *et al.*, 1994; Mackay *et al.*,

1994). Glycopeptides also bind to D-ala-D-ala residues in completed peptidoglycan layers however this is not thought to inhibit peptidoglycan synthesis. Diffusion of glycopeptide molecules to the plasma membrane is presumed to occur nearly unhindered through the cell wall of susceptible staphylococci. In contrast, VISA and the clinically resistant subpopulations of hVISA strains synthesise additional quantities of D-ala-D-ala murein monomer residues which the glycopeptide molecules bind with, preventing them from reaching the sites of emerging cell wall precursors (Hanaki *et al.*, 1998b; Boyle-Vavra *et al.*, 2001). Additional quantities of synthesised peptidoglycan give VISA strains their characteristic irregularly shaped thickened cell wall (Cui *et al.*, 2003) and reduced cross-linking of peptidoglycan strands leads to further exposure of D-ala-D-ala residues (Hanaki *et al.*, 1998a; Hanaki *et al.*, 1998b). The altered cross-linking is due to reduced amounts of L-glutamine available for amidation of D-glutamate in the pentapeptide bridge during cell wall synthesis (Walsh and Howe, 2002) and this results in the increased quantities of D-ala-D-ala residues. In addition, it is understood that glycopeptide molecules bound in this manner become part of the mechanism of resistance by contributing to steric hindrance to free glycopeptide molecules which subsequently prevents them penetrating the outer cell wall to reach their target (Sieradzki *et al.*, 1999a). This mechanism has been designated affinity trapping (Hiramatsu, 1998; Rotun *et al.*, 1999; Marchese *et al.*, 2000). The clinical resistance phenotype expressed by VISA and hVISA subpopulations tend to be lost after time in the absence of selective antibiotic pressure (Pfeltz *et al.*, 2000; Boyle-Vavra *et al.*, 2001).

The second form of vancomycin resistance, which is observed in VRSA strains, is widely considered the result of the acquisition, from a vancomycin-resistant *Enterococcus faecalis*, of a 57.9 kbp multiresistance conjugative plasmid designated pLW1043, within which the transposon Tn1546 (*vanA*) is integrated (Weigel *et al.*, 2003). The enterococcal plasmid encoding *vanA* also encodes a sex pheromone that is synthesised by *S. aureus*, which supports the theory of conjugal transfer of *vanA* (Showsh *et al.*, 2001). Resistance in VRSA strains is caused by alteration of the terminal peptide to D-alanine-D-lactate (D-ala-D-lac) instead of D-ala-D-ala (figure 1.4). This effectively alters the target of glycopeptide antibiotics and renders them ineffective. Synthesis of D-ala-D-lac occurs only with exposure to low concentrations

of vancomycin and has limited additional biosynthetic demands so that VRSA remain ecologically fit (Gonzalez-Zorn and Courvalin, 2003). Recent work has highlighted that cell wall biosynthesis in VRSA strains is entirely dependant on PBP2 and that PBP2a, the product of *mecA*, appears to be unable to utilise the cell wall precursor produced in the vancomycin-resistant cells for transpeptidation (Severin *et al.*, 2004).

Figure 1.4 Mechanism of resistance to vancomycin in VRSA strains. Adapted from (Murray, 2000).

1.2.5 Community-acquired MRSA

Methicillin-resistant *S. aureus* have been primarily associated with nosocomial infections which mainly occurred in large tertiary hospitals and in intensive care units, where colonised and infected patients and colonised care workers were a significant

source of cross-infection. In recent years there has been an emerging problem of MRSA in the community. Community-acquired MRSA strains were first reported in Western Australia in 1993 (Udo *et al.*, 1993) but were not widely considered of great concern until 1999 after four paediatric deaths in Minnesota and North Dakota, USA were reported (Anonymous, 1999). It was after this report that interest was generated concerning the prevalence and origin of CA-MRSA.

1.2.5.1 Defining community acquired MRSA

The characteristics which distinguish CA-MRSA from HA-MRSA have not been standardised and at least eight different classifications have been described (Salgado *et al.*, 2003). The most commonly used description of a CA-MRSA implies that it is known that the microorganism was acquired in the community. This term, however, is often used to indicate detection of colonisation or infection in the community, rather than actual acquisition of MRSA in the community. Methicillin-resistant *S. aureus* colonisation can persist for months to years (Sanford *et al.*, 1994) which unless clinical infection arises, acquisition of MRSA may go undetected. This can often make it difficult to determine whether MRSA were acquired in the hospital or the community (Salgado *et al.*, 2003). Certain risk factors are associated with acquisition of MRSA, which include recent hospitalisation, recent surgery, recent outpatient visit, recent nursing home admission, recent antibiotic exposure, chronic illness, intravenous drug use and close contact with an individual with one or more of the aforementioned risk factors (Salgado *et al.*, 2003). It is therefore considered appropriate to evaluate risk factors before classifying an MRSA isolate as CA-MRSA.

In definition, a nosocomial infection is an infection acquired in hospital, and which is not in the incubation phase upon the patient's admission to hospital; however, a nosocomial infection may be caused by a colonising organism which the patient carried before hospital admission (Garner *et al.*, 1988). Typically this means that most bacterial nosocomial infections will become evident within 48 hours or more after admission to a hospital. Conversely, if symptoms occur outside of the hospital or within 48 hours of admission to hospital, then the infection is defined as "community-acquired."

There is evidence that the prevalence of CA-MRSA is high, however, a global analysis of 57 studies on the incidence of CA-MRSA among hospital patients or among community members reported that most individuals with CA-MRSA had more than one health care-associated risk, which suggests that the prevalence of MRSA among persons without risk factors is in fact very low (≤0.24%) (Salgado *et al.*, 2003). The high incidence of MRSA colonisation among members of closed populations, such as Australian Aboriginal (Udo *et al.*, 1993) and Native American (Groom *et al.*, 2001) communities, may be associated with risk factors for spread in the community that include overcrowding, high rates of skin infections and frequent use of broad-spectrum antibiotics (Maguire *et al.*, 1998).

A number of studies suggest that CA-MRSA among non-hospitalised patients are due to the introduction of HA-MRSA into the community (Salgado *et al.*, 2003; Coombs *et al.*, 2004; O'Brien *et al.*, 2004). In contrast other studies have reported that the clones found among CA-MRSA are different from any major HA-MRSA clones (Groom *et al.*, 2001; Naimi *et al.*, 2001; Dufour *et al.*, 2002; Okuma *et al.*, 2002), suggesting that CA-MRSA may arise sporadically through acquisition of *mecA* (Salmenlinna *et al.*, 2002). A CA-MRSA has even been implicated in a hospital outbreak (O'Brien *et al.*, 1999). Hiramatsu proposes that the essence of the issue is not the emergence of CA-MRSA but the wide dissemination of a successful type of SCC*mec* i.e. SCC*mec* type IV (Hiramatsu *et al.*, 2001).

Most CA-MRSA isolates carry the type IV SCC*mec* element (Okuma *et al.*, 2002; Vandenesch *et al.*, 2003), which does not encode any antibiotic resistance genes other than *mecA*. A major characteristic of CA-MRSA is susceptibility to most non-β-lactam antibiotics and thus unlike many HA-MRSA are non-multidrug resistant (Naimi *et al.*, 2001). Type IV SCC*mec* is the most widely disseminated and most successful type of SCC*mec* element found in twice as many clones as any other SCC*mec* type (Hiramatsu *et al.*, 2001; Robinson and Enright, 2003). Recently, a novel type V SCC*mec* element from an Australian CA-MRSA was reported that also only encoded the resistance gene *mecA* (Ito *et al.*, 2004).

1.2.5.2 Community-acquired MRSA and Panton-Valentine leucocidin

Of increasing concern are reports of highly virulent CA-MRSA carrying the genes for Panton-Valentine leucocidin (PVL), which have been implicated with severe necrotising pneumonia and skin and soft-tissue infections (Gillet *et al.*, 2002; Diep *et al.*, 2004). The PVL genes are encoded by two cotranscribed genes, *luk*S-PV and *luk*F-PV, which are carried by a prophage that can integrate into the *S. aureus* chromosome (Prevost *et al.*, 1995). The genes encode a two component cytolytic toxin with high affinity for human leukocytes. Until recently detection of the PVL genes was infrequent (<5%) amongst both HA-MRSA and CA-MRSA isolates (Prevost *et al.*, 1995; Lina *et al.*, 1999). Furthermore, based on there unrelated genetic backgrounds the fact the isolates were geographically dispersed there is significant evidence that these strains have multiple clonal origins (Vandenesch *et al.*, 2003). *Staphylococcus aureus* carrying the type IV SCC*mec* element and PVL has raised concern that a highly virulent pathogen well adapted to the hospital and community setting is emerging.

1.3 Mechanisms of antibiotic resistance

The mechanisms by which *S. aureus* acquire resistances genes may be classified into two main categories. These include chromosomal gene mutation and acquisition of resistance genes as a result of conjugation (cell to cell contact), transduction (bacteriophage-mediated) and transformation (direct uptake of free, non-self replicating DNA). *Staphylococcus aureus* can acquire exogenous antibiotic resistance genes by horizontal transfer of genomic islands, phage-mediated transduction, or from mobile genetic elements such as plasmids and transposons. In addition there are three general mechanisms by which resistance to antibiotics occur; (i) antibiotic is prevented from reaching its target; (ii) antibiotic is rendered inactive before reaching its target; (iii) alteration of the target, which affects antibiotic-target interaction (Chambers, 2004). Methicillin-resistant *S. aureus* isolates are more likely to be resistant to other antimicrobial agents than MSSA isolates (Ben-David and Rubinstein, 2003). A summary of antibiotic resistance mechanisms reported in MRSA strains is depicted in table 1.2.

1.3.1 Fluoroquinolones

Fluoroquinolones are synthetic antibacterials introduced in the 1980s and the first quinolones to have activity against staphylococci. They target the essential type II DNA topisomerases, namely DNA gyrase and topoisomerase IV (Drlica and Zhao, 1997). These enzymes are made up of 2 units each of GyrA and GyrB, in the case of DNA gyrase, and GrlA and GrlB, in the case of topoisomerase IV. Both are involved in maintaining chromosomal DNA integrity during negative supercoiling and decatenation. Negative supercoiling is initiated by DNA gyrase and is required to release topological pressure that builds up along the chromosomal DNA during transcription and replication. Decatenation is carried out by topoisomerase IV and is a vital process required for the separation of daughter chromosomes during cell division. The exact mechanism of action fluoroquinolones exert is unknown, however, there is evidence that they act as poisons trapping DNA gyrase and topoisomerase on DNA, which leads to a lethal release of double-strand DNA breaks (Drlica and Zhao, 1997).

Resistance to fluoroquinolones can occur in *S. aureus* due to two mechanisms. The first mechanism is due to chromosomal mutations in the DNA gyrase genes *gyrA* and *gyrB* and the topoisomerase IV genes *grlA* and *grlB*. This is essentially a mutational target alteration as the antibacterial will no longer bind to either enzyme. Uniquely fluoroquinolone resistance has not been observed in a plasmid mediated form in *S. aureus* isolates. The second mechanism observed in staphylococci is due to a chromosomally encoded multidrug efflux pump, designated NorA (Neyfakh *et al.*, 1993).

Table 1.2 Mechanisms and genetic determinants of resistance in *S. aureus* to commonly used antimicrobials. Adapted from ((Lowy, 2003) and (Chambers, 2004))[a].

1.3.2 Macrolides, Lincosamides and Streptogramin B

Macrolides, lincosamides and streptogramin B (MLS) are separate classes of antibiotics with similar target sites that overlap and focus on the 50S ribosomal subunit. Typically, MLS antibiotics bind reversibly to the peptidyl transferase centre of the 50S ribosomal subunit and inhibit RNA-dependant protein synthesis (Morrissey and Farrell, 2003). Several categories of phenotypic resistance patterns have been described in *S. aureus*. These include M-type, resistance to 14 and 15 membered ring macrolides; MS-type, resistance to 14 and 15 membered ring macrolides and streptogramin B; and MLS-type, resistance to 14, 15 and 16 membered ring macrolides, lincosamides and streptogramin B.

There have been three mechanisms of MLS resistance reported in *S. aureus*. The first mechanism is due to methylation of a common binding site on the ribosome altering the target site. There have been five methylase genes described in *S. aureus* which are designated, *ermA* (Murphy, 1985), *ermB* (Wu *et al.*, 1999), *ermC* (Projan *et al.*, 1987), *ermF* (Chung *et al.*, 1999) and *ermY* (Matsuoka *et al.*, 2002). The most common genes in clinical isolates of *S. aureus* are *ermA* which is encoded on a transposon, Tn554 that is associated with MRSA strains with the type II and III SCC*mec* element and *ermC*, which has been found on plasmids (Westh *et al.*, 1995; Lina *et al.*, 1999). The expression of methylase genes is either constitutive or inducible and as such directly affects the resistance phenotype. Strains that are constitutively resistant are MLS-type and those that are inducible may only be M-type, however, inducible strains readily mutate in the presence of MLS antibiotics and become constitutively resistant (Weisblum, 1985). The second mechanism is due to an ATP-dependant efflux pump encoded by the *msrA* gene. Active efflux will only confer MS-type resistance and is rare in clinical *S. aureus* isolates (Martineau *et al.*, 2000). The third mechanism has also only been described rarely in clinical *S. aureus* isolates and involves the inactivation of macrolides by esterases encoded by *ereA* and *ereB* (Wondrack *et al.*, 1996).

1.3.3 Tetracyclines

Tetracyclines are a class of broad-spectrum bacteriostatic antibiotics that were first discovered in the late 1940s. There are three generations of tetracyclines that include first generation lead compounds, second generation semi-synthetic analogues and third generation glycylcyclines. Tetracyclines target a high affinity site on the small 30S subunit of the bacterial ribosome inhibiting protein synthesis (Schnappinger and Hillen, 1996). The binding of tetracyclines to this target is reversible, hence their bacteriostatic rather than bacteriocidal effect (Chopra *et al.*, 1992).

The main mechanisms of resistance to tetracyclines observed clinically in *S. aureus* isolates are active efflux and ribosome protection. Two plasmid encoded genes, *tet*(K) and *tet*(L), have been attributed to active efflux of tetracycline antibiotics and two genes, *tet*(M) and *tet*(O) either chromosomally or transposon encoded have been attributed to ribosome protection (Chopra and Roberts, 2001). The *tet*(K) and *tet*(L) genes are found on small plasmids such as pT181. These genes encode membrane-associated proteins that export tetracyclines from the bacterial cell, however, glycylcyclines are not transported by these proteins (Chopra and Roberts, 2001). The *tet*(M) and *tet*(O) genes encode ribosomal protection proteins, which act within the bacterial cytoplasm conferring tetracycline resistance by binding to the bacterial ribosome without affecting the rate or extent of protein synthesis.

1.3.4 Rifampicin fusidic acid and mupirocin

Rifampicin is a semisynthetic derivative of rifamycin B, which is a member of the rifamycin family of antibiotics produced by *Amycolatopsis mediterranea*. Uniquely, rifampicin binds to bacterial RNA polymerase β subunit, encoded by the *rpoB* gene, thus preventing transcription (Aboshkiwa *et al.*, 1995). Resistance occurs when mutations in the *rpoB* gene lead to structural changes in the RNA polymerase β subunit target resulting in reduced binding affinity for the antibiotic (Morrow and Harmon, 1979).

Fusidic acid is a steroid-like antibiotic, first isolated from *Fusidium coccineum* (Godtfredsen *et al.*, 1962). Like rifampicin, fusidic acid has a unique target and interferes with elongation factor G to inhibit bacterial protein synthesis (Tanaka *et al.*, 1968). In *S. aureus*, resistance is caused by mutations in the gene *fusA*, which result in structurally altered elongation factor G resulting in decreased fusidic acid binding (Chopra, 1976). Resistance can develop rapidly in *S. aureus* when rifampicin or fusidic acid are used in monotherapy. For this reason these antibiotics are often used in conjunction with each another (O'Neill *et al.*, 2001).

Mupirocin also known as pseudomonic acid, is a topical antibiotic derived from the fermentation of *Pseudomonas fluorescens* (Fuller *et al.*, 1971). Mupirocin is an analogue of the amino acid isoleucine and inhibits protein synthesis by binding irreversibly to the enzyme isoleucyl tRNA synthetase (IleS) (Hughes and Mellows, 1978). Two forms of resistance have been described in *S. aureus*. Low level resistance to 8-256mg/L of mupirocin is due to a structural mutation in IleS (Antonio *et al.*, 2002). High level resistance to $\geq$ 512mg/L of mupirocin and is due to the acquisition of a new *mupA* gene that encodes a second novel IleS (Hodgson *et al.*, 1994). Spread of the *mupA* gene amongst *S. aureus* is typically plasmid mediated (Rahman *et al.*, 1990). The significance of either form of resistance in a clinical setting is unclear as mupirocin is often used in concentrations of 20000mg/L, which far exceeds the minimum inhibitory concentrations (MICs) reported in resistant *S. aureus* strains.

1.3.5 Aminoglycosides

The aminoglycosides are a diverse group of broad-spectrum bacteriocidal antibiotics, which disrupt protein synthesis by binding to the 16S rRNA component of the ribosomal 30S subunit. Resistance in clinical *S. aureus* isolates is due to enzymatic modification of aminoglycoside molecules reducing the binding affinity to the target RNA (Kotra *et al.*, 2000). The enzymes responsible are N-acetyltransferases (AAC), O-nucleotidyltransferases (ANT) and O-phosphotransferases (APH) which all modify specific amino or hydroxyl groups on aminoglycosides. Each of these enzymes can be further distinguished based on their site of modification (Vanhoof *et al.*, 1998). In staphylococci resistance to gentamicin, tobramycin and kanamycin is mediated by

AAC(6')-APH(2'') encoded by the *aac(6')-Ie+aph(2'')* gene. This gene is commonly located on the transposon Tn*4001* and is widely distributed both chromosomally and on many plasmid types (Ubukata *et al.*, 1984).

Aminoglycoside resistance has also been attributed to a novel mechanism of antibiotic resistance, namely small colony variants (SCV) of *S. aureus*. Isolates of SCV exhibit an altered phenotype to their parent strain, which aids their survival intracelluarly and may cause persistent recurrent infection. The SCV membrane has a reduced electrochemical gradient that decreases the uptake of antimicrobial agents such as aminoglycosides that require a charge differential to be active (von Eiff and Becker, 2003). This mechanism of resistance has only been observed in *S. aureus* and MRSA isolates.

1.3.6 Sulphonamides and trimethoprim

The sulphonamide, sulphamethoxazole and trimethoprim are synthetic agents often used in combination as they have a synergistic interaction against *S. aureus in vitro* and are effective against MRSA (Bushby and Hitchings, 1968; Then *et al.*, 1992). Sulphonamides, including sulphamethoxazole, and trimethoprim inhibit folic acid biosynthesis. Sulphonamides are structural analogues of p-aminobenzoic acid (PABA), the natural substrate of the bacterial enzyme dihydropterate synthetase (DHPS), which is involved in the production of folic acid. In a later step of folic acid biosynthesis, dihydrofolate reductase (DHFR) forms tetrahydrofolate from dihydrofolate. It is at this stage trimethoprim inhibits this process as it is an analogue of dihydrofolate.

Resistance to sulphonamides in *S. aureus* occurs due to mutations in the *dhps*(*folP*) gene. This effectively alters the target preventing, sulphonamides from binding to DHPS but allowing the enzyme to function normally. Trimethoprim resistance is commonly attributed to an altered DHFR encoded by the *dfrA* gene located on transposon Tn*4003*. Typically, this transposon is carried by various plasmids but can also be located chromosomally (Burdeska *et al.*, 1990; Dale *et al.*, 1995).

1.4 Typing methods

A number of phenotypic and genotypic techniques may be employed for the typing of *S. aureus* and MRSA to aid in infection control and the analysis of bacterial populations and evolution. Criteria exist for interpreting typeability, reproducibility, discriminatory power, ease of interpretation and ease of use for different techniques (Maslow *et al.*, 1993; Tenover *et al.*, 1994).

1.4.1 Phenotypic methods

Typing methods that assess phenotypic differences rely on testing for a gene product that is expressed. Although phenotypic methods are limited by the propensity of organisms to alter the expression of their genes, these methods were first employed to discriminate between *S. aureus* strains and still prove to be useful tools.

1.4.1.1 Antibiotic sensitivity testing

One of the most useful phenotypic techniques routinely carried out in most microbiology laboratories is antibiotic susceptibility testing (AST) which is also called resistotyping. This technique may be used to determine the sensitivity of a microorganism to a panel of antibiotics; the pattern of resistance and sensitivity being recorded as an antibiogram. Generally, AST is used within microbiology laboratories to identify empirical therapy for a MRSA infection, however, it is also possible to use the antibiogram to differentiate between strains of the same species (Mulligan and Arbeit, 1991). Investigations of localised outbreaks of MRSA using a quantitative disk diffusion method have demonstrated comparable results with ribotyping (Rossney *et al.*, 1994; Rossney *et al.*, 1994a). This approach is particularly useful for identifying clonally unrelated isolates of MRSA, as antibiotic resistance can vary considerably from strain to strain. The technique is highly quality-controlled, easy to perform, gives rapid results and is inexpensive, however, poor discriminatory ability between isolates of the same clone and lack of reproducibility are disadvantages. From sharing the same environment and thus similar antibiotic selective pressures unrelated strains may share the same antibiogram. Conversely, strains that are the same clone may have

different antibiograms due to the loss or acquisition of a mobile genetic element carrying resistance genes (Kuroda *et al.*, 2001) or even a single nucleotide mutation (Aubry-Damon *et al.*, 1998). The identification of a new pattern of antibiotic resistance among isolates cultured from different patients is often the first indication of an outbreak.

1.4.1.2 Biotyping

Biotyping utilises the phenotypic responses an organism expresses to a panel of tests and may include biochemical tests, morphological differences and environmental tolerances (Arbeit, 1997). For instance tests may include fermentation of mannitol, small colony variants and tolerance to pH.

Biotyping can be used to assess the relationships between strains but often suffers from poor reproducibility, mainly because a microorganism can alter the expression of many cellular products (Tenover *et al.*, 1997). Variations in inoculum size, incubation conditions and the age of cultures may all effect phenotypic expression (Tenover *et al.*, 1997). Although biotyping may be used to differentiate between MRSA strains studies have shown that biochemical tests may yield so many distinct subtypes that it is not possible to group outbreak-associated isolates (Tenover *et al.*, 1994).

1.4.1.3 Serotyping

Serotyping uses antibodies to detect different antigenic determinants on the surface of bacteria. Most *S. aureus* isolates are covered by a polysaccharide capsule, of which 11 different serotypes have been identified (Fournier, 1990). Types 5 and 8 are the most commonly occurring types and account for 75 percent of human infections and almost all MRSA strains (Schlichting *et al.*, 1993; Wilkinson, 1997; Lowy, 1998). The poor discriminatory power of serotyping has meant that this technique is not widely used for typing MRSA.

1.4.1.4 Phage typing

Phage typing has been used for decades to characterise strains of *S. aureus* by their differential susceptibility to bacteriophage infection and was first used specifically for *S. aureus* in 1972 (Parker, 1972). The technique is based on the observation that most *S. aureus* isolates carry bacteriophages that lyse some, but not all, epidemiologically unrelated strains. If isolates of the same strain share an identical lysis pattern it is assumed that epidemiologically they are the same. Using an internationally agreed panel of diverse bacteriophages it is possible to differentiate between distinct *S. aureus* strains during an epidemiological study. This technique first identified the epidemic-MRSA (EMRSA) strains, EMRSA-15 and EMRSA-16, predominantly found in UK hospitals.

Although this method has been widely used as a high-throughput and cheap phenotypic method it has largely been replaced in recent years by molecular typing techniques. The enormous effort and technical skill required to maintain high quality phage sets, poor discriminatory ability between MRSA strains (Tambic *et al.*, 1997) and limitations in reproducibility (O'Neill *et al.*, 2001a) have all contributed to this change.

1.4.1.5 Multilocus enzyme electrophoresis

Multilocus enzyme electrophoresis (MLEE) is used to compare variation in the electrophoretic mobility of essential metabolic "housekeeping" enzymes, which are visualised on starch gels providing a series of allele numbers for each locus studied (Enright and Witte, 2004). Allele numbers are generated by comparing enzyme electrophoretic mobility with standard enzyme extracts for each allele, which are run on the same gel. The technique has contributed to the understanding of bacterial population biology (Kreiswirth *et al.*, 1993) but has not been widely employed as an epidemiological typing tool. Although MLEE can be highly discriminatory if sufficient loci are examined the technique is extremely labour intensive, requires the running of many allelic standards on each gel and requires specialised reagents for

each enzyme stained. The technique, however, is reproducible, robust and results are relatively easy to interpret and may be represented on dendrograms.

1.4.2 Genotypic methods

Developments in molecular biology have allowed the relationships between *S. aureus* strains to be assessed genotypically. Currently, there are multiple-DNA-based methods for the molecular typing of *S. aureus* which can be broadly separated into techniques that require restriction endonuclease digests of DNA and PCR-based methods. Both typically provide distinctive banding patterns for differentiating unrelated strains. A third category designated, probe mediated techniques may combine features of other methods. No single technique yet exists that is universally applicable for the molecular typing of *S. aureus* strains and thus many of these methods are used in conjunction with each other.

1.4.2.1 Restriction endonuclease analysis of plasmid DNA

Plasmids are autonomously replicating extra-chromosomal DNA and arguably the best understood mobile genetic elements in bacteria. Currently, it is estimated that more than 90% of MRSA strains carry plasmids and approximately 50% of MSSA isolates lack them (Coia *et al.*, 1988; Hartstein *et al.*, 1989; Hartstein *et al.*, 1995).

The analysis of plasmid DNA was the first DNA-based technique to be applied in the study of the epidemiology of *S. aureus* and MRSA (Meyers *et al.*, 1976; McGowan *et al.*, 1979; Locksley *et al.*, 1982). The number and size of plasmid DNA carried by different isolates can be resolved rapidly and inexpensively by electrophoresis, to differentiate *S. aureus* strains. Plasmid DNA can naturally exist in three variable states, supercoiled, nicked and linear, all of which have different electrophoretic properties. Due to this it is possible for the same plasmid DNA to resolve as three separate bands of varying molecular size making it difficult to discriminate accurately between different plasmid types. This problem can be circumvented by digesting plasmid DNA with a restriction endonuclease and analysing the number and size of restriction fragments. Restriction endonuclease analysis of plasmid DNA can

determine the variable position and frequency of restriction sites in two unrelated plasmids of the same molecular size allowing them to be differentiated (Hartstein *et al.*, 1995).

Not all *S. aureus* isolates carry plasmids and this can limit the application of REAP, however, the method can be useful during acute outbreaks for typing if isolates carry plasmids and in conjunction with AST for identifying resistance plasmids. There have been multiple studies reported where REAP has been used in conjunction with PFGE to ascertain both chromosomal and plasmid genotypes (Zuccarelli *et al.*, 1990; Tenover *et al.*, 1994; Hartstein *et al.*, 1995).

1.4.2.2 Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) is undoubtedly the most widespread genotyping tool in developed countries and is considered the gold standard for DNA fingerprinting of MRSA (Murchan *et al.*, 2003). Briefly, PFGE involves embedding microorganisms in agarose and lysing them *in situ* using lytic enzymes; in the case of MRSA using lysozyme, lysostaphin and proteinase K. The lysis releases the chromosomal DNA, which remains suspended in the surrounding agarose. The chromosome is then digested with a restriction endonuclease that cuts infrequently; in the case of MRSA this is usually *Sma*I (Tenover *et al.*, 1995). The large DNA fragments are prevented from undergoing autodigestion after the restriction digest as they remain embedded in the agarose plug. Slices of the agarose containing the chromosomal DNA fragments are inserted into the wells of an agarose gel and the restricted DNA fragments are resolved into a pattern of distinct bands. This process is implemented using apparatus that switches the direction of current across the gel according to a predetermined pattern. In this way large linear fragments of DNA are better able to move through the agarose gel matrix along the path of least resistance. The DNA restriction profiles of each isolate can then be visualised and compared with one another to determine relatedness. The PFGE method is an extremely useful epidemiological tool, however, it is relatively time consuming, taking upwards of six days to get profiles using some protocols (Matushek *et al.*, 1996) and it is also

expensive due to the cost of reagents and the requirement of specialised equipment (Tenover *et al.*, 1998).

1.4.2.3 Multilocus sequence typing

Multilocus sequence typing is used to compare the variable and conserved sequences of DNA from amplified internal fragments of six or seven "housekeeping" genes and may thus be used to classify microorganisms (Maiden *et al.*, 1998). Housekeeping genes encode proteins that are essential for cell viability. The sequence of each gene fragment is assigned a distinct allele identification number and the combination of the numbers is used to generate distinct sequence types. Isolates that share the same allelic profile are considered clonally related.

The technique has been used successfully to type MRSA strains (Enright *et al.*, 2000) for which a database of strain information exists. Sequence types are easily stored in computer databases and laboratories can compare strain relationships via the internet (Spratt, 1999). Multilocus sequence typing is highly discriminatory and results correlate well with other molecular typing techniques, such as PFGE (Enright *et al.*, 2000). The technique has been used successfully to investigate bacterial phylogeny and the evolution of population lineages, however, MLST is considered technically demanding and is generally unsuitable for typing strains in hospital outbreaks and epidemics (Enright *et al.*, 2002; Robinson and Enright, 2003).

1.4.2.4 *Spa* typing

Protein A is a cell wall constituent of *S. aureus* encoded by the *spa* gene. *Spa* typing (protein A gene typing) uses PCR to amplify a hypervariable repetitive section of the *spa* gene, called region X which contains various numbers of degenerate 24 bp repeats (Tang *et al.*, 2000). Using DNA sequencing it is possible to determine the number and sequence of the repeats and to differentiate between *S. aureus* strains by comparing the differences in these repeats (Shopsin *et al.*, 1999). The technique is rapid and relatively easy to perform.

The discriminatory ability of this technique is poor as there is only one gene target and variation in *spa* is limited. In addition, *spa* types do not correlate with the clonal relationships defined by other molecular typing techniques, however, the method does represent a suitable alternative first approach for investigating an epidemic outbreak (Montesinos *et al.*, 2002).

1.4.2.5 Random amplification of polymorphic DNA

Random amplification of polymorphic DNA (RAPD) also called arbitrarily primed PCR (AP-PCR) has been employed to successfully type a number of Gram-negative and Gram-positive organisms including *S. aureus* (Hilton *et al.*, 1996; van Leeuwen *et al.*, 1996; Hilton *et al.*, 1997a; Perry *et al.*, 2003).

The RAPD method requires the use of a single short primer, typically 10 bp, the sequence of which is not directed towards any specific sequence of the target DNA template. An advantage of this method is that no prior knowledge of the template DNA sequence is required.

The first round of PCR is carried out under low annealing temperatures. This is done to promote low stringency binding of the primer to the template DNA. The process promotes the annealing of the short primer to sequences of DNA which are not entirely complementary to that of the primer sequence. Subsequent rounds support high stringency binding so that the primer can only anneal to a complementary sequence on the template; in this way the reaction supports the amplification of the template product from the first round of cycles. The PCR products are resolved into a pattern of discrete bands using gel electrophoresis. Polymorphisms within the template vary and the proximity, number and locations of priming sites between strains contribute to the unique DNA fingerprints.

One criticism of RAPD has been its lack of reproducibility, however, if critical factors affecting the reaction are addressed this need not be a problem. To ensure the reproducibility of the reaction template concentration, primer design and concentration, brand and type of DNA polymerase, reaction conditions and buffer

composition must be optimised. This can ensure that RAPD gives a high level of typability, discriminatory power and reproducibility.

1.4.2.6 Multiplex PCR for mec element type assignment

Currently, three PCR techniques have been applied to the characterisation of the SCC*mec* element. Two of the techniques amplify the heterogeneous *ccr* gene and *mec* gene complexes to discern the SCC*mec* element type (Ito *et al.*, 2001; Okuma *et al.*, 2002). The remaining technique is a multiplex PCR that characterises the SCC*mec* element types based on the structural features of the typical SCC*mec* elements carried by several MRSA clones (Oliveira and de Lencastre, 2002). Combined with MLST, SCC*mec* type assignment has been used to estimate that SCC*mec* has been acquired by *S. aureus* in nature on 20 separate occasions (Robinson and Enright, 2003) and to propose a new nomenclature for MRSA clones (Enright *et al.*, 2002).

The technique is useful when combined with other molecular typing techniques to discern SCC*mec* lineage but has a limited discriminatory ability when used alone.

1.4.2.7 Ribotyping

Ribotyping is a hybridisation-mediated typing procedure that uses ribosomal RNA as a probe. The technique requires the restriction of chromosomal DNA by a frequently cutting restriction enzyme, such as *EcoRI*. The DNA fragments are then separated by size using electrophoresis and subsequently transferred onto a nylon membrane. Labelled probes of restricted ribosomal gene fragments are then used to target the chromosomal DNA. This detects polymorphisms in the number and sites of the ribosomal operon targets. All staphylococci carry five to seven ribosomal operons that may vary in sequence and the position of restriction sites thus it is possible to identify the differences between unrelated strains (Blumberg *et al.*, 1992; Arbeit, 1997). Radiolabelling of probes with ^{32}P is commonly used, however, the technique has been successfully performed with a biotinylated probe that is believed to be safer and more stable (Preheim *et al.*, 1991).

Although ribotyping is more reproducible and discriminatory than phenotypic methods (Blumberg *et al.*, 1992) the technique is the least discriminatory of all molecular typing techniques (Tenover *et al.*, 1994). The technique is also technically demanding and time consuming and for these reasons has not been widely adopted for the typing of MRSA.

1.4.2.8 Binary typing

Binary typing is a probe-mediated method combined with reverse hybridisation, in principle very similar to Southern hybridisation. The technique uses a set of *S. aureus* specific DNA probes generated using RAPD and selected for their discriminatory ability (van Leeuwen *et al.*, 1996; van Leeuwen *et al.*, 1999). Each DNA probe corresponds to a specific fraction of the *S. aureus* genome. During typing, *S. aureus* genomic DNA is subjected to a hybridisation assay and subsequent analysis reveals the presence or absence of hybridisation for each probe providing a simple yes/no binary code. The codes have been shown to be stable within a strain, constant within an outbreak strain and unique for epidemiologically unrelated strains (van Belkum *et al.*, 1997; van Leeuwen *et al.*, 1998; van Leeuwen *et al.*, 1999; Zadoks *et al.*, 2000). This has demonstrated the technique to be technically reproducible, biologically reproducible and to have sufficient resolution as a typing method. In addition, binary codes can be stored in a computer database and exchanged between laboratories allowing strain relationships to be studied nationally and internationally (van Leeuwen *et al.*, 2002).

The method has been reported to be more discriminatory than PFGE, reproducible and to transfer successfully to other laboratories (van Leeuwen *et al.*, 1999; van Leeuwen *et al.*, 2002). The main disadvantage to this technique is the length of time that is required to complete the whole typing process, however, once the technique is set up in a laboratory the method is reported to be more rapid than PFGE.

1.5 The *S. aureus* Genome

Five complete *S. aureus* genomes have been published to date that include N315, Mu50, MW2, MRSA252 and MSSA476 (Kuroda *et al.*, 2001; Baba *et al.*, 2002; Holden *et al.*, 2004).

Two other *S. aureus* genomes are also publicly available COL (http://www.tigr.org) and NCTC 8325 (http://www.genome.ou.edu/staph). In addition, whole genome sequences of two strains of staphylococcal species are available for comparison with the *S. aureus* genome. These are *Staphylococcus epidermidis*, ATCC12228 and RP62A, available at (http://www.ncbi.nlm.nih.gov accession numbers NC_004461 and accession number NC_002976) respectively. At present there are more complete genomes available for *S. aureus* than any other bacterial species. Genes that govern virulence and resistance are found on the chromosome and extrachromosomal elements. A summary of the major genetic elements are included in tables 1.3 and 1.4.

The *S. aureus* strains sequenced to date are comprised of the following: N315 is isolate number 325 from Nagasaki University Hospital, Japan and is a HA-MRSA; Mu50 is a HA-VISA strain from Japan that is related to N315; MW2 is a CA-MRSA from North Dakota, USA responsible for a fatal paediatric bacteraemia and carries the genes for PVL (Anonymous, 1999); MRSA252 is a typical epidemic MRSA-16 (EMRSA-16) strain from Oxford, UK; MSSA476 is a CA-MSSA from Oxford, UK; COL is an early MRSA from Colindale, UK; and NCTC8325 is a widely used *S. aureus* laboratory strain from Colindale, UK.

1.5.1.1 Staphylococcal cassette chromosome

The five *S. aureus* genomes published to date have a genome consisting of a circular chromosome approximately 2.8mbp in size, with prophages, vSa islands, plasmids, insertion sequences and transposons. All possess an SCC element; N315, Mu50, MW2, and MRSA252 carry a SCC*mec* element; MSSA476 carries a unique SCC*mec*-like-element designated SCC_{476} (Holden *et al.*, 2004). The SCC_{476} of MSSA476 encodes a protein with 43.7% amino acid homology to the fusidic acid-resistance

determinant Farl which does appear to confer fusidic acid-resistance (O'Brien *et al.*, 2002). The SCC*mec* of MRSA252 has been designated type II and is similar to those reported in N315 and Mu50 (Katayama *et al.*, 2000; Ito *et al.*, 2001). The structure of SCC*mec* in MW2 is type IVa and that of COL is type I (Hiramatsu *et al.*, 2001; Baba *et al.*, 2002).

1.5.1.2 Genome structure

Based on a comparison of GC content *S. aureus* is classified with low GC content Gram-positive eubacteria, which includes the bacterial species *Bacillus subtilis*, *Lactococcus lactis*, *Streptococcus pyogenes* and *Clostridium perfringens* (Baba *et al.*, 2004). Of these species *B. subtilis* has been identified as the most closely related to date (Kuroda *et al.*, 2001).

It is estimated that *S. aureus* has between 2400-2700 open reading frames (*orfs*) that may encode functional proteins, which were either vertically transmitted from ancestral bacterial species, generated *de novo* by gene duplication, or horizontally acquired during various evolutionary stages of speciation (Baba *et al.*, 2004). The *S. aureus* chromosome is divided into two functional domains. The first is designated the genetic backbone and encodes functions that are essential for maintenance and the propagation (house-keeping) of the microorganism. This backbone was transmitted vertically for innumerable generations starting from ancestral cells. The second consists of genomic islands/islets that have been acquired laterally, which encode function for successful vegetation and propagation in the specific environments that *S. aureus* may inhabit (Baba *et al.*, 2004). The term genomic island has been used in place of the term pathogenicity island as new islands have been identified that do not carry associated with pathogenicity (Baba *et al.*, 2002). Well established transposons (Tn) and insertion sequences (IS) are excluded from genomic island terminology.

Genomic islands are defined as relatively large chromosomal regions that have been acquired from other bacteria by lateral gene transfer. Typically, genomic islands encode most of the genetic material that is involved in *S. aureus* virulence and drug resistance. These located at multiple loci in the *S. aureus* chromosome and are

dispersed throughout the backbone of the chromosome. The genomic islands identified to date for *S. aureus* are sized between 3.1-45.9 kb (Baba *et al.*, 2004). Some virulence factors are found on the chromosome at loci that are not part of genomic islands and exist as single genes among house-keeping genes, which are well conserved amongst *S. aureus*. Such virulence genes are designated genomic islets as they are smaller in size, may exist in several allelic forms and may be exchanged among *S. aureus* strains by lateral gene transfer. Prophages and νSa islands are classified as genomic islands. A prophage is a latent bacteriophage in which the viral genes have been incorporated into the host chromosome without disrupting bacterial cell function; three prophage families designated ΦSa1-ΦSa3 have been defined to date (Baba *et al.*, 2004). The prophage ΦSa1mu has only been found on the Mu50 chromosome and the PVL toxin genes *lukF-PV* and *lukS-PV* have been found to be carried by ΦSa2mw in strain MW2 (Finck-Barbancon *et al.*, 1991; Baba *et al.*, 2004). These have been used as examples of how bacteriophages play a role in the short term evolution of *S. aureus* clones (Lindsay and Holden, 2004). Genomic islands that are unique to *S. aureus* and structurally unrelated to either prophages or SCC are designated νSa islands; ν is derived from the Greek word [υισοσ] meaning island. Such elements have been identified as encoding pathogenic factors such as superantigens and cytolytic toxins (Baba *et al.*, 2004). The strain MW2 carries a unique island designated νSa3mw that carries two new allelic forms of enterotoxin genes, *sel2* and *sel4* (Baba *et al.*, 2002). Two other classes of ν islands have been identified in all the *S. aureus* strains tested and have been designated νSaα and νSaβ (Baba *et al.*, 2004). These are thought to have been integrated into the chromosome of evolutionary early *S. aureus*.

Multiple Tn and IS have been identified in the *S. aureus* genome. Such mobile genetic elements often encode antimicrobial resistance genes and are found on the *S. aureus* chromosome and the SCC element (table 1.5). Strain MRSA252 was found to carry in addition to SCC*mec* two copies of Tn*554* encoding erythromycin resistance (Holden *et al.*, 2004). In addition to Tn and IS, plasmids are an important vehicle for the introduction of IS and Tn into the *S. aureus* genome.

Plasmids are laterally acquired genetic elements that are capable of self replication in the cytoplasm of the host cell and may be grouped into three classes according to their sizes and capacity of self-transfer (Novick, 1989); Class I plasmids are small sized, found in many Gram-positive bacteria, typically transfer readily among other Gram-positive species and often carry a resistance gene such as tetracycline, chloramphenicol or erythromycin; Class II plasmids are bigger in size and carry multiple antibiotic resistance genes often located on transposons which typically confer resistance to β-lactamase labile penicillins (Tn*552*), aminoglycosides (Tn*4001*), macrolides (Tn*551*) and various heavy metals; Class III plasmids are the biggest in size, are capable of intercellular self-transfer, are thus designated conjugative plasmids and unlike other plasmid classes carry transfer gene complexes which are essential for conjugative transmission (Morton *et al.*, 1993). The Class III plasmids were first identified in outbreaks of gentamicin resistance and are responsible for the transmission of gentamicin resistance between *S. aureus* and coagulase-negative staphylococci (Archer and Johnston, 1983). Plasmids carried by *S. aureus* typically range in size from 1 to 60 kb. The strain MSSA476 has been identified as carrying a plasmid designated pSAS1 encoding a β-lactamase (pSAS19) (Holden *et al.*, 2004). A summary of reported Staphylococcal plasmids is included in table 1.6.

The sequencing of seven *S. aureus* genomes has provided insight into the structure of the chromosome and extrachromosomal elements of both MRSA and MSSA strains Such work has contributed to our understanding of *S. aureus* evolution, virulence and resistance to antimicrobial agents. It is expected that several more *S. aureus* genomes will be sequenced in the future. Currently, the genome of *S. haemolyticus* is being sequenced (Baba *et al.*, 2004). This will allow the direct comparison of the *S. aureus* genome with that of a closely related yet less virulent species.

Table 1.3 The major genetic elements in sequenced S. aureus strains. (Adapted from (Baba *et al.*, 2004; Holden *et al.*, 2004)).

Table 1.4 The major genetic elements in sequenced S. aureus strains. (Adapted from (Baba *et al.*, 2004; Holden *et al.*, 2004)).

Table 1.5 Transposon and insertion sequences found in *S. aureus*. Adapted from (Baba *et al.*, 2004).

Table 1.6 Staphylococcal plasmids*. (Adapted from (Baba *et al.*, 2004)).

1.6 Virulence and pathogenesis

Staphylococcus aureus is considered a serious pathogen due to its array of multifactorial virulence mechanisms that cause an array of human diseases. Such virulence factors can be generally separated into two main classes; surface-associated factors and secreted factors (table 1.7); surface-associated factors provide the mechanisms for adherence, attachment and immune evasion; and secreted factors that may cause tissue destruction counteracting host cell responses (Wright and Novick, 2003). The expression of most virulence factor genes is regulated by the accessory gene regulator (*agr*) loci, which is also involved in quorum sensing (Ji *et al.*, 1995).

Table 1.7 Potential virulence determinants of *S. aureus*. Adapted from (Lee and Bohach, 2004).

1.6.1 Surface-associated factors

It is widely accepted that *S. aureus*, during the exponential phase of its growth cycle *in vitro*, produces surface proteins (adhesins) that mediate adherence to extracellular matrix proteins in a host, other bacterial cells and even inert surfaces (Foster and Hook, 1998). These proteins are collectively known as, microbial surface components recognising adhesive matrix molecules (MSCRAMMS). Many of them share biological and structural features and have certain domains in common. Most staphylococcal surface proteins are covalently anchored to the cell wall by sortase A (SrtA), the presence of which is important in virulence. If mutations occur in SrtA a

defective surface protein linkage is often observed that gives rise to a "bald" phenotype which exhibits reduced virulence (Mazmanian *et al.*, 1999).

Probably the most studied surface protein in staphylococci is staphylococcal protein A (Spa) which plays a role in hiding *S. aureus* from the innate immune system during early infection (Patel *et al.*, 1987; Patel *et al.*, 1992). Protein A on the surface of *S. aureus* binds immunoglobulin G (IgG) in an orientation opposite to that required for its function. In this manner protein A inhibits phagocytosis and disguises *S. aureus* from the innate immune response by preventing opsonisation-dependant activation of the complement cascade (Wright and Novick, 2003).

Formation of a polysaccharide capsule is another process by which *S. aureus* is able to evade the host immune system. Capsules produced by *S. aureus* are typically very thin (<0.05 μm) and known as microcapsules (Wright and Novick, 2003). Approximately 90% of clinical isolates of *S. aureus* possess a microcapsule, which is implicated with masking complement factor C3b that binds to the *S. aureus* cell wall, blocking phagocytosis and enhancing virulence (Cunnion *et al.*, 2001).

The extracellular matrix proteins of a host are abundant with fibronectin and fibrinogen, which are important for wound healing and often absorbed on foreign surfaces such as catheters and prosthetic devices (Francois *et al.*, 1996).The binding of *S. aureus* to fibronectin is mediated by the production of two proteins, FnbA and FnbB. The protein FnbA also mediates binding to fibrinogen (Wann *et al.*, 2000). The Fnb proteins are speculated to be important in the establishment of *S. aureus* following entry into subcutaneous tissue and with biolfilm formation in conjunction with other surface proteins such as, biofilm associated protein (Bap) and extracellular polysaccharide adhesin (PIA) (Cucarella *et al.*, 2001). The formation of biofilms by *S. aureus* and other staphylococci play an important role in catheter related sepsis (Casey *et al.*, 2003). There are two other distinct fibrinogen binding proteins known as clumping factors that are designated ClfA and ClfB. These two proteins bind to different regions of the fibrinogen molecule and mediate binding to biomaterials coated with fibrinogen (McDevitt *et al.*, 1994; McDevitt *et al.*, 1995; Ni Eidhin *et al.*, 1998). Clumping factor A is also thought to bind platelets, which may inhibit the release of antimicrobial peptides activated by platelet aggregation and thus protect *S.*

aureus during the early stages of infection (Siboo *et al.*, 2001). A single protein, designated Cna, binds to collagen and has been associated with deep tissue infections, such as osteomyelitis (Elasri *et al.*, 2002), keratitis (Rhem *et al.*, 2000) and septic arthritis (Patti *et al.*, 1994).

1.6.2 Secreted factors

Staphylococcus aureus produces an array of extracellular factors such as enzymes and toxins, which are directly and indirectly linked to pathogenisity.

The enzyme coagulase is produced almost exclusively by *S. aureus* and is frequently used clinically to differentiate it from other staphylococci. Coagulase binds prothrombin to form a complex known as staphylothrombin, which can convert fibrinogen to fibrin (Kawabata *et al.*, 1985). This process has been postulated to promote abscess pocket formation that may protect *S. aureus* from host immune cells and antibiotic therapy, however, the role of coagulase in pathogenesis is not fully understood.

A recently discovered small extracellular protein called chemotaxis inhibitory protein (CHIP) has been observed to interfere with the mobilisation of polymorphonucleocytes. In addition, CHIP blocks the activation of the C5a complement cascade thus protecting *S. aureus* from the hosts immune cells (Veldkamp *et al.*, 2000).

Staphylococcus aureus produces and array of proteases. This group of enzymes have a number of postulated roles that include: evading host defences by degrading antibacterial proteins, during invasion; degrading matrix components including staphylococcal adhesions; and degradation of host proteins to amino acids for nutrition (Wright and Novick, 2003). The most important of the staphylococcal proteases is a serine protease designated SSpA (also known as V8), which can cleave Fnb proteins and other adhesins to release the microorganism from its attachment site enabling it to spread (McGavin *et al.*, 1997; Karlsson *et al.*, 2001).
There are at least three lipid esterases and one fatty acid esterifying enzyme (FAME) produced by *Staphylococcus aureus* all of which possess different substrate

specificities. Two well reported lipases are glycerol ester hydrolase and lipase (butyryl esterase), designated Geh and Lip respectively. The suggested roles of lipases are in nutrition, impairing the host immune system by degrading bacteriocidal fatty acids and promoting the interstitial spread (Wright and Novick, 2003). Strains of *S. aureus* that produce FAME have been shown greater virulence in a murine model of infection (Mortensen *et al.*, 1992).

Almost all *S. aureus* strains produce a calcium-dependant thermostable DNA nuclease designated, Nuc and over 90% produce a hyaluronate lyase, designated HysA. The enzyme Nuc is able to degrade both double stranded DNA and RNA, while HysA uses extracellular polysaccharide as a substrate. These two enzymes are both putative virulence factors, however, there is evidence that HysA may enable *S. aureus* to spread during invasion (Farrell *et al.*, 1995).

Staphylococcus aureus produces a number of toxins that demonstrate amongst other effects cytotoxic and cytolytic properties. The enzyme α-haemolysin (Hla) also called α-toxin, demonstrates cytolytic pore forming properties that can kill several cell types including, erythrocytes, mononuclear immune cells, epithelial cells, endothelial cells and platelets (Wright and Novick, 2003). The enzyme β-haemolysin (Hlb) also known as β-toxin, disrupts the cell membranes of erthrocytes and other mammalian cells including immune cells (Walev *et al.*, 1996; Marshall *et al.*, 2000).

Two closely related toxins are γ-haemolysin (Hlg) and PVL, which are formed from a combination of three gene products, HlgA (LukS-like), HlgC (LukS-like) and HlgB (LukF) (Cooney *et al.*, 1993; Konig *et al.*, 1997). Both Hlg and PVL are composed of one LukS-like and one LukF subunit. Approximately 99% of clinical *S. aureus* isolates carry the *hlg* locus, which encodes a pair of these subunits that form Hlg (Prevost *et al.*, 1995). Only a small subset of 2-5% of isolates carry the gene combination required to produce PVL, which has been demonstrated to lyse neutrophils and macrophages (Ferreras *et al.*, 1998; Szmigielski *et al.*, 1998). Recently PVL has been implicated with highly virulent *agr* group III CA-MRSA, which cause cutaneous infections and a fulminant necrotising pneumonia (Gillet *et al.*, 2002). The genes for Hlg are usually chromosomally encoded, however, the PVL genes are

generally carried by a phage known as PVL-phage (Zou *et al.*, 2000; Narita *et al.*, 2001).

Staphylococcus aureus produces an array of pyrogenic exotoxins known as superantigens that may cause food poisoning, staphylococcal scarlet fever and exfoliative dermatitis. These toxins are able to bind to major histocompatibility complex class II proteins, on antigen presenting cells, during an interaction with CD4 T-cell receptors, on T-cells. This instigates T-cells to overexpress various interleukins and cytokines, which can cause an acute systemic illness known as toxic shock syndrome (TSS) (McCormick *et al.*, 2001). The toxin commonly associated with TSS is toxic shock syndrome toxin 1 (TSST-1). Other toxins such as, enterotoxins A-D have been implicated with food poisoning. This condition, however, is not associated with a superantigen effect but with a specific interaction of the toxin structure with the parasympathetic nerve endings of the gastrointestinal tract (McCormick *et al.*, 2001).

1.7 Colonisation and infection

Staphylococcus aureus is a well-adapted human pathogen which is responsible for a variety of suppurative infections and toxinoses in humans. It causes a broad range of skin and soft-tissue infections such as folliculitis, furunculosis, cellulitis, and necrotising fasciitis; more serious infections such as pneumonia, mastitis, phlebitis, meningitis, and urinary tract infections; and deep-seated infections, such as osteomyelitis and endocarditis (John, 2004). *Staphylococcus aureus* is also a major cause of nosocomial infection of surgical wounds and infections associated with indwelling medical devices, such as catheters. *Staphylococcus aureus* can also cause food poisoning by releasing enterotoxins into food, and toxic shock syndrome by release of superantigens into the blood stream (John, 2004).

The most clinically significant of *S. aureus* infections are bacteraemic infections related to catheterisation, endocarditis, metastatic infections, sepsis and the condition known as toxic shock syndrome (Steinberg *et al.*, 1996), however, almost all areas of the human body are potential infection sites. Infection with *S. aureus* is initiated by contamination and breach of the skin or mucosal barriers, which allows the organism

access to adjoining tissues or the blood stream. After such a breach, *S. aureus* can go on to infect any tissue of the body. In a recent SENTRY Antimicrobial Surveillance Program report, which included the USA, Canada, Latin America, Europe and the Western Pacific region between 1997 and 1999, *S. aureus* was found to be the most prevalent cause of bloodstream infection, skin and soft-tissue infection, and pneumonia in almost all of these geographic areas (Diekema *et al.*, 2001).

Individuals that are colonised by *S. aureus* are at a greater risk of subsequent infection (Wenzel and Perl, 1995; Kluytmans *et al.*, 1997; Weems and Beck, 2002). Approximately 35% of normal healthy individuals carry *S. aureus* resident in the anterior nares (Noble, 1997). It has been established that methicillin-resistant *S. aureus* (MRSA) strains are descended from a group of select methicillin-sensitive *S. aureus* (MSSA) strains (Enright *et al.*, 2000), and that both MRSA and this group of MSSA are responsible for persistent colonising and invasive disease (Day *et al.*, 2001). Recent evidence suggests that *S. aureus* infections, requiring hospitalisation, usually arise from a patient's own predominant nasal strain (von Eiff *et al.*, 2002). At particular risk of infection are sufferers of type I diabetes, intravenous drug users (Bassetti and Battegay, 2004), patients undergoing haemodialysis (Yu *et al.*, 1986), surgical patients (Kluytmans *et al.*, 1995) and patients with the acquired immune deficiency syndrome (Weinke *et al.*, 1992). Typically, the therapeutic outcome of infections caused by MRSA is worse than infections caused by MSSA (Cosgrove *et al.*, 2003).

1.7.1 Skin and soft tissue infections

Staphylococcus aureus is a common cause of skin and subcutaneous infections which have a range of manifestations depending on the histological site of involvement; infections of the epidermis include impetigo and ecthyma, the dermis is a site for cellulites and necrotising fasciitis, and the pilosebaceous unit is associated with folliculitis, furunculosis and carbunculosis (Baddour, 2000; Chiller *et al.*, 2001).

Skin infections due to *S. aureus* are predominant in intravenous drug users (Lowy and Miller, 2002), HIV-infected patients (Bar *et al.*, 2003) and also in individuals with atopic dermatitis (Hoeger *et al.*, 2000). The elderly are especially at risk mainly due to

increased skin dryness from ageing and skin that becomes emaciated or folded due to age or long term residence in a care facility (Laube and Farrell, 2002). Conditions such as impetigo and staphylococcal scalded-skin syndrome (SSSS) predominantly affect the epidermis of young children, however, disease can also occur in immunocompromised adults (Opal *et al.*, 1988), diabetics and may be associated with MRSA (Ito *et al.*, 2002). More recently, community-acquired MRSA (CA-MRSA) that carry the genes for Panton-Valentine leucocidin (PVL) have been associated with severe skin and soft tissue infections as well as more serious infections, such as, necrotising pneumonia (Dufour *et al.*, 2002; Gillet *et al.*, 2002; Vandenesch *et al.*, 2003).

1.7.1.1 Impetigo

Impetigo affects the upper layers of the epidermis and can present in two general forms. The first form is characterised by honey-coloured stuck-on crusts that are formed by excess serous exudates that dry at the site of infection (Noble, 1997). The second form is bullous impetigo and is characterised by small blisters that form and burst. This latter form is also, in extreme cases, associated with SSSS a potentially life threatening disease (Ladhani *et al.*, 1999).

1.7.1.2 Staphylococcal scalded-skin syndrome

Staphylococcal scalded-skin syndrome refers to a group of primarily cutaneous diseases which predominantly affect the epidermis of young children. These include toxic epidermal necrolysis, bullous impetigo and staphylococcal scarlet fever (Barg and Harris, 1997). The condition is caused by staphylococcal exfoliatin toxins (epidermolytic toxins) which are serine proteases (Dancer *et al.*, 1990). Two exfoliatin toxins are associated with SSSS; Exfoliatin A (ETA) which is chromosomally encoded, and Exfoliatin B (ETB), which is plasmid encoded (Noble, 1997).

1.7.1.3 Folliculitis, furunculosis and carbunculosis

Staphylococcus aureus is often implicated with subepidermal infection of the pilosebaceous unit, the most common being the progressive condition of folliculitis,

which can lead to furuncles (boils) and carbuncles (Noble, 1997). This type of infection has been associated with wearing tight clothing when boils occur on the neck or perineum, and connected to shaving when boils occur in the axillae (Noble, 1997). It is estimated that 65% of furuncles, principally those of the head and neck, are caused by an indistinguishable *S. aureus* strain from that in the patient's nose and that perineal carriage is associated with boils on the legs (Kay, 1962). Recurrent infection is limited to approximately 2-3% of the population (Steele, 1980; Zimakoff *et al.*, 1988) and severe or long continued recurrent infection has been associated with defects in cell-mediated immunity (Rebora *et al.*, 1980; Busch, 1990) or a deficiency of iron metabolism (Weijmer *et al.*, 1990). There have been no specific pathogenisity factors identified that are responsible for the pathology of boils. The most common treatment for furuncles is surgical drainage as antibiotic treatment is often ineffective due to the enclosed nature of boils.

1.7.1.4 Surgical site infections

It is well established that *S. aureus* is the most common cause of surgical site infections and that nasal carriers are more susceptible to wound infection than are noncarriers (Williams *et al.*, 1959). *Staphylococcus aureus* infection has emerged as a significant problem associated predominantly with cardiac surgery (Kluytmans *et al.*, 1995) with evidence supporting a link between nasal carriage and sternal colonisation establishing an "endogenous" infection pathway in cardiothoracic wounds (Jakob *et al.*, 2000). It has also been demonstrated that MRSA surgical site infections are more likely than MSSA to produce a fatal outcome and that the cost of MRSA infections in the United States was $40, 000 in excess of that for MSSA (Engemann *et al.*, 2003).

1.7.2 Bacteraemia and sepsis

Bacteraemia due to *S. aureus* is extremely common and occurs when an infected site inoculates the blood stream with the microorganism. Approximately one third of all *S. aureus* bacteraemia have no discernable origin (John, 2004). Increased mortality from bacteraemia are associated with factors that include an age of more than 50 years, nonremovable foci of infection, and serious underlying cardiac, neurologic, or respiratory disease (Lowy, 1998). Staphylococcal bacteraemia has a high frequency of

complications, ranging from 11-53%. In addition as many as 31% of patients that do not have evidence of endocarditis do have evidence of metastatic infection (Libman and Arbeit, 1984; Mylotte *et al.*, 1987; Sanabria *et al.*, 1990; Jernigan and Farr, 1993; Ing *et al.*, 1997). Catheterisation has been associated with an increased incidence of bacteraemic infection (Steinberg *et al.*, 1996) but the rate of complications is typically lower than that for other cases of bacteraemia (24%), as is the overall mortality rate (15%) (Jernigan and Farr, 1993). Symptoms such as fever that persist for more than 72 hours after a catheter has been removed may result in a patient being at an increased risk of complications (Raad and Sabbagh, 1992). Some evidence suggests that the incidence of endocarditis in patients with catheters is low, ranging between 0-18% (Jernigan and Farr, 1993), however, other studies have suggested that the incidence may be as high as 55% (Espersen and Frimodt-Moller, 1986).

In a minority of bacteraemias or localised infections caused by *S. aureus*, sepsis may occur. At particular risk are the elderly, immunosuppressed and patients undergoing chemotherapy or invasive procedures (Lowy, 1998). Sepsis caused by *S. aureus* presents symptoms which are similar to that of Gram-negative sepsis and include fever, hypotension, tachycardia and tachypnoea. Of all Gram-positive pathogens *S. aureus* is the most common cause of sepsis, which in severe cases may cause multiorgan dysfunction, disseminated intravascular coagulation, lactic acidosis and even death (Bone, 1994).

1.7.3 Endocarditis

Endocarditis is an inflammation of the inside lining of the heart chambers and heart valves, which may be caused by infection with a number of microorganisms including *S. aureus*. If endocarditis is caused by a bacterial infection then it is referred to as infective endocarditis (IE). It is estimated that *S. aureus* accounts for 25-35% of all IE cases (Sanabria *et al.*, 1990; Sandre and Shafran, 1996). Infective endocarditis occurs in intravenous drug users, the elderly, patients with prosthetic valves, and hospitalised patients. Initially, IE may be difficult to diagnose as symptoms can be limited to fever and malaise, however, unlike less virulent pathogens, *S. aureus* endocarditis is often characterised by a rapid onset, high fever, frequent involvement of normal cardiac valves, and the absence of physical stigmata of the disease on initial presentation

(Chambers *et al.*, 1983). In cases of IE associated with intravenous drug use, the disease is frequently right-sided, patients are often young, rates of mortality are low, and the majority of patients do not have antecedent valvular disease. This prognosis is typically worse for intravenous drug users that have advanced disease associated with human immunodeficiency virus (HIV) infection (Pulvirenti *et al.*, 1996). In cases of IE that are not related to drug use, the disease is often left-sided, patients are older, the rate of mortality is high (20-44%), and the disease usually involves previously damaged cardiac valves (Chambers *et al.*, 1983; Sanabria *et al.*, 1990; Ing *et al.*, 1997).

Staphylococcus aureus is one of the most common causes of prosthetic-valve endocarditis (PVE). One of the major sources of bacterial inoculation is intravenous catheterisation. Recent studies have reported that certain regulatory genes such as *agr* may be related to types of human *S. aureus* disease. There are four *agr* groups designated I-IV and those of groups I and II have been reported to be associated more frequently with suppurative infections and endocarditis (Jarraud *et al.*, 2002). In addition, among blood culture isolates MRSA were found to be more likely than MSSA to be *agr* group II (Sakoulas *et al.*, 2003).

1.7.4 Metastatic infections

With any *S. aureus* infection it is highly possible that the microorganism may spread to other sites of the human body. Particularly vulnerable are the bones, joints, kidneys and lungs (Libman and Arbeit, 1984; Musher *et al.*, 1994; John, 2004). Suppurative collections at infected sites serve as potential foci for recurrent infections, which may be exacerbated if the microorganism is a MRSA strain.

1.7.5 Toxic shock syndrome

Toxic shock syndrome (TSS) caused by *S. aureus* was first identified in the early 1980's after the introduction of super absorbent tampons. The condition is caused by *S. aureus* strains that produce an exotoxin designated, toxic shock syndrome toxin 1 (TSST-1). The TSST-1 may serve as a superantigen during infection, activating an array of T-lymphocytes (Matsuda *et al.*, 2003) via a Vβ T-cell receptor response that

may cause clinical manifestations which include high fever, hypertension, rash and multiorgan damage (Lowy, 1998). Since the reduction in use of superabsorbent tampons the incidence of TSS has decreased, however, nonmenstrual TSS has been reported and may be associated with localised infection and surgery.

1.8 Aims and objectives

Methicillin-resistant *Staphylococcus aureus* is a significant cause of nosocomial and community morbidity and mortality which has become a worldwide problem exacerbated by the emergence of MR-MRSA, VISA and VRSA. Between 2001 and 2002 there was an increase in the number of MR-MRSA isolates from the University Hospital Birmingham, NHS Foundation Trust, UK (UHB). In addition, increased reports of highly virulent CA-MRSA had raised concerns over the emergence of such microorganisms in the Birmingham, UK area. Advances in molecular biology have made it possible to differentiate MRSA strains at a genomic level. Multiple DNA-based techniques are available for typing MRSA, however, a single technique has not been identified that is universally applicable. To investigate the MRSA population of the UHB and South Birmingham area, it was necessary to type clinical isolates using a number of methods. Many molecular typing techniques produce complex banding patterns which are often interpreted using traditional statistical methods. Applying alternative statistical analysis to such data it may be possible to identify discriminatory bands for use with a new typing system. There is considerable speculation over the fitness cost to *S. aureus* that carry SCC*mec* and other resistance genes. To investigate the survival characteristics of MRSA and MSSA under different conditions a number of growth and survival experiments were performed.

The aims of this study were to:

- Determine the antibiotic sensitivity of clinical MRSA isolates from the UHB and South Birmingham, UK to identify potential MR-MRSA, VISA and VRSA strains.
- Characterise clinical MRSA isolates using a gold standard molecular typing technique and compare strain types with respective antibiotic sensitivity data.

- Determine if MRSA isolates of different molecular types are better able to survive in different environments in comparison to *S. aureus*.
- Optimise a rapid inexpensive molecular typing technique comparable to the gold standard method and compare strain types with respective antibiotic sensitivity data.
- Characterise strains using molecular typing methods, complementary to those already performed.
- Analyse complex banding patterns, generated using PFGE, with principal components analysis to identify the most discriminatory bands within profile types and compare the technique with a standard statistical method.
- Determine the most descriptive information from molecular typing and investigate the feasibility of a reverse hybridisation method based on an existing protocol.

CHAPTER 2 BACTERIAL STRAINS AND REAGENTS

2.1 Bacterial isolates

A total of 130 MRSA isolates were investigated. Ninety-four of the isolates were from blood cultures collected within the University Hospital Birmingham, NHS Trust, UK (UHB). Isolates of MRSA obtained from patients with no evidence of infection within 48 hours of admission to the UHB were designated hospital-acquired. Thirty-six of the isolates were samples sent to the UHB by general practitioners in the South Birmingham area isolated from soft tissue infections from non-hospitalised patients. Isolates of MRSA obtained from patients who had no recent history of hospitalisation prior to evidence of infection were designated community-acquired. All HA-MRSA were isolated from different patients within the UHB between September 2001 and September 2002. All CA-MRSA were isolated between September 2002 and February 2003. Unless otherwise stated all 130 isolates of MRSA were subjected to analysis.

During this thesis an isolate is defined as a population of microbial cells in pure culture derived from a single colony on an isolation plate and identified to the species level (Riley, 2004). During this thesis a strain is identified as a group of isolates exhibiting phenotypic and/or genotypic traits belonging to the same lineage, distinct from those of other isolates of the same species (Riley, 2004).

2.2 Additional bacterial isolates

Staphylococcus aureus strains from the American Type Culture Collection (ATCC) and National Collection of Type Cultures (NCTC) were used where stated.

Staphylococcus aureus: Type strains, *S. aureus* NCTC 6571, *S. aureus* NCTC 8325, were included for antibiotic sensitivity testing and PFGE, respectively.

Methicillin-resistant *Staphylococcus aureus*: COL, PER34, N315, ANS46, HU25 and MW2, were included as controls for SCC*mec* type assignment (Oliveira and de Lencastre, 2002).

Staphylococcus aureus: Type strain, *S. aureus* ATCC 49775, was included as a control for PVL gene detection (Lina *et al.*, 1999).

Heterogeneous vancomycin-intermediate *S. aureus*: Mu3 ATCC 700698 (Hiramatsu *et al.*, 1997b) was included for vancomycin sensitivity testing.

Vancomycin-intermediate *S. aureus*: Mu50 ATCC 700699 (Hiramatsu *et al.*, 1997a) was included for vancomycin sensitivity testing.

Methicillin-resistant *Staphylococcus aureus*: Witte 1-Witte 20 obtained from Dr. W. van Leeuwen and described in (van Leeuwen *et al.*, 2001), were included for binary typing.

2.3 Storage and culture of bacterial isolates

All isolates were stored at -70°C on cryobeads (Microbank™, Pro-Lab Diagnostics, Canada). All *S. aureus* isolates were grown on brain heart infusion (BHI) agar under aerobic conditions for 16 hours at 37°C unless otherwise stated.

2.4 Reagents and media

All chemicals were purchased from Sigma Company, UK and media from Oxoid Ltd, UK except where otherwise stated.

CHAPTER 3 PHENOTYPIC RESISTANCE CHARACTERISTICS OF MRSA

3.1 Introduction

One of the most useful phenotypic techniques routinely carried out in diagnostic microbiology laboratories for the screening of bacterial infections is AST. The technique is used to determine the pattern of resistance/sensitivity of a microorganism to a panel of antibiotics. The subsequent results may be recorded as an antibiogram and used to identify therapy for an infection and to discriminate between different bacterial strains of the same species (Mulligan and Arbeit, 1991). The identification of a new pattern of antibiotic resistance among isolates cultured from different patients is often the first indication of an outbreak. Antibiograms are routinely used in UK hospitals as the primary surveillance tool for MRSA outbreaks.

The emergence of MRSA strains with reduced susceptibility to vancomycin in several developed countries has raised concerns over outbreaks of VISA and VRSA strains in health care institutions in the UK. Standard AST has failed to identify VISA and VRSA strains in some instances (Hiramatsu *et al.*, 1997b), which has led to the development of more sensitive screening methods (Walsh *et al.*, 2001).

Increased use of antibiotics promoted the emergence of MRSA that are resistant to almost all available therapies, however, little is known about how well MRSA compete in the environment in comparison to other *S. aureus*. It has been suggested that MRSA are less "streamlined" than *S. aureus* strains and that without the selective pressure of antibiotics would compete poorly in the natural environment (Gudmundsson *et al.*, 1991; Andersson and Levin, 1999; Bjorkman and Andersson, 2000; Bjorkman *et al.*, 2000).

In this chapter isolates of MRSA were subjected to antibiotic sensitivity testing using a standard disc diffusion method. In addition, MRSA strains were screened for reduced sensitivity to vancomycin using a method recommended by the European Antimicrobial Resistance Surveillance System (Walsh *et al.*, 2001). Preliminary

investigations studying environmental survival of MRSA and *S. aureus* during exposure to a number of stressful conditions including, desiccation, high and low pH and increasing NaCl concentrations were also undertaken.

3.2 Materials and methods

3.2.1 Antibiotic sensitivity testing

The antibiogram profile of all isolates was determined against a panel of ten antibiotics using the British Society for Antimicrobial Chemotherapy (BSAC) disc diffusion method (Andrews, 2001).

Suspensions of each MRSA isolate were prepared in 5mL sterile phosphate-buffered saline (PBS) equivalent to a 0.5 MacFarland standard. A 2μL loop of suspension was inoculated onto a 90mm IsoSensitest agar (ISA) plate and streaked across the entire surface of the plate. A sterile swab was subsequently used to inoculate the entire surface of the plate. Preparation for testing flucloxacillin was performed by suspending a sterile swab into the cell suspension and inoculating the entire surface of the plate. Antibiotic discs were applied to each plate using a disc dispenser, ensuring all discs were delivered firmly to the agar surface. Plates were incubated at 37°C for 18 hours. Plates were assessed to confirm that growth on the plates was semi-confluent and reading of the zones of inhibition was performed using a template. The panel of antibiotics tested, concentrations and limits for the zones of inhibition are summarised in table 3.1. The Oxford *S. aureus* NCTC 6571 was used as a sensitive control to test disc content, incubation conditions and media performance. Each observed pattern of antibiotic resistance was allocated a unique three digit antibiogram code number (Table 3.1). Isolates resistant to six or more antibiotics were designated MR-MRSA and were allocated the number six as a prefix to the three digit antibiogram code.

Table 3.1 Example of method used to assign antibiogram code number.

Antibiotic	Value assigned if resistant	Example	Result
Flucloxacillin	4	R	
Rifampicin	2	R	6
Tetracyclin	1	S	
Erythromycin	4	S	
Fusidic acid	2	S	1
Gentamicin	1	R	
Mupirocin High	4	S	
Mupirocin Low	2	R	3
Trimethoprim	1	R	

Table 3.2 Antibiotic panel and size of zones of inhibition used for determining antibiotic sensitivity.

Antibiotic	Concentration in disc (μg)	Resistant (≤ mm)	Sensitive (≥ mm)
Flucloxacillin	1	14	15
Erythromycin	5	19	20
Trimethoprim	5	19	20
Rifampicin	2	29	30
Fusidin	10	29	30
Tetracycline	10	19	20
Mupirocin low	5	21	22
Mupirocin high	200	9	10
Gentamicin	10	19	20
Vancomycin	5	11	12

3.2.2 Vancomycin E-test

Reduced sensitivity of MRSA isolates to vancomycin was determined using a method of screening for VISA and VRSA reported by (Hiramatsu *et al.*, 1997b) and a modified macromethod E-test protocol recommended by the European Antimicrobial Resistance Surveillance System (EARSS at http://www.earss.rivm.nl/) from (Walsh *et al.*, 2001).

Isolates of MRSA were grown in 5mL BHI broth and incubated at 37°C for 16 hours with shaking. A 10μL aliquot of stationary phase culture was pipetted onto a Mueller Hinton (MH) agar plate containing 4μg/mL of vancomycin, incubated at 37°C and

inspected for growth at 24 and 48 hours. If confluent growth was observed after 24 hours the strain was considered a VRSA strain. If a countable number of colonies (1 to 30) was observed after 48 hours the strain was considered a possible hVISA. If cell growth was not apparent after 48 hours the strain was considered susceptible. The strain NCTC 6751 was used as a vancomycin sensitive negative control, Mu3 as a hVISA positive control and Mu50 as a VISA positive control.

A suspension of each MRSA strain was prepared in BHI broth to an inoculum density of 2 MacFarland. A 200μL aliquot of suspension was pipetted onto a 90mm BHI agar plate and streaked evenly with a sterile swab. The plate was allowed to dry. The E-test strip (AB biodisk, Solna, Sweden) was applied to the plate using a pair of sterile tweezers. Plates were incubated at 35°C for 48 hours. Strains of the hVISA Mu3, VISA Mu50 and Oxford *S. aureus* NCTC 6571 were used as controls. A cut off point of 8μg/mL of vancomycin was used to determine resistance. The E-test ellipse for each test was scrutinised for microcolonies and small colony variants as recommended (Walsh *et al.*, 2001).

3.2.3 Survival and growth of *S. aureus* and MRSA

3.2.3.1 Calibration Curve

The isolate B07T1 was inoculated onto a MH agar plate and incubated for 16 hours at 37°C. A single colony taken directly from the plate was used to inoculate 10mL of MH broth which was then incubated at 37°C with shaking. The absorbance at 600nm (A_{600}) of the culture was measured every 30 minutes in a spectrophotometer (Novaspec II, Pharmacia Biotech, UK) until the culture reached log phase (approximately three hours and an optical density (OD) of 0.3). An aliquot of 500μL of log phase culture was pipetted into a flask of 50 mL MH broth. The A_{600} was measured at time zero and the flask were subsequently placed in a shaking water bath at 37°C. Every 40 minutes the A_{600} of the culture was measured and the OD recorded. Samples were diluted according to the guidelines in table 3.2. After appropriate dilutions, 6 x 20μL of culture were dropped onto separate sections of a MH agar plate using the Miles & Misra technique (Miles and Misra, 1938), and incubated for 16

hours at 37°C. The number of colonies in each of the six 20μL drop areas was counted and an average taken to determine the colony forming units per mL (cfu/mL). The log cfu/mL were plotted against the corresponding OD readings. The method was performed in triplicate.

Table 3.3 Dilutions for calibration curve

Optical density (600nm)	Dilutions
0.000	Neat, 10^{-1}, 10^{-2}
0.001	10^{-1}, 10^{-2}, 10^{-3}
0.010	10^{-2}, 10^{-3}, 10^{-4}
0.100	10^{-3}, 10^{-4}, 10^{-5}
0.200	10^{-3}, 10^{-4}, 10^{-5}
0.300	10^{-3}, 10^{-4}, 10^{-5}
0.400	10^{-4}, 10^{-5}, 10^{-6}
0.500	10^{-4}, 10^{-5}, 10^{-6}
0.600	10^{-6}, 10^{-7}, 10^{-8}
0.700	10^{-6}, 10^{-7}, 10^{-8}
0.800	10^{-6}, 10^{-7}, 10^{-8}
0.900	10^{-6}, 10^{-7}, 10^{-8}
1.000	10^{-7}, 10^{-8}, 10^{-9}
1.100	10^{-7}, 10^{-8}, 10^{-9}
1.200	10^{-7}, 10^{-8}, 10^{-9}
1.300	10^{-8}, 10^{-9}, 10^{-10}

3.2.3.2 Comparison of growth of different MRSA strains

Ten MRSA strains were selected based on their antibiogram profile, PFGE type (chapter 4), SCC*mec* type and REAP type (chapter 5) for growth comparisons (table 3.3). The methicillin sensitive *S. aureus* strain NCTC 8325 was included as a control strain, as it does not carry the SCC*mec* element. The isolates were prepared as described in section 3.2.3.1 until the reading of time point zero. The A_{600} of the culture was measured every one hour in a spectrophotometer (Novaspec II, Pharmacia Biotech, UK) and the OD recorded. All ten strains were each tested in triplicate. The OD readings were used to determine the equivalent cfu/mL at each time point.

Table 3.4 Strains of MRSA selected for growth comparisons. (PFGE, chapter 4 and SCC*mec* type, chapter 5)

3.2.3.3 Statistical analysis of results

The data for the growth curves was analysed with a repeated measures analysis of variance test (RE-ANOVA) using Statistica, Version 6.0 (Statsoft, Tulsa, USA).

3.2.4 Effect of desiccation on the survival of MRSA

Three plastic (polystyrene) trays with lids were cleaned with 10% ($^{w}/_{v}$) SDS and disinfected with 70% ($^{v}/_{v}$) industrial methylated spirits (IMS), which was allowed to evaporate. The trays were subsequently rinsed in sterile double distilled water (SDW) to remove IMS residue. The dry plastic trays were then exposed to UV light for one hour. Three grids consisting of 45, 10 x 10mm squares were marked out on each plastic tray. The three plastic trays were each divided into three areas, one area per strain. The strains A07T1, C09T1 and NCTC 8325 were chosen for testing (table 3.3).

Isolates were inoculated onto separate BHI agar plates and incubated for 16 hours at 37°C. A single colony taken directly from a plate was used to inoculate 10mL of BHI broth which was incubated for 16 hours at 37°C with shaking. The A_{600} of each culture was measured and adjusted to OD 1.15, which is approximately 12 log cfu/mL as calculated from a calibration curve (figure 3.2). A 50µL aliquot of each culture was pipetted on to every 10 x 10mm square in the area of tray allocated for the strain and allowed to dry. The plastic tray was covered with a lid to reduce the possibility of

airborne contamination and incubated at room temperature (21°C). An inoculated 10 x 10mm square was swabbed with a sterile cotton swab dampened in 500µL of SDW. The swab was resuspended in the 500µL of SDW and agitated to allow cell recovery. Each of the suspensions was serially diluted in SDW and inoculated onto BHI agar using the Miles-Misra method (section 3.2.3.1). The agar plates were incubated at 37°C for 16 hours and the colony forming units were counted. Swabs were taken at time zero after the plates were dry, and every seven day interval in triplicate for each test strain.

The data were analysed as described in section 3.2.3.3. The D-values for each strain were calculated. The D-value is the time taken in days for the bacterial population to reduce by one log order.

3.2.5 Exposure of MRSA strains to varied pH

Cultures of strains A07T1, C09T1 and NCTC 8325 were grown to log phase growth as described in section 3.2.3.1. A 100µL aliquot of log phase culture was pipetted into a series of 50mL flasks containing 10mL MH broth (Gudmundsson *et al.*, 1991) adjusted by titration with HCL (acid) or NaOH (alkaline) to the following pH levels: pH 2.0, pH 3.0, pH 4.0, pH 5.0, pH 6.0, pH 7.0 (control), pH 8.0, pH 9.0, pH 10.0 and pH 11.0. Flasks were incubated for 16 hours at 37°C with shaking. Repeats were carried out in triplicate. The A_{600} of each flask was measured to detect growth using uninoculated broth adjusted to the respective pH as a blank. The data were analysed as described in section 3.2.3.3. A 100µL aliquot was spread on a MH agar plate for each strain at each pH and subsequently incubated at 37°C for 16 hours to determine cell viability.

3.2.6 Growth of MRSA at high and low pH

Cultures of strains A07T1, C09T1 and NCTC 8325 were grown to log phase growth as described in section 3.2.3.1. A 500µL aliquot of log phase culture was pipetted into 250mL flasks containing 50mL MH broth adjusted to pH 4 and pH 9.5 for each strain. The A_{600} was measured at time zero and the flask was placed in a shaking water bath at 37°C. Subsequently, the A_{600} of the culture was measured every hour and the OD

recorded. Repeats were carried out in triplicate. The results were analysed as described in section 3.2.3.3.

3.2.7 Minimum inhibitory NaCl concentration

Cultures of strains A07T1, C09T1 and NCTC 8325 were grown to log phase growth as described in section 3.2.3.1. An aliquot of 100μL of log phase culture was pipetted into a series of flasks containing 10mL MH broth adjusted to the following NaCl concentrations: 5.0%, 7.5%, 10.0%, 12.5%, 15.0%, 17.5%, and 20% ($^{w}/_{v}$). Flasks were incubated for 16 hours at 37°C with shaking. A 100μL aliquot was spread on a MH agar plate for each strain at each NaCl concentration and subsequently incubated at 37°C for 16 hours to determine cell viability. Repeats were carried out in triplicate. The turbidity of each culture was assessed and results were recorded as minimum inhibitory NaCl concentration.

3.3 Results

3.3.1 Antibiotic resistance

The antibiotic resistance profiles (table 3.4) indicated that there were 27 MR-HA-MRSA isolates and one MR-CA-MRSA isolate resistant to all antibiotics investigated with the exception of rifampicin, tetracycline and vancomycin. Seventeen MR-HA-MRSA isolates and one MR-CA-MRSA isolate were sensitive to mupirocin at high concentration in addition to rifampicin, tetracycline and vancomycin. The non-multidrug-resistant (NMR) HA-MRSA isolates and CA-MRSA isolates were separated into eleven antibiogram types. All HA-MRSA were resistant to methicillin and sensitive to gentamicin, mupirocin (low and high concentrations) and vancomycin with the exception of two isolates which demonstrated resistance to gentamicin. Two of the HA-MRSA isolates were resistant to rifampicin and a further two were resistant to tetracycline. Ten of the HA-MRSA and nine CA-MRSA isolates were resistant to flucloxacillin only.

Table 3.5 Antibiotic resistance phenotypes of 130 MRSA isolates.

[a]MR	[b]Antibiotic resistance phenotype/antibiogram code											Number of isolates	
	Fl	Em	Tp	Rf	Fu	Gm	Tc	Mu^L	Mu^H	Vm	Code	Hospital	Community
-	R	S	S	S	S	S	S	S	S	S	400	10	9
-	R	S	R	S	S	S	S	S	S	S	401	2	1
-	R	S	R	S	R	S	S	S	S	S	421	2	0
-	R	R	S	S	S	S	S	S	S	S	440	21	18
-	R	R	R	S	S	S	S	S	S	S	441	5	6
-	R	R	R	S	S	R	S	S	S	S	451	2	0
-	R	R	R	S	R	S	S	S	S	S	461	2	0
-	R	R	S	S	R	S	R	S	S	S	560	2	0
-	R	R	R	S	R	S	R	S	S	S	561	1	0
-	R	R	S	R	R	S	S	S	S	S	640	1	0
-	R	R	R	R	R	S	S	S	S	S	661	2	0
MR	R	R	R	S	R	R	S	R	S	S	6473	17	1
MR	R	R	R	S	R	R	S	R	R	S	6477	27	1
Totals												**94**	**36**

[a]MR designates multidrug-resistance. (Isolates resistant to 6 or more antibiotics).
[b]Antibiotic abbreviations: Fl (flucloxacillin), Em (erythromycin), Tm (trimethoprim), Rf (rifampicin), Fu (Fusidin), Gm (gentamicin), Tc (tetracycline), Mu^L (mupirocin-low), Mu^H (mupirocin-high) and Vm (vancomycin).

3.3.2 Screening for hVISA and VISA

Screening for hVISA and VISA strains using the method of (Hiramatsu *et al.*, 1997b) revealed all 130 MRSA isolates tested were sensitive to vancomycin at a concentration of 4μg/mL after incubation at 37°C for 48 hours.

3.3.3 Vancomycin E-tests

The vancomycin macromethod E-test protocol of (Walsh *et al.*, 2001) identified no hVISA or VISA strains amongst the 130 MRSA strains tested. The E-test results obtained for Mu50, Mu3, NCTC 6571 and a typical clinical isolated from the UHB is depicted in figure 3.1. Results are summarised in table 3.5.

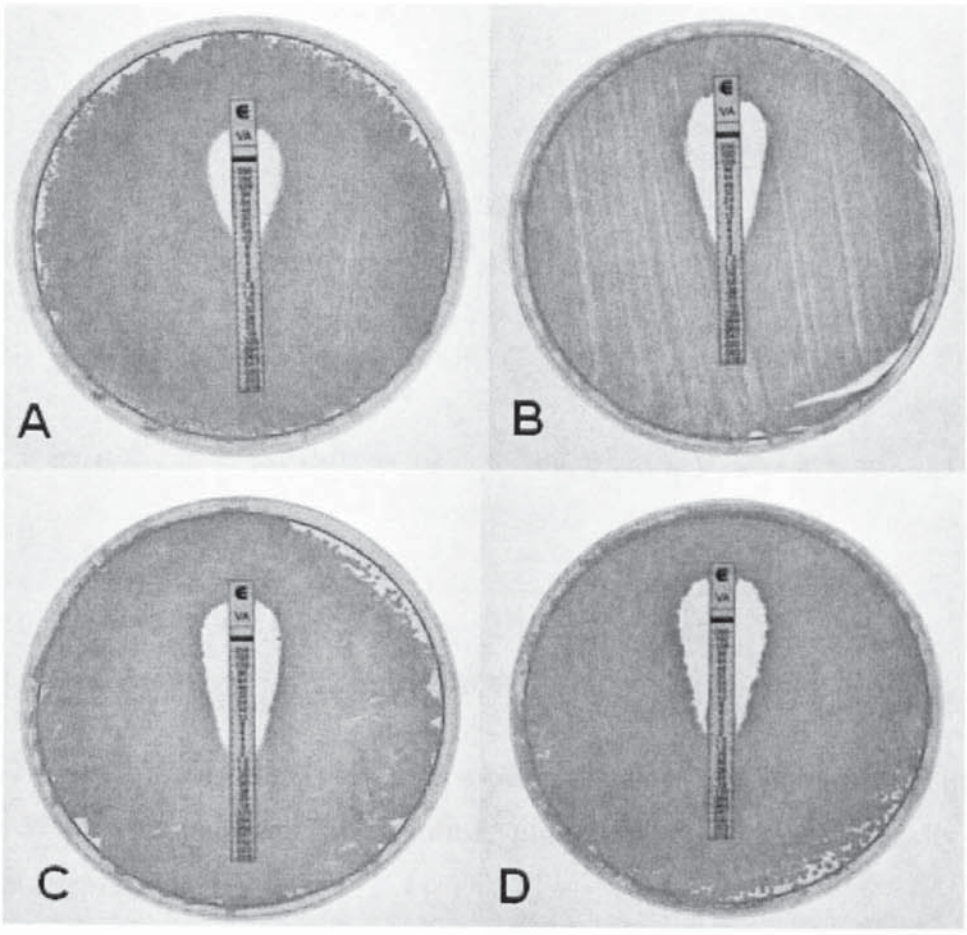


Figure 3.1 Etest E-test results obtained for Mu50 (A), Mu3 (B), NCTC 6571 (C) and a typical clinical isolated from the UHB (D).

Table 3.6 Distribution of E-test readings of 130 MRSA isolates and three control organisms including: Oxford *S. aureus* NCTC 6751 (O), Mu3 and Mu50.

	E-test reading µg/mL									
	0.5	0.75	1.0	1.5	2.0	3.0	4.0	6.0	8.0	12.0
Number of isolates	0	35	39	32	24	0	0	0	0	0
Control isolates		O					Mu3			Mu50

3.3.4 Calibration curve

A calibration curve for the growth of a clinical MRSA strain was calculated by plotting OD against corresponding log cfu/mL results (figure 3.2). The regression line passed through each of the data points and possessed an R^2 value of high significance (0.9976). The regression equation was accepted for calculating log cfu/mL from OD readings.

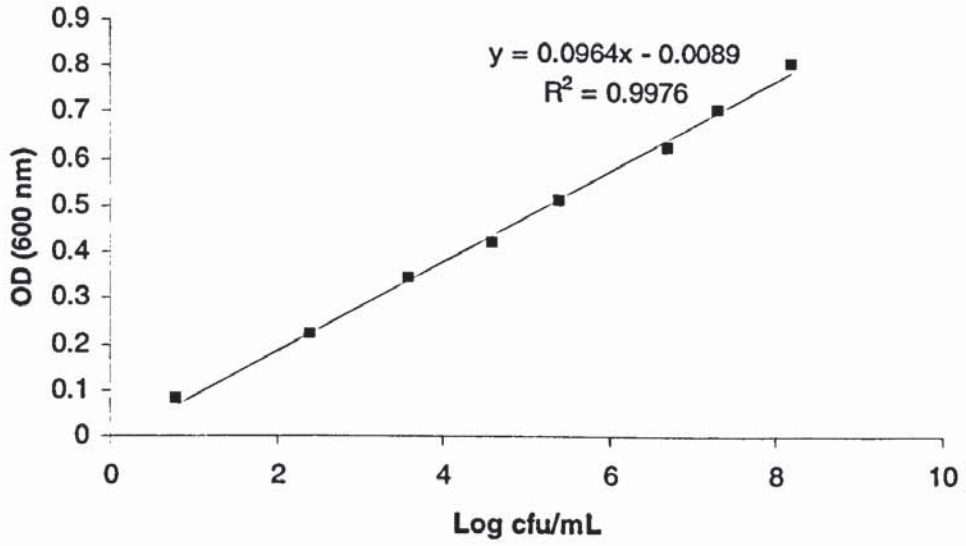


Figure 3.2 Growth calibration curve for a clinical isolate of MRSA grown in BHI broth at 37°C with shaking. Log cfu/mL plotted against optical density with regression analysis.

3.3.5 MRSA growth curves

The growth curves of nine MRSA strains (table 3.3) in comparison with the MSSA strain NCTC 8325 are shown in figures 3.3.1, 3.3.2, 3.3.3 and 3.3.4. Analysis using RE-ANOVA indicated that there were no significant differences between the rates of growth between MRSA strains of different antibiogram type, SCC*mec* type, PFGE type and REAP type, however, there was a significant difference between all nine MRSA strains and NCTC 8325 ($p=<0.0001$, 95% confidence interval). Post Hoc analysis indicated that MRSA strains were growing significantly faster between 100 and 300 minutes during the log phase in comparison to NCTC 8325.

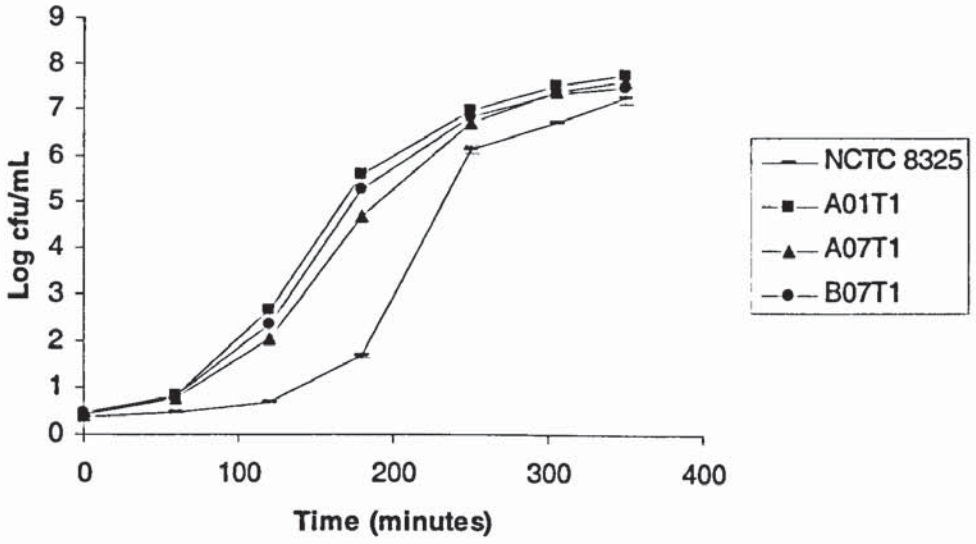


Figure 3.3.1 Growth in MH broth at 37°C with shaking of MR-MRSA strains A01T1, A07T1 and B07T1 (antibiogram type, 6477 and 6473, SCC*mec* type II, PFGE type F, G and H respectively) in comparison with the MSSA strain NCTC 8325. Error bars represent ± standard error of the mean.

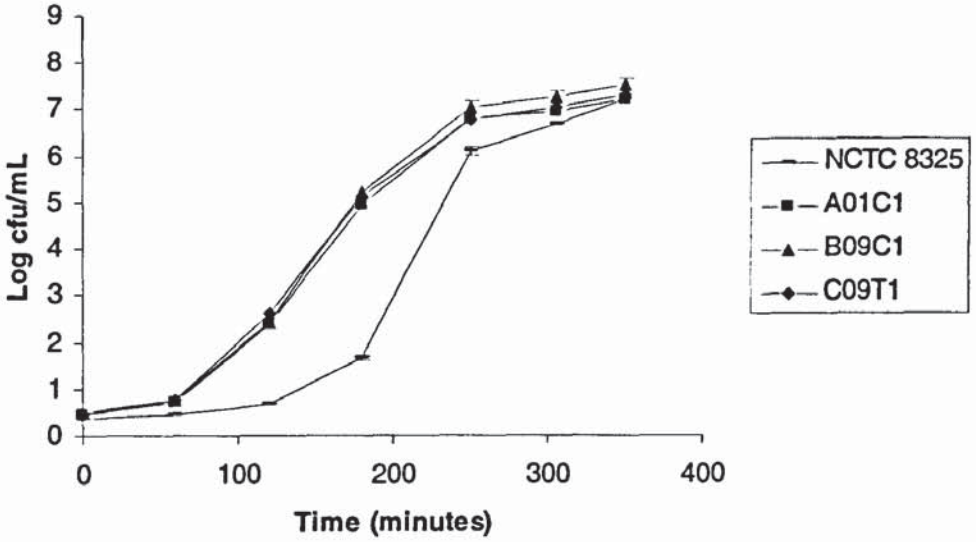


Figure 3.3.2 Growth in MH broth at 37°C with shaking of MRSA strains A01C1, B09C1 and C09T1 (antibiogram type 400, SCC*mec* type IV, PFGE type A, B and C respectively) in comparison with the MSSA strain NCTC 8325. Error bars represent ± standard error of the mean.

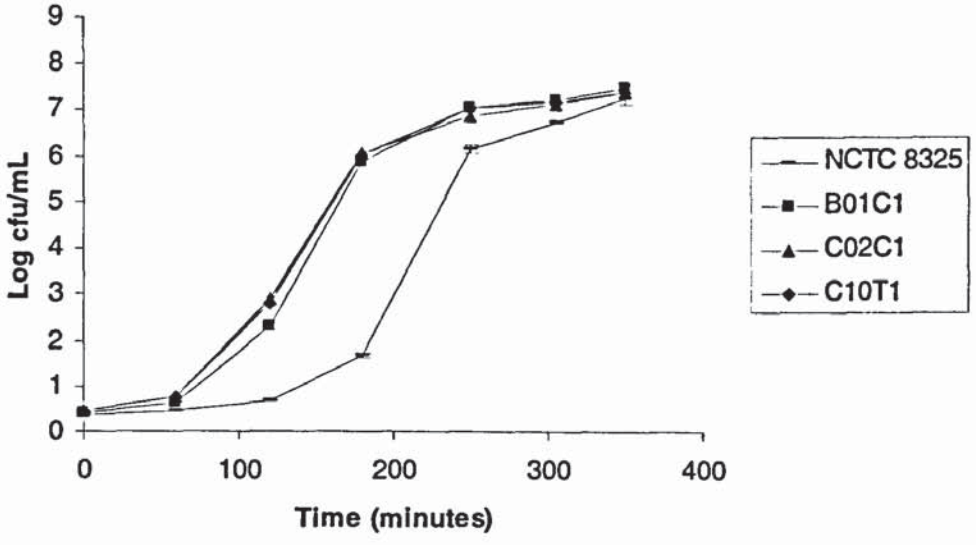


Figure 3.3.3 Growth in MH broth at 37°C with shaking of MRSA strains B01C1, C02C1 and C10T1 (antibiogram type 440, SCC*mec* type IV, PFGE type A, B and C respectively and REAP type p5) in comparison with the MSSA strain NCTC 8325. Error bars represent ± standard error of the mean.

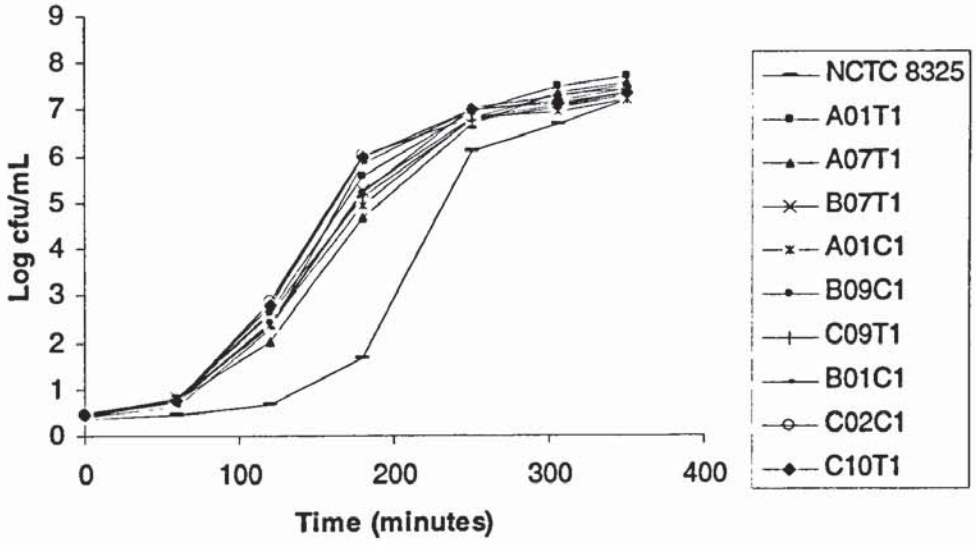


Figure 3.3.4 Growth in MH broth at 37°C with shaking of nine MRSA strains in comparison with the MSSA strain NCTC 8325. Error bars represent ± standard error of the mean.

3.3.6 Desiccation

The log cfu/mL recovered for strains NCTC 8325, A07T1 and C09T1 from a plastic (polystyrene) surface that was initially inoculated with approximately 12 log cfu/mL are shown in figures 3.4. Analysis of the data using RE-ANOVA indicated that there was a significant difference in the log cfu/mL recovered for the three strains from each plastic tray ($p=<0.0001$, 95% confidence interval) over an eight week period. The D-value for each strain was calculated to be 18.4, 11.5 and 9.5 days for NCTC 8325, A07T1 and C09T1 respectively.

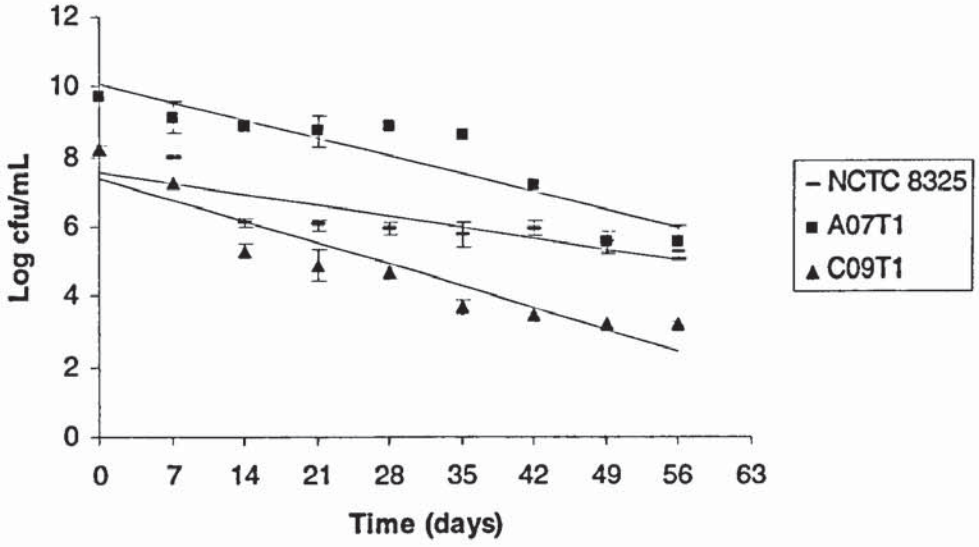


Figure 3.4 Log cfu/mL recovered for strains NCTC 8325 (MSSA), A07T1 (MR-MRSA, SCC*mec* type II) and C09T1 (MRSA, SCC*mec* type IV) from a plastic (polystyrene) surface over an eight week period. Error bars represent ± standard error of the mean.

3.3.7 Tolerance of MRSA to a range of pH levels

The growth of strains NCTC 8325, A07T1 and C09T1 grown in MH broth adjusted to pH levels ranging from pH 2 to pH 12 is shown in figure 3.5. Analysis using RE-ANOVA indicated that there was no significant difference between the log cfu/mL for each isolate at pH levels ranging from pH 5 to pH 9. All three strains were able to survive 16 hours of exposure to each pH level except pH $\leq$3 and pH $\geq$11. The growth of the strains at pH 4 and pH 10 was significantly lower when compared to the next nearest pH level in both cases (p=<0.0001, 95% confidence interval).

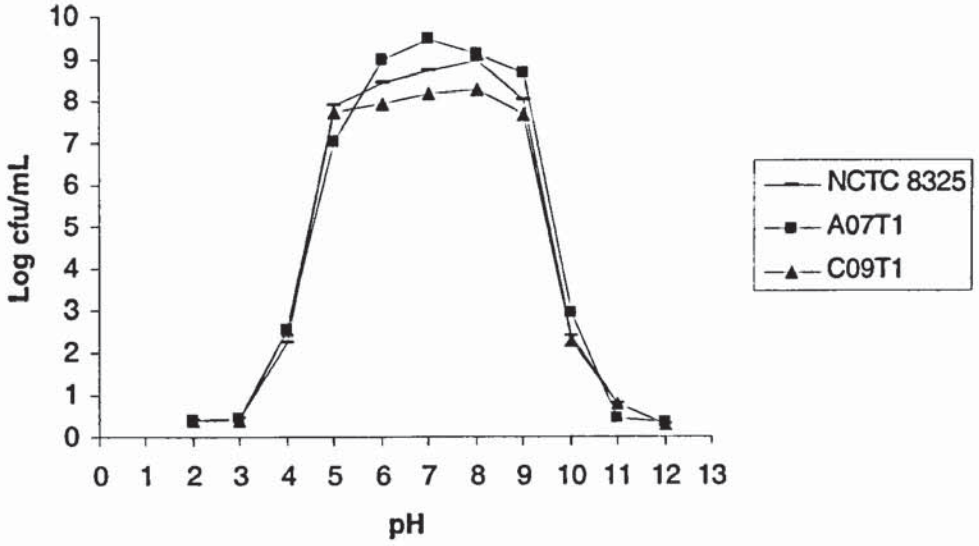


Figure 3.5 Growth in MH broth adjusted to pH levels ranging from pH 2 to pH 12 at 37°C for 16 hours of strains NCTC 8325 (MSSA), A07T1 (MR-MRSA, SCC*mec* type II) and C09T1 (MRSA, SCC*mec* type IV). Error bars represent ± standard error of the mean.

3.3.7.1 Growth of MRSA at pH 4 and pH 9.5

The growth curves of strains NCTC 8325, A07T1 and C09T1 grown in standard MH broth at pH 7 and adjusted to pH 4 and pH 9.5 are shown in figures 3.6.1 and 3.6.2. Analysis using RE-ANOVA indicated that there was a significant difference in growth between strains grown at pH 7 and pH 4 (p=<0.0001, 95% confidence interval). At pH 4 strains A07T1 and C09T1 were able to grow successfully but significantly slower than at pH 7. Strain NCTC 8325 was not able to grow at pH 4. At pH 9.5 all three strains grew significantly less compared with growth at pH 7 (p=<0.0001, 95% confidence interval), however, strain A07T1 was able to grow significantly more than strains C09T1 and NCTC 8325.

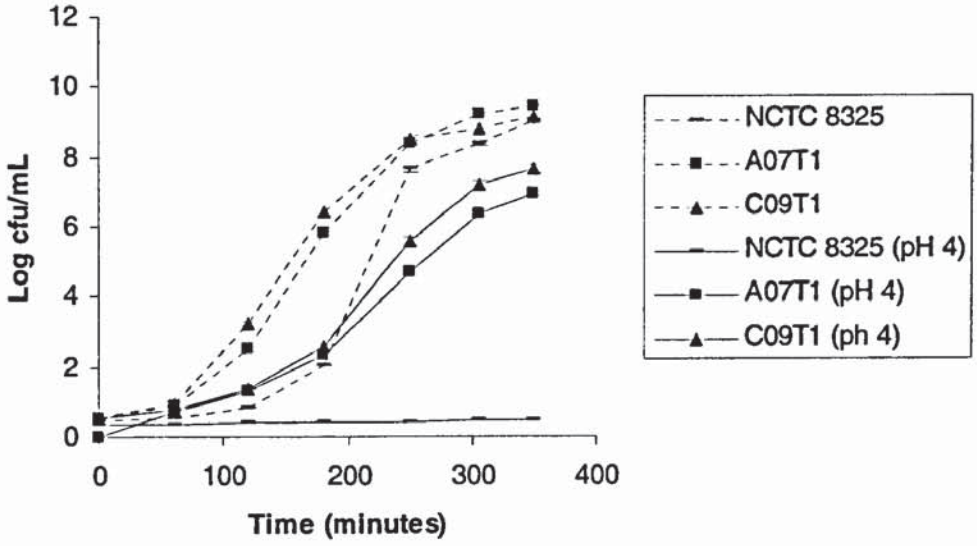


Figure 3.6.1 Growth in MH broth at pH 7 and adjusted to pH 4 of strains NCTC 8325 (MSSA) A07T1 (MR-MRSA, SCC*mec* type II) and C09T1 (MRSA, SCC*mec* type IV). Error bars represent ± standard error of the mean.

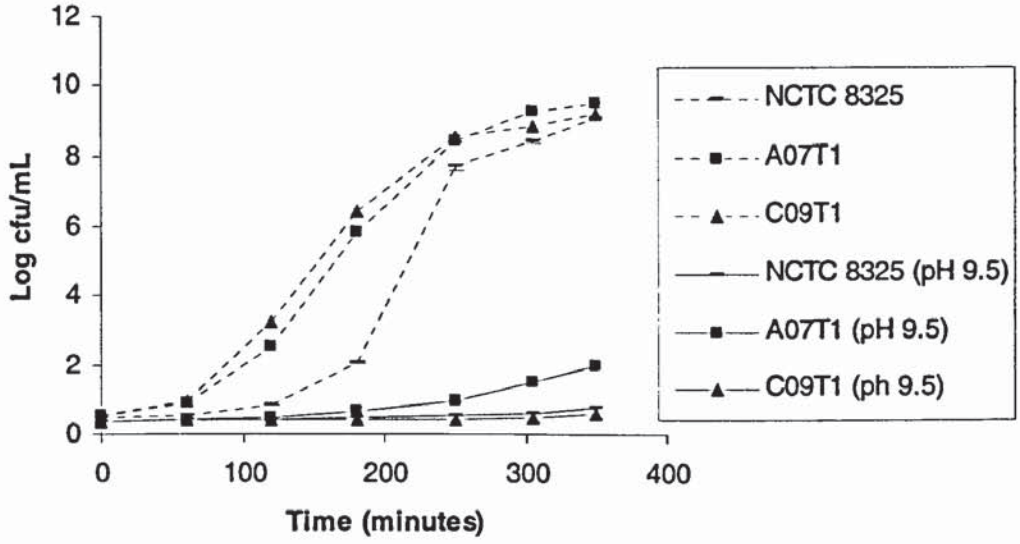


Figure 3.6.2 Growth in MH broth at pH 7 and adjusted to pH 9.5 of strains NCTC 8325 (MSSA) A07T1 (MR-MRSA, SCC*mec* type II) and C09T1 (MRSA, SCC*mec* type IV). Error bars represent ± standard error of the mean.

3.3.8 Minimum inhibitory NaCl concentration

The minimum inhibitory NaCl concentration for strains NCTC 8325, A07T1 and C09T1 was 17.5% (table 3.6). At 15% NaCl concentration all three strains had grown and viable cells could be recovered.

Table 3.7 Growth of strains NCTC 8325 (MSSA), A07T1 (MR-MRSA, SCC*mec* type II) and C09T1 (MRSA, SCC*mec* type IV) in MH broth adjusted to NaCl concentrations ranging from 5 to 20% at 37°C for 16 hours.

NaCl concentration (% $^{w}/_{v}$)	NCTC 8325	A07T1	C09T1
5.0	+	+	+
7.5	+	+	+
10.0	+	+	+
12.5	+	+	+
15.0	+	+	+
17.5	-	-	-
20.0	-	-	-

+ indicates growth. – indicates no growth.

3.4 Discussion

The aim of this chapter was to characterise isolates based on antibiotic sensitivity, to identify potential hVISA, VISA and VRSA isolates and to investigate the stress responses of MRSA strains in comparison to an *S. aureus* strain.

3.4.1 Antibiotic sensitivity

Antimicrobial sensitivities for the isolates were determined using the disc diffusion method. At the time of testing, disc diffusion was the standard method used in the UHB and thus protocols and reagents were readily available. In addition, the method is well documented, results are easily interpreted and, if necessary estimates, of minimum inhibitory concentrations (MICs) may be calculated using a standard curve (Andrews, 2001). All 130 isolates from the UHB were confirmed as methicillin resistant as determined by resistance to flucloxacillin. All MRSA isolates were later confirmed to carry the *mecA* gene as confirmed by SCC*mec* type assignment (chapter 5).

A total of 46 isolates (35%) were resistant to six or more antibiotics and designated MR-MRSA. Two isolates from this total were CA-MRSA. This confirmed the findings of routine antibiotic sensitivity testing performed previously at the UHB. The MR-MRSA isolates were all sensitive to vancomycin, tetracycline and rifampicin. Eighteen of the MR-MRSA isolates were also sensitive to mupirocin at high concentrations, however, whether this is clinically relevant is uncertain. Mupirocin is typically administered topically at concentrations of 20000mg/L which far exceeds the 20 and 500μg amounts tested using disc diffusion (Morrissey and Farrell, 2003). In addition there are few reports concerning clinical outcomes in patients treated with mupirocin that have resistant strains (Henkel and Finlay, 1999). Resistance to the aminoglycoside, gentamicin, was a distinguishing feature of MR-MRSA isolates. Only two NMR-HA-MRSA isolates were identified with gentamicin resistance. Gentamicin resistance is mediated by a modifying enzyme AAC(6')-APH(2'') encoded by the *aac(6')-Ie+aph(2'')* gene commonly located on the transposon Tn*4001*, which is distributed both chromosomally and on plasmids (Ubukata *et al.*,

1984). Gentamicin resistance is also associated with the SCC*mec* elements type II and III, which is discussed in chapter 5. The remaining HA-MRSA and CA-MRSA isolates shared various antibiogram patterns as illustrated in table 3.4. Typically, CA-MRSA isolates remain sensitive to most non-β-lactam antibiotics (Naimi *et al.*, 2001), however, the majority of CA-MRSA isolates from the South Birmingham area were resistant to erythromycin and/or trimethoprim in addition to flucloxacillin.

3.4.2 Vancomycin E-test

Sensitivity to vancomycin was tested by both disc diffusion and E-test. Testing for vancomycin resistance using the disc diffusion method was routinely performed in the UHB between 2001 and 2002, however, it has been demonstrated that such laboratory tests may not detect hVISA, VISA and VRSA strains (Hiramatsu *et al.*, 1997b). As a large tertiary care hospital the UHB routinely administers vancomycin for systemic MRSA infections and therefore may be at a higher risk of promoting a population of MRSA strains with reduced sensitivity to glycopeptides. The vancomycin macromethod E-test described by (Walsh *et al.*, 2001) indicated that none of the MRSA isolates from the UHB were clinically resistant to vancomycin and furthermore remained highly sensitive to the drug. Resistant subpopulations, in the form of microcolonies that may grow in the elliptical zone of inhibition, were not detected for any of the isolates excluding the hVISA control strain Mu3.

3.4.3 Calibration curve

A calibration curve was constructed to provide a simple and accurate method for calculating log/cfu from OD measurements. The calibration curve was constructed using data from three separate experiments. The highly significant R^2 value (0.9976) and the fact the line of regression past through each of the data points indicated that the calibration curve was suitable for accurately calculating log/cfu from OD measurements. All growth curves, including the calibration curve and pH experiments were performed using MH broth as a growth medium. Mueller-Hinton agar and broth are standard media for antibiotic sensitivity testing and batch quality is rigorously controlled in comparison to BHI medium. In addition, MH broth contains no added NaCl and fewer pH buffering components compared to BHI broth, which may have

affected the results of experiments involving NaCl and pH tolerance. Other groups performing similar work have also employed MH broth (Gudmundsson *et al.*, 1991).

3.4.4 Growth curves

It has been suggested that antibiotic resistance acquired by microorganisms may carry a biological cost (bioburden) (Gudmundsson *et al.*, 1991). For example, mutations in the *rpoB* gene encoding the β–subunit responsible for resistance to rifampin in *S. aureus* has been reported to affect transcriptional efficiency and to reduce growth rate (Wichelhaus *et al.*, 2002). Acquisition of resistance genes or chromosomal gene mutations that confer resistance to different groups of antibiotics may therefore affect rates of growth in *S. aureus*. The SCC*mec* element is a large 21-67 kb fragment of DNA that carries the *mecA* gene that confers methicillin resistance to *S. aureus* and inserts near the origin of replication on the *S. aureus* chromosome (*orfX*). In addition, there is evidence that plasmid carriage may increase bioburden when selective pressures such as antibiotics in the environment are no longer present (Bouma and Lenski, 1988; Smith and Bidochka, 1998). The size of the SCC*mec* element and its proximity to *orfX* coupled with plasmid carriage may affect the rate of growth of MRSA strains when compared to MSSA strains. The MRSA strains used for growth experiments were therefore selected based on the following criteria: antibiogram type; SCC*mec* type (chapter 5); PFGE type (chapter 4); and REAP type (chapter 5).

The relative fitness of nine MRSA strains in comparison to the *S. aureus* strain NCTC 8325 were compared by observing the rate of growth over time. It was expected that the rate of growth for MRSA strains would be significantly slower compared to a MSSA strain, due to the increased bioburden of the former; however, during 100-300 minutes of the log phase it was observed that the MRSA strains rate of growth was significantly faster than that of the MSSA strain. There was no significant difference between the growth of MRSA strains that carried the SCC*mec* type II element and those that carried the SCC*mec* type IV element. In addition, there was no significant difference between MRSA strains carrying plasmids and strains that did not. The repeatability of the growth curves, which were tested at separate times in triplicate, suggests that the observed difference is not due to experimental error. In addition, it is unlikely that differences between batches of growth media have affected results.

Mueller-Hinton broth was chosen specifically for this study because the nutrient formulation is identical between batches due to the medium being used internationally for antimicrobial sensitivity testing (Anonymous, 2000). During this study MH broth was not supplemented with either Ca^{2+} or Mg^{2+}, whether the absence of these divalent cations significantly affects the growth of certain *S. aureus* strains in the absence of antibiotics is not known. Cations are often important for the optimal activity of specific enzymes and their absence may affect the metabolism of certain *S. aureus* strains and thus growth rate. The observed significant difference in the growth curves may be due to the growth rate of the *S. aureus* strain NCTC 8325. It is possible that the strain NCTC 8325 does not grow as fast as MRSA strains. To validate these findings it will be necessary to perform further growth curves using a larger number of *S. aureus* strains. It may also be necessary to supplement MH broth with Ca^{2+} or Mg^{2+} at the recommended concentrations to exclude there possible effect on growth rate. Typically, a well documented strain such as NCTC 8325, is used for comparisons during growth curve studies, however, during this type of study it may also be important to include clinical *S. aureus* isolates. Inclusion of clinical MSSA strains that do not carry the SCC*mec* element and are isolated in the same time frame and similar environs as MRSA strains may provide a better representation of any observed growth rate differences. If however, the observation that certain MRSA may have a significantly increased growth rate between 100 and 300 minutes of the log phase, compared with *S. aureus* strains, then it may be that MRSA are at an advantage to colonise a host faster than a MSSA. Presently, it is only possible to speculate the bioburden of antibiotic resistance to MR-MRSA and MRSA when compared to MSSA strains that reside in both hospital and community environs (Andersson and Levin, 1999; Bjorkman and Andersson, 2000; Bjorkman *et al.*, 2000).

3.4.5 Desiccation and survival

The ability of *S. aureus* to persist in the environment, especially within hospitals, is of major concern but not fully understood (Smith *et al.*, 1996; O'Connell and Humphreys, 2000). To determine the death kinetics of MRSA and *S. aureus* in the environment *ex vivo*, the rates of recovery of three organisms from a plastic (polystyrene) surface were assessed. The results indicated that rates of recovery for all three strains declined over time and persisted for at least 56 days (8 weeks). Although

each strain was adjusted to the same OD prior to inoculation of the surface the log cfu/mL recovered for strain A07T1 (MR-MRSA) were significantly higher ($p<0.0001$) than for C09T1 (MRSA) and NCTC 8325. To determine the rate of decline for each strain the decimal reduction time (D-value) was calculated. The D-value is the number of days required for a bacterial population to drop by one log order and is independent of the starting cell density of a culture. The results indicated that the D-value for strain NCTC 8325 was almost twice as long as for strain C09T1 and A07T1, therefore, this may indicate that *S. aureus* strains that are not MRSA are better able to survive desiccation for longer periods of time. Variations in the rates of recovery for each strain on each sampling day are very low, as indicated by the size of the standard error bars in figure 3.4, which suggests consistent rates of recovery during sampling. In addition, if all the results for A07T1 are reduced by log orders then the pattern of decline is similar to that of C09T1. It would be necessary to use more test strains in future studies to confirm the findings of this study. In addition, it may be necessary to prepare bacterial cells by washing with a media that does not provide any nutrition such as saline, prior to the inoculation of a test surface. Broths may act as a supporting medium maintaining the viability of bacterial cells for extended periods of time even in desiccated conditions (Adams and Moss, 2000). A number of studies have reported the persistence of MRSA strains on a selection of surfaces after desiccation, including cotton bed sheets, glass and surfaces contaminated with dried blood, however, death rates demonstrate variation depending on the type of surface (Beard-Pegler *et al.*, 1988; Smith *et al.*, 1996). It was observed that *S. aureus* remained viable for between three and six months on cotton and dried blood (Smith *et al.*, 1996) but populations rapidly declined on stainless steel (Kusumaningrum *et al.*, 2003). A number of studies have also tested cross-contamination from one surface to another via cleaning apparatus such as sponges and cloths (Scott and Bloomfield, 1990; Kusumaningrum *et al.*, 2003), however, it is not certain how significant these findings are as a recent study has demonstrated a significant ($p<0.0001$) negative linear trend between inoculum size and bacterial transfer rates (Montville and Schaffner, 2003). This indicates that it may be necessary to test a range of inoculum sizes when investigating cross-contamination and death kinetics. Rates of recovery from contaminated surfaces and cross-contaminated surfaces may provide a better indication of how well MRSA strains are able to persist in a non-static environment over time. A recent study in Germany investigated the role

that homes may play in the recolonising of health care workers with MRSA after treatment to eradicate the microorganism (Kniehl *et al.*, 2005). Even after a thorough eradication regimen MRSA was still detectable for up to two years in home environments that had become heavily contaminated (Kniehl *et al.*, 2005). This type of study demonstrates the persistent nature of MRSA and the importance of sanitation for health care workers in both hospital and community environments. Interestingly, few studies have investigated the possible role non-health care workers visiting hospitals play in the introduction of MRSA to the hospital environment (Afif *et al.*, 2002).

Further studies testing the dynamics of surface contamination and cross-contamination coupled with human colonisation may provide a better indication of MRSA survival and transmission in an array of environments. In addition, an array of surfaces should be tested as death kinetics associated with different materials may be different (Scott and Bloomfield, 1990; Smith *et al.*, 1996; Kusumaningrum *et al.*, 2003). The ability of MRSA to persist, both in stressful environments and on and in the human body is of particular importance regarding the development of antibacterial surfaces (O'Connell and Humphreys, 2000; Bright *et al.*, 2002; Lewis and Klibanov, 2005), wound dressings (Strohal *et al.*, 2005) and both implanted and prosthetic components (Francois *et al.*, 1996; Alt *et al.*, 2004).

3.4.6 Tolerance to varying pH levels

The ability of MRSA and *S. aureus* to survive exposure to levels of pH in the range of pH 2 to pH 12 was assessed to determine the most acidic and alkaline conditions that MRSA and *S. aureus* can tolerate. Tolerance to extremes in pH levels may be clinically relevant in terms of MRSA strains being resistant to low pH in the macrophage phagosome (Cotter and Hill, 2003) or the high pH of some cleaning products (Adams and Moss, 2000). All three strains were able to survive exposure to pH levels as low as pH 4 and as high as pH 10, which is typical of *S. aureus* (Adams and Moss, 2000). There was no significant difference in the cell density of the strains between pH 5 to pH 9. In addition, there was no significant difference when results for cell densities at pH 5 were compared with pH 7. The pH of abscess fluid from suppurative infections typically ranges between pH 5.5 to pH 6.8 (Gudmundsson *et*

al., 1991). As the difference between cell density at pH 5 and pH 7 was not significant after incubation for 16 hours it may be that the growth of MRSA or MSSA within an abscess is not significantly affected by a change in pH. When growth curves for strains A07T1 and C09T1 were performed at pH 4 in comparison to pH 7 there was a significant difference in the rate of growth. Although, A07T1 and C09T1 grew significantly slower at pH 4 in comparison to pH 7 after 16 hours both strains had grown to between 10 and 12 log cfu/mL. In addition, the *S. aureus* strain NCTC 8325 did not appear to grow which was contradictory to previous observations. It has been observed that a fine precipitate may occur in MH broth at low and high pH. The precipitate may affect absorbance readings and ultimately the interpretation of cell numbers in the culture. Although MH broth adjusted to the relevant pH levels were used as blanks during testing, in future experiments it may be necessary to plate all samples to determine accurate cell numbers rather than to rely on OD readings. To make a stronger conclusion further testing would be required. When strains were grown at pH 9.5 in comparison to pH 7 only strain A07T1 was able to grow. Again this result is contradictory, as previous testing had indicated that all three strains were able to grow at pH 10. If MRSA strains are able to grow during sustained exposure to pH levels as low as pH 4 and as high as pH 10 there may be opportunities for MRSA strains to survive on surfaces even after cleaning has taken place. Detergents and disinfectant preparations often contain acids to remove mineral deposits from surfaces and alkalis to assist in solubilising organic material such as fats and proteins (Adams and Moss, 2000). In addition, disinfectants, unlike antibiotics, do not act upon a specific aspect of microbial cell metabolism but have a broadly based inhibitory effect. Acid-anionic surfactants are combinations of acid, usually phosphoric acid, with surface-active agents and are effective only below pH 2.5. Although, the *S. aureus* and MRSA strains tested during this study did not survive below pH 4, other studies have demonstrated that *S. aureus* may tolerate up to pH 2 if continually exposed to sub lethal pH levels (Cotter and Hill, 2003). In the case of quaternary ammonium compounds (QACs), they act as surfactants, disrupting the cell membranes integrity and are generally effective over a wide pH range (Adams and Moss, 2000). The resistance of MRSA strains to both QACs and antiseptics has been associated with *qac* genes (Noguchi *et al.*, 1999; Sidhu *et al.*, 2002). Known *qac* resistance genes reported in clinical staphylococci, *qacA*, *qacB*, and *qacC/smr*, are generally plasmid borne, encode a multidrug efflux pump and are widely distributed in the environment

(Leelaporn *et al.*, 1995; Mayer *et al.*, 2001). It is highly probable that resistance arises due to exposure of *S. aureus* to sublethal concentrations of a disinfectant (van Schaik and Abee, 2005), therefore, cleaning products that are not prepared to sufficient concentrations to kill, may allow the microorganism to persist in the surrounding environment.

3.4.7 Salt tolerance

Staphylococcus aureus, is considered a highly osmotolerant bacterium that is able to tolerate and grow in an environment with a water activity (a_w) as low as 0.86, the equivalent of 3.5M (20% ($^w/_v$)) NaCl (Scott, 1953). Two parameters that are related to osmotolerance are water activity and osmotic pressure. Water activity is a measurement of the available water in a solution in comparison to pure water. Pure water is described as having water activity that is equal to 1 ($a_w = 1$) and thus the water activity of a solution is expressed as a ratio of the relative water available in the substance in relation to pure water. This ratio is generally expressed as a number smaller than one e.g. $a_w = 0.9$. In addition water activity depends on the number, rather than the size, of molecules or ions present in a solution. Thus a compound such as NaCl, which dissociates into two ions in solution, is more effective at reducing water activity than a compound such as sucrose on a mole-to-mole basis (Adams and Moss, 2000). Typically, as the water activity of a solution decreases the osmolarity increases.

The biochemical reactions that take place in the cytoplasm of a bacterial cell do so in an aqueous environment, therefore, an important aspect related to the survival and growth of a microorganism is the ability to regulate the flow of water in and out of the bacterial cell. Typically, water molecules will flow from an environment with a high water activity to an environment with a low water activity (Adams and Moss, 2000). The water activity of the cytoplasm in a bacterial cell is maintained to be lower than the surrounding environment, ensuring that sufficient water molecules flow into the bacterial cell to maintain biological functions. This is achieved by the production of increasing concentrations of solutes in the cytoplasm, known as compatible solutes, which do not interfere with cytoplasmic function (Adams and Moss, 2000). Typically, *S. aureus* undergoes an extensive programme of gene and protein expression and

utilises the amino acid and amino acid-like compounds, glycine, taurine, choline, proline and betaine as compatible solutes to maintain turgor pressure in high osmolarity environments (Graham and Wilkinson, 1992; Townsend and Wilkinson, 1992; Pourkomailian and Booth, 1994; Amin *et al.*, 1995). As water is drawn into the cytoplasm the osmotic pressure within the bacterial cell increases and is regulated by the rigidity of the bacterial cell wall. Bacteria may become stressed if there is a net flow of water out of the cytoplasm, leading to plasmolysis, or a net flow into the cell leading to lysis (Adams and Moss, 2000). The former is prevented from occurring by increasing the concentration of compatible solutes into the cytoplasm, ensuring that the water activity is consistently lower than that of the surrounding environment. The latter is prevented by the presence of the bacterial cell wall and the controlled flow of water out of the bacterial cell. The osmotolerance of *S. aureus* is an important determinant of its ability to grow in foods and on human skin, from where it may spread and cause disease. Observing variations in NaCl tolerance may give some indication of the osmotolerance of *S. aureus* and MRSA strains in the environment.

During this discussion water activity measurements are given as approximate values based on the findings of other studies. The tolerance of MRSA and MSSA to reduced water activity was assessed using MH broth adjusted to salt concentrations ranging from 5 to 20% ($^{w}/_{v}$), which represented Aw = 0.98 to 0.83 respectively (Scott, 1953; Adams and Moss, 2000). All strains were found to have a NaCl, MIC of 15% ($^{w}/_{v}$) (A_w 0.86), which is typical of *S. aureus* (Wilkinson, 1997). These preliminary findings suggest that the majority of strains from the UHB and South Birmingham area are highly tolerant to a reduced water activity environment. Other studies have demonstrated that MRSA strains have NaCl MICs that range between 9.5 and 14%, however, these were tested using nutrient agar, which contains 5% NaCl as standard (Adhikari *et al.*, 2002) but tolerance to NaCl levels between 0 and 20% ($^{w}/_{v}$) is not unusual in *S. aureus* (Adams and Moss, 2000). To study salt tolerance a Tn917-*lacZ*-induced NaCl-sensitive mutant has been created in *S. aureus* NCTC 8325-4 (Vijaranakul *et al.*, 1997). It was observed that the mutant could be rescued by glycine, choline and betaine. Subsequent, cloning and sequencing revealed that the transposon insertion disrupted a branched chain amino acid transporter gene (Vesterholm-Nielsen *et al.*, 1999). In addition, a NaCl-sensitive mutant that could not be rescued by osmoprotectants has been created in the same *S. aureus* strain by

inserting a Tn917-*lacZ*-induced in the *arsR* gene of the *ars* operon (Scybert *et al.*, 2003). It has been suggested that the *arsR* gene may encode a membrane efflux protein that has Na^+ efflux activity. Disruption of this gene may impede the ability of *S. aureus* to remove Na^+ from the cytoplasm leading to cell death (Scybert *et al.*, 2003).

This study only investigated NaCl MICs for a small selection of MRSA strains. Further investigation of a larger selection of strains may provide greater differences in MIC results. Investigating the minimum bactericidal concentration (MBC) may be more discriminatory. The MBC is expected to be higher than the MIC since it is the minimum concentration that kills a microorganism and not just the inhibition of growth. In addition, it may be important to use a defined media containing known concentrations of compatible solutes and to accurately measure the water activity of growth media using an industrial probe designed for such measurements (Townsend and Wilkinson, 1992).

3.4.8 Future studies

As well as studying the phenotypic responses of MRSA and MSSA to environmental stress it may be necessary to study sigmaB factors. SigmaB factors have been identified in *S. aureus* and *B. subtilis* and are associated with stress responses that may aid survival (Cotter and Hill, 2003). SigmaB mutants of *B. subtilis* display a 50 to 100 fold reduced ability to survive heat (54°C), ethanol (9% ($^v/_v$)), NaCl (10% ($^w/_v$)), acid (pH 4.3) and shocks such as freezing, desiccation, and exhaustion of glucose or phosphate (Volker *et al.*, 1999). One important finding made during this study was the fact that NCTC 8325 is a sigmaB defective mutant and may not respond well to all environmental stresses (Kullik *et al.*, 1998). In future studies of stress responses it may be necessary to use a number of alternative *S. aureus* strains as controls to compare with MRSA strains.

3.4.9 Conclusion

In summary, AST has identified that a large proportion of MRSA strains in the UHB are multidrug resistant. Although such strains retain sensitivity to only rifampicin,

tetracycline and vancomycin, reassuringly all the MRSA strains tested remain highly sensitive to vancomycin, as determined by the macromethod of E-testing. Preliminary investigation of phenotypic stress responses in MRSA strains have highlighted potential problems that must be addressed during future investigations. Although, phenotypic methods such as AST have successfully differentiated MRSA strains from the UHB and South Birmingham area based on their antibiogram profile it is necessary to characterise the strains using genotypic methods. Molecular typing techniques allow for the differentiation of strains at a genomic level and are therefore unaffected by varying levels of gene expression unlike phenotypic methods. In addition, genotypic methods are typically more discriminatory than phenotypic methods and in some instances may be performed in less time.

CHAPTER 4 GENOTYPIC CHARACTERISATION OF MRSA USING PFGE AND RAPD

4.1 Introduction

Traditionally, characterisation of MRSA within health care institutions was performed using an array of phenotypic methods, which were inexpensive but limited by the number of characteristics that could be examined and provided misleading results due to variation in gene expression. Many phenotypic methods have been superseded by molecular techniques that allow the specific comparison of genotypic relationships between bacteria of the same species. Comparatively, genotypic methods are more discriminatory, can be applied to a broader range of bacterial species and may, in some cases, be performed in less time (Tenover *et al.*, 1997). Genotypic methods can be broadly separated into techniques that require restriction endonucleases and those that use PCR.

Pulsed-field gel electrophoresis is a technique that requires restriction endonucleases and is the most widely employed genotypic method used in reference and clinical laboratories for typing MRSA (Murchan *et al.*, 2003). The technique remains the "gold standard" for the molecular typing of MRSA and other bacterial species (Schlichting *et al.*, 1993; Leonard *et al.*, 1995; Prevost *et al.*, 1995; Liu *et al.*, 1996), is highly reproducible when the same protocol is followed and comprehensive criteria exist for the standardised interpretation of profiles (Tenover *et al.*, 1995; Cookson *et al.*, 1996). However, PFGE is time consuming often taking up to six days to obtain profiles and is also expensive due to the cost of reagents and the requirement of specialised equipment . Comparatively, PCR based techniques are less expensive than PFGE and provide results in a shorter time frame. Random amplification of polymorphic DNA (RAPD), also called arbitrary primed PCR, is a PCR based technique that has been employed to successfully type Gram-negative and Gram-positive bacterial species (Hilton *et al.*, 1996; Hilton *et al.*, 1997; Hilton *et al.*, 1997a; Hermans *et al.*, 2001; Hermans *et al.*, 2001a; Hopkins and Hilton, 2001a; Perry *et al.*, 2003) including MRSA (van Leeuwen *et al.*, 1996; Pereira *et al.*, 2002). Random amplification of polymorphic DNA uses a single short arbitrary primer that anneals to

numerous locations on the template DNA, priming the subsequent amplification of an array of products that serve as a DNA fingerprint. The method requires no prior knowledge of the target DNA. Random amplification of polymorphic DNA may lack reproducibility if critical factors affecting the reaction are not addressed. To ensure the reproducibility of the reaction factors such as, template DNA concentration, primer concentration and buffer composition must be optimised. This may ensure RAPD gives a high level of typability, discriminatory power and reproducibility.

In this chapter the typing of 130 clinical MRSA isolates by PFGE and an optimised RAPD reaction is described. The two methods are compared for typeability and discriminatory power and assessed for their application in a clinical setting.

4.2 Materials and methods

4.2.1 Pulsed-field gel electrophoresis

Harvesting and preparation of cell blocks prior to lysis was performed as described by Lang *et al.* (Lang *et al.*, 1999). An isolated colony was inoculated into 5ml BHI broth and incubated with shaking at 37°C for 16 hours. The wet weight of a pellet from 0.5mL of this culture was determined. The pellet was resuspended in the required amount of NET-100 (10mM Tris-HCl, pH 8, 100mM EDTA, pH 8, and 100mM NaCl) to give a final cell suspension of 20mg/mL. A 0.5 ml aliquot of the resuspended cells was mixed with an equal volume of 0.9% ($^{w}/_{v}$) chromosomal grade agarose (Bio-Rad Laboratories Ltd, UK) at 50°C. The cell/agarose suspension was loaded into block moulds (Bio-Rad Laboratories Ltd, UK) and allowed to solidify at 4°C. The prepared blocks were incubated for 24 hours at 37°C in 3ml lysis solution (6mM Tris-HCl pH 7.6, 100mM EDTA pH 8, 100mM NaCl, 0.5% ($^{w}/_{v}$) lauroyl sarcosine, 1mg/mL lysozyme) with 20 units of lysostaphin. The initial lysis solution was removed and the blocks were incubated for 48 hours at 50°C in 3mL ESP (0.5M EDTA pH 9, 1.5mg/ml proteinase K and 1% ($^{w}/_{v}$) lauroyl sarcosine). The blocks were washed at room temperature twice for two hours followed by two, one hour washes using TE buffer (10mM Tris-HCl and 1mM EDTA, pH 8). A portion of each agarose block (1 x 1 x 9mm) was digested with 20 units of *Sma*I (Roche, UK) in 0.1mL buffer for 16 hours at 25°C. The digested DNA samples were subjected to pulsed-field gel electrophoresis (CHEF Mapper system, Bio-Rad, UK) with the following running parameters: initial pulse five seconds; final pulse 40 seconds; voltage, 200V or 6V/cm; time 20 hours; and temperature, 12°C (Bannerman *et al.*, 1995). Wells three and eight of all PFGE gels carried a *Sma*I chromosomal digest from *S. aureus* strain NCTC 8325 as a control and a molecular weight marker (Tenover *et al.*, 1995; Murchan *et al.*, 2003). Gels were stained with 1μg/mL of ethidium bromide for 45 minutes and destained for 45 minutes in distilled water. Gels were visualised under UV illumination and photographed using the GeneGenius Bio Imaging System (Syngene, UK). Images of PFGE profiles were compared visually and by use of Gel Compar II software (Applied Maths, Belgium). The similarity between the isolates was calculated by the Dice coefficient (tolerance of 1% and optimisation of 0.5%), and

a dendrogram was constructed using un-weighted pair groups using mathematical averages (UPGMA) clustering. The PFGE profiles were assigned letters according to their position in the dendrogram. Unique PFGE profiles were assigned the letter U followed by a number according to their position in the dendrogram. The interpretation of differences in banding patterns was performed using guidelines by Tenover *et al.* (Tenover *et al.*, 1995).

4.2.2 Typeability and discriminatory power of PFGE

The typeability of PFGE was calculated as the percentage number of strains which could be assigned to typing groups. The discriminatory power was determined using Simpson's index of diversity (D) (Hunter and Gaston, 1988):

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} n_j (n_j - 1)$$

where N is the total number of strains in the sample population, s is the total number of types described, and n_j is the number of strains belonging to the jth profile type.

4.2.3 DNA extraction for RAPD

4.2.3.1 Method 1 (purification of DNA by phenol extraction and ethanol precipitation)

An isolated colony was inoculated into 5mL BHI broth and incubated overnight with shaking at 37°C. A 1.5mL aliquot of the over night culture was centrifuged at 5500 x g for 4 minutes and the supernatant discarded. The pellet was resuspended in 300µL of TE buffer (1mM EDTA, pH 8 and 10mM Tris-HCl, pH 8) containing lysostaphin (80µg/mL) and incubated for 15 minutes at 37°C and then at 75°C for 10 minutes. An aliquot of 100µL of 10% ($^{w}/_{v}$) SDS and 5µL of RNAase A (10mg/mL) was added to the sample followed by an incubation period of ten minutes at 37°C. A 1µL aliquot of proteinase K (20mg/mL) was added and the sample was incubated for two hours at 65°C. A fresh 1.5mL centrifuge tube containing a 100µL pellet of silicon gel and 300µL of sterile, double-distilled water (SDW) was prepared, to which 400µL of the

sample was added. An aliquot of phenol: chloroform: isoacyl alcohol 25:24:1 saturated with 10mM Tris-HCl and 1mM EDTA, pH 8 (PCI) was added to the sample followed by centrifugation at 13000 x g for 5 minutes. Avoiding the silicon and protein, 400µL of the aqueous phase of the sample was dispensed into a fresh 1.5mL centrifuge tube containing 40µL of 3 M sodium acetic acid, pH 4. The sample was mixed by inversion and left at room temperature for 20 minutes. An 880µl aliquot of refrigerated absolute ethanol was added to the sample and this preparation was stored at –20°C overnight. After this time the sample was centrifuged at 13000 x g for 20 minutes. The sample was washed twice with 1mL of 70% (v/v) ethanol and centrifuged between washes at 13000 x g for 5 minutes. The resulting pellet was allowed to dry at room temperature for one hour. The pellet was resuspended in 200µL of SDW and stored for 16 hours at 4°C and subsequently at –20°C until required.

4.2.3.2 Determination of DNA quantity and purity

Preparations of DNA were examined by gel electrophoresis to ensure DNA was not excessively sheared. A 10µL aliquot of DNA preparation was mixed with 2µL of 6x loading buffer (0.25% (w/v) bromophenol blue, and 30% (v/v) glycerol and loaded into wells on a 2% (w/v) agarose gel containing 1µg/mL ethidium bromide. One well was loaded with 5µL of BIOzyme standard ladder 100-5000bp (GeneFlow, UK). Electrophoresis was performed in 1 x TAE running buffer (40mM Tris-HCl, 1mM EDTA and 0.1% (v/v) glacial acetic acid, pH 8) at 100V for one hour. Gels were visualised under UV using the GeneGenius Bio Imaging System (Syngene, UK).

The quantity and purity of DNA was determined using a UV spectrophotometer (Camspec, UK). A 5µL aliquot of DNA sample was mixed with 995µL of SDW in a quartz crystal cuvette and the A_{260} was measured (1 A_{260} unit is equal to 50µg/mL DNA) to determine the quantity of DNA. The extracted DNA was adjusted to a concentration of 5ng/µL with SDW. A measurement at A_{280} was taken and the ratio of A_{260} and A_{280} used to determine purity. Readings between 1.7-1.9 indicated DNA of high purity and these were taken forward for analysis.

4.2.3.3 Method 2 (DNA extraction by boiling of whole cells)

Isolates were inoculated onto BHI agar plates and incubated for 16 hours at 37°C. A single loop of colonies taken directly from the plate was suspended in 1mL of TESS buffer (50mM Tris-HCl, 5mM EDTA, 50mM NaCl and 50mM sucrose, pH 8), centrifuged for four minutes at 5500 × *g* and the supernatant discarded. The pellet was washed twice with 1mL of SDW. The cells were subsequently resuspended in 1mL of SDW and transferred to a 1mL sterile plastic cuvette. The A_{600} of the suspension was measured and adjusted to OD 1.7 with SDW. A 0.1mL aliquot of the adjusted suspension was transferred to a 0.2mL PCR tube and incubated at 94°C in a GeneAmp® PCR system 9700 (Applied Biosystems, UK) for 12 minutes. The heated cell suspension was subsequently centrifuged at 3000 × g for ten seconds. The resulting supernatant containing extracted DNA was cooled to room temperature and used as template in a multiplex PCR.

4.2.4 RAPD reaction

The RAPD reaction was carried out in a 25µL volume containing 19.15µL SDW, 2.5 µL 10x PCR Buffer (section 4.2.5.2), 0.6µL 100 µM primer (section 4.2.5.2), 0.5µL 10 mM dNTPs (Promega, UK), 0.25µL of 1.25 units/µL *Taq* DNA polymerase (Promega, UK) and 2µl of 5ng/µL DNA template (section 4.2.6.2). Amplification was performed on a GeneAmp® PCR system 9700 (Applied Biosystems, UK) using the following conditions: four minutes 30 seconds at 94°C followed by five cycles of 30 seconds at 94°C, two minutes at 20°C, one minute at 72°C followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 30°C, one minute at 72°C and concluded with a final extension of five minutes at 72°C and storage at 4°C until analysis. Amplicons were separated by agarose gel electrophoresis as described in section 4.2.3.2. Photography and analysis was performed as described in section 4.2.1.

4.2.5 RAPD optimisation

4.2.5.1 Strain selection

Three HA-MRSA strains representative of the three largest groups of PFGE profiles, were selected for optimising the RAPD reaction. The first strain selected was C05T1 (PFGE type C, type IV SCC*mec*) the second strain was B07T2 (PFGE type F, SCC*mec* II) the third strain was A04T1 (PFGE type G, type II SCC*mec*); SCC*mec* type assignment is described in chapter 5.

4.2.5.2 Selecting PCR buffer and primer

The optimum buffer composition for the RAPD reaction was determined using the Opti-prime™ buffer matrix (Schoettlin *et al.*, 1994). Each buffer (table 4.1) was tested with template DNA from C05T1, B07T2 and A04T1 and with each of the primers in table 4.2. The reaction was performed as described in section 4.2.4. The buffers and primers producing clearly distinguishable profiles with a suitable number of amplicons were selected for use in subsequent RAPD reactions. The selected primers were optimised for use in separate RAPD reactions.

Table 4.1 Opti-prime™ buffer matrix (Schoettlin *et al.*, 1994).

Tris-HCl 10 mM	$MgCl_2$ mM	KCl 25 mM	KCl 75 mM
pH 8.3	1.5	Buffer 1	Buffer 2
pH 8.3	3.5	Buffer 3	Buffer 4
pH 8.8	1.5	Buffer 5	Buffer 6
pH 8.8	3.5	Buffer 7	Buffer 8
pH 9.2	1.5	Buffer 9	Buffer 10
pH 9.2	3.5	Buffer 11	Buffer 12

Table 4.2 Primers used for RAPD typing (MWG biotech, Germany).

Primer	Sequence (5'-3')	Reference
GEN	GTT TGC CTC C	(Dautle *et al.*, 2002)
EP015	ACA ACT GCT C	(Tambic *et al.*, 1997)
KAY1	AGC AGC CTG C	(Tambic *et al.*, 1997)
1254	CCG CAG CCA A	(Akopyanz *et al.*, 1992)
797	AGC GTC ACT G	(Pereira *et al.*, 2002)

4.2.6 Concentration of primer

The optimum concentration of the selected primers to be included in the RAPD reactions was determined using template DNA from the strains, C05T1, B07T2 and A04T1 prepared using method 1 (section 4.2.3.1). The reactions were performed in duplicate as described in section 4.2.4 with primer stocks at concentrations of 50, 100, 200 and 300μM. The primer concentration producing clearly distinguishable profiles was selected.

4.2.6.1 Concentration of dNTPs

The optimum concentration of dNTPs to be included in the RAPD reactions was determined using template DNA from the strains, C05T1, B07T2 and A04T1 prepared using method 1 (section 4.2.3.1). The reactions were performed in duplicate as described in section 4.2.4 with dNTP stocks at concentrations of 5, 10, 20, 30 mM. The dNTP concentration producing clearly distinguishable profiles was selected.

4.2.6.2 Template DNA from boiled whole cells

The RAPD reaction was carried out on strains C05T1, B07T2 and A04T1 using template DNA prepared using method 1 (section 4.2.3.1) and template DNA prepared using method 2 (section 4.2.3.3). This was to determine the suitability of the boiled whole cells method for subsequent RAPD reactions. Profiles were compared to determine whether banding patterns remained stable.

4.2.7 RAPD intra- and inter-reproducibility

To confirm the intra- and inter- reproducibility of the optimised RAPD reactions freshly prepared chromosomal DNA from strain A04T1 was used as a control for every set of reactions. Every month freshly extracted chromosomal DNA from the strains C05T1, B07T2 and A04T1 underwent RAPD analysis in separate reactions with both primers to assess the stability of the profiles. Chromosomal DNA was prepared for each control reaction as described in section 4.2.3.3.

4.2.8 RAPD typing of MRSA strains

Using the optimised RAPD reactions all 130 MRSA strains were analysed. The strain A04T1 was used as a positive control for every set of reactions.

4.2.9 Typeability and discriminatory power of RAPD

Typeability and discriminatory power of RAPD analysis was determined as described in section 4.2.2.

4.3 Results

4.3.1 PFGE

Analysis of PFGE data revealed 31 different PFGE profiles. Eight PFGE clusters comprising of multiple isolates were designated types A-H. Two MR-HA-MRSA isolates, twelve HA-MRSA isolates and nine CA-MRSA isolates gave unique profiles and given the designation U followed by a number relating to the position of the isolate in the dendrogram (figure 4.1). The MR-HA-MRSA and MR-CA-MRSA isolates were separated into three clusters designated F-H with two unique types. The NMR-HA-MRSA and NMR-CA-MRSA isolates were separated into four main clusters designated A-E with 23 unique types.

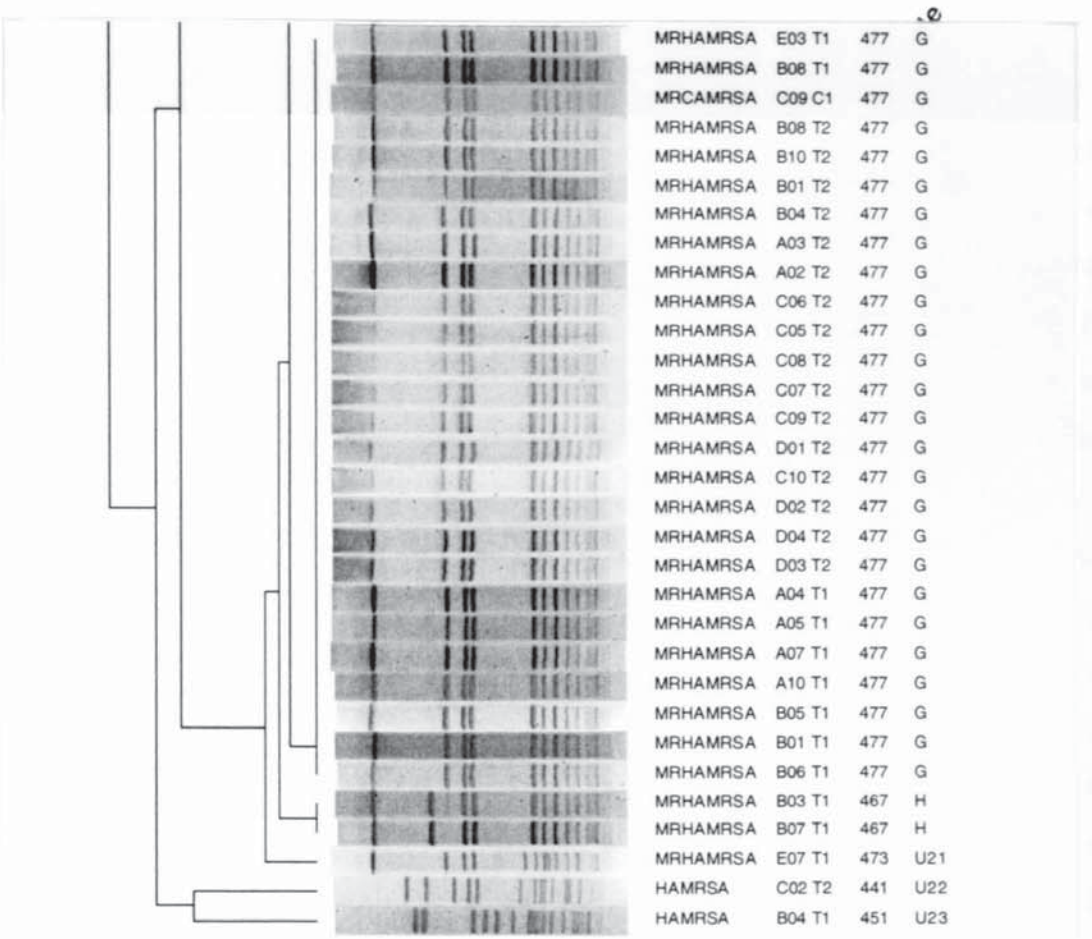
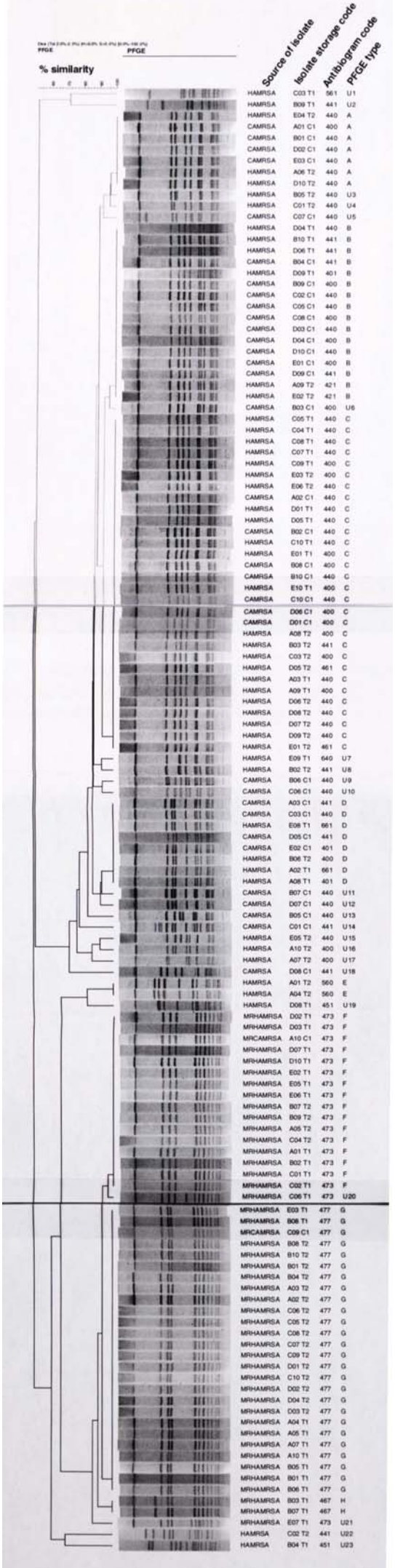


Figure 4.1 Dendrogram of 94 HA- and 36 CA-MRSA strains depicting PFGE profiles. Similarity calculated by Dice coefficient (tolerance of 1% and optimisation of 0.5%) and represented by UPGMA clustering.


PFGE
PFGE
% similarity
Source of isolate
Isolate storage code
Antibiogram code
PFGE type
HAMRSA C03 T1 561 U1
HAMRSA B09 T1 441 U2
HAMRSA E04 T2 440 A
CAMRSA A01 C1 400 A
CAMRSA B01 C1 440 A
CAMRSA D02 C1 440 A
CAMRSA E03 C1 440 A
HAMRSA A06 T2 440 A
HAMRSA D10 T2 440 A
HAMRSA B05 T2 440 U3
HAMRSA C01 T2 440 U4
CAMRSA C07 C1 440 U5
HAMRSA D04 T1 440 B
HAMRSA B10 T1 441 B
HAMRSA D06 T1 441 B
CAMRSA B04 C1 441 B
HAMRSA D09 T1 401 B
CAMRSA B09 C1 400 B
CAMRSA C02 C1 440 B
CAMRSA C05 C1 440 B
CAMRSA C08 C1 400 B
CAMRSA D03 C1 440 B
CAMRSA D04 C1 400 B
CAMRSA D10 C1 440 B
CAMRSA E01 C1 400 B
CAMRSA D09 C1 441 B
HAMRSA A09 T2 421 B
HAMRSA E02 T2 421 B
CAMRSA B03 C1 400 U6
HAMRSA C05 T1 440 C
HAMRSA C04 T1 440 C
HAMRSA C08 T1 440 C
HAMRSA C07 T1 440 C
HAMRSA C09 T1 400 C
HAMRSA E03 T2 400 C
HAMRSA E06 T2 440 C
CAMRSA A02 C1 440 C
HAMRSA D01 T1 440 C
HAMRSA D05 T1 440 C
CAMRSA B02 C1 440 C
HAMRSA C10 T1 440 C
HAMRSA E01 T1 400 C
CAMRSA B08 C1 400 C
CAMRSA B10 C1 440 C
HAMRSA E10 T1 400 C
CAMRSA C10 C1 440 C
CAMRSA D06 C1 400 C
CAMRSA D01 C1 400 C
HAMRSA A08 T2 400 C
HAMRSA B03 T2 441 C
HAMRSA C03 T2 400 C
HAMRSA D05 T2 461 C
HAMRSA A03 T1 440 C
HAMRSA A09 T1 400 C
HAMRSA D06 T2 440 C
HAMRSA D08 T2 440 C
HAMRSA D07 T2 440 C
HAMRSA D09 T2 440 C
HAMRSA E01 T2 461 C
HAMRSA E09 T1 640 U7
HAMRSA B02 T2 441 U8
CAMRSA B06 C1 440 U9
CAMRSA C06 C1 440 U10
CAMRSA A03 C1 441 D
CAMRSA C03 C1 440 D
HAMRSA E08 T1 661 D
CAMRSA D05 C1 441 D
CAMRSA E02 C1 401 D
HAMRSA B06 T2 400 D
HAMRSA A02 T1 661 D
HAMRSA A08 T1 401 D
CAMRSA B07 C1 440 U11
CAMRSA D07 C1 440 U12
CAMRSA B05 C1 440 U13
CAMRSA C01 C1 441 U14
HAMRSA E05 T2 440 U15
HAMRSA A10 T2 400 U16
HAMRSA A07 T2 400 U17
CAMRSA D08 C1 441 U18
HAMRSA A01 T2 560 E
HAMRSA A04 T2 560 E
HAMRSA D08 T1 451 U19
MRHAMRSA D02 T1 473 F
MRHAMRSA D03 T1 473 F
MRCAMRSA A10 C1 473 F
MRHAMRSA D07 T1 473 F
MRHAMRSA D10 T1 473 F
MRHAMRSA E02 T1 473 F
MRHAMRSA E05 T1 473 F
MRHAMRSA E06 T1 473 F
MRHAMRSA B07 T2 473 F
MRHAMRSA B09 T2 473 F
MRHAMRSA A05 T2 473 F
MRHAMRSA C04 T2 473 F
MRHAMRSA A01 T1 473 F
MRHAMRSA B02 T1 473 F
MRHAMRSA C01 T1 473 F
MRHAMRSA C02 T1 473 F
MRHAMRSA C06 T1 473 U20
MRHAMRSA E03 T1 477 G
MRHAMRSA B08 T1 477 G
MRCAMRSA C09 C1 477 G
MRHAMRSA B08 T2 477 G
MRHAMRSA B10 T2 477 G
MRHAMRSA B01 T2 477 G
MRHAMRSA B04 T2 477 G
MRHAMRSA A03 T2 477 G
MRHAMRSA A02 T2 477 G
MRHAMRSA C06 T2 477 G
MRHAMRSA C05 T2 477 G
MRHAMRSA C08 T2 477 G
MRHAMRSA C07 T2 477 G
MRHAMRSA C09 T2 477 G
MRHAMRSA D01 T2 477 G
MRHAMRSA C10 T2 477 G
MRHAMRSA D02 T2 477 G
MRHAMRSA D04 T2 477 G
MRHAMRSA D03 T2 477 G
MRHAMRSA A04 T1 477 G
MRHAMRSA A05 T1 477 G
MRHAMRSA A07 T1 477 G
MRHAMRSA A10 T1 477 G
MRHAMRSA B05 T1 477 G
MRHAMRSA B01 T1 477 G
MRHAMRSA B06 T1 477 G
MRHAMRSA B03 T1 467 H
MRHAMRSA B07 T1 467 H
MRHAMRSA E07 T1 473 U21
HAMRSA C02 T2 441 U22
HAMRSA B04 T1 451 U23

4.3.2 Effects of reaction buffer and primer on RAPD

Two separate RAPD reactions were optimised using primers 1254 and 797 with buffer 3. To allow discrimination between unrelated strains and to improve discriminatory capacity of RAPD analysis a sufficient number of amplicons are required, however, not so many that profile analysis is too difficult or time consuming. Profiles obtained by RAPD analysis from the chromosomal DNA of three HA-MRSA strains (C05T1, B07T2 and A04T1) using 12 different buffers and two primers, 1254 and 797, are shown in figures 4.2.1, 4.2.2, and 4.2.3. The buffers with low KCl concentration (25mM) and lower pH (pH 8.3 and pH 8.8) supported product amplification (lanes 1, 3, 5 and 7). In addition it was observed that pH (pH 8.3) and high $MgCl_2$ concentration (3.5mM) supported profile stability (lane 3). A high pH of 9.2 and high KCl concentration (75mM) did not support product generation (lanes 2, 4, 6, 8, 10 and 12). The RAPD reactions that included buffer 3 with primer 1254 or primer 797 provided sufficient amplicons and stable profiles. The primers GEN, EP015 and KAY1 did not generate a sufficient number of stable amplicons when tested. Based on these observations two RAPD reactions were optimised: reaction 1 (buffer 3 with primer 1254); and reaction 2 (buffer 3 with primer 797).

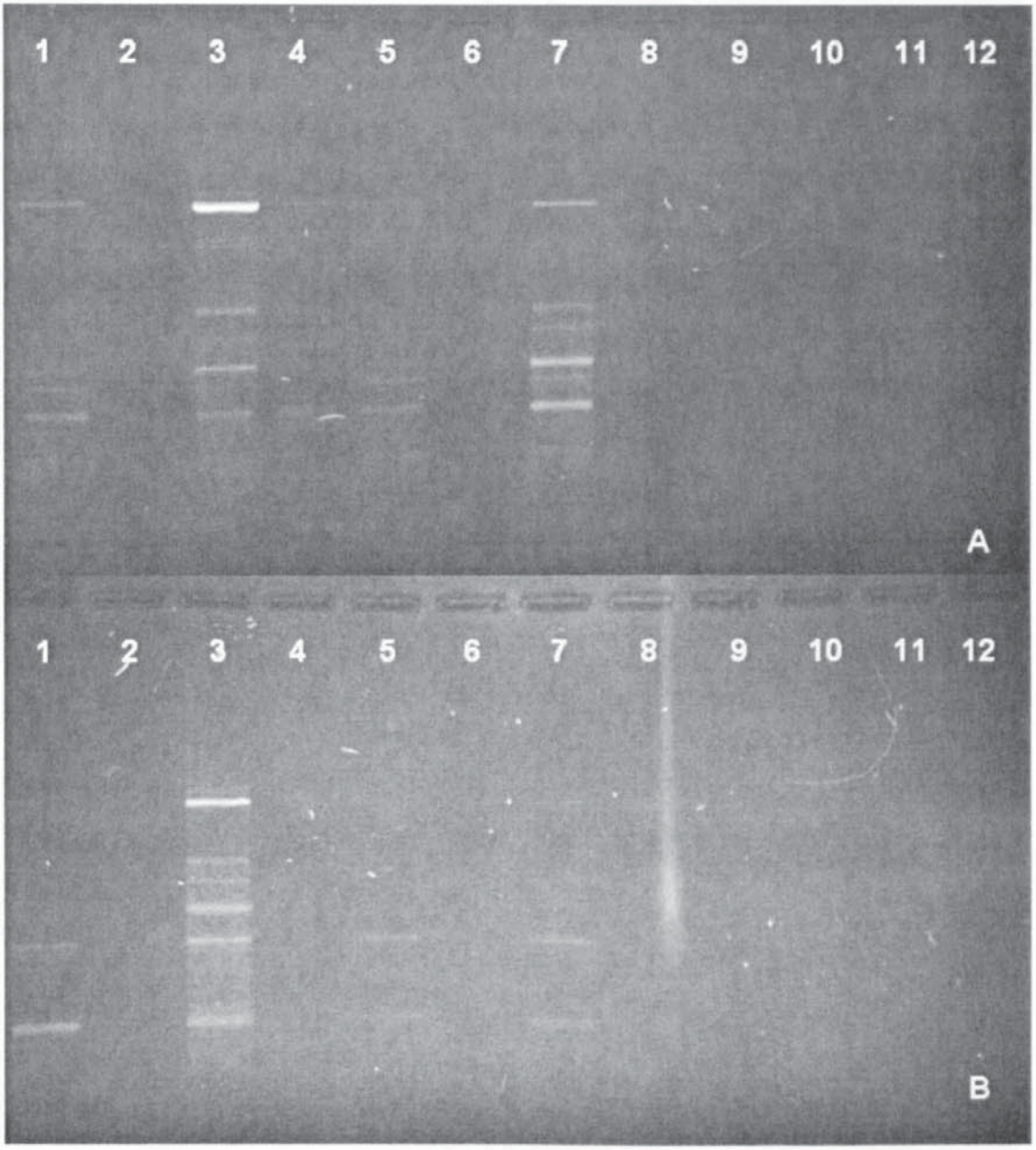


Figure 4.2.1 RAPD profiles of strain A04T1 (PFGE type G, type II SCC*mec*) using the 12 buffers detailed in table 4.1. Lanes labelled according to buffer number. Gel A using primer 1254 and gel B using primer 797.

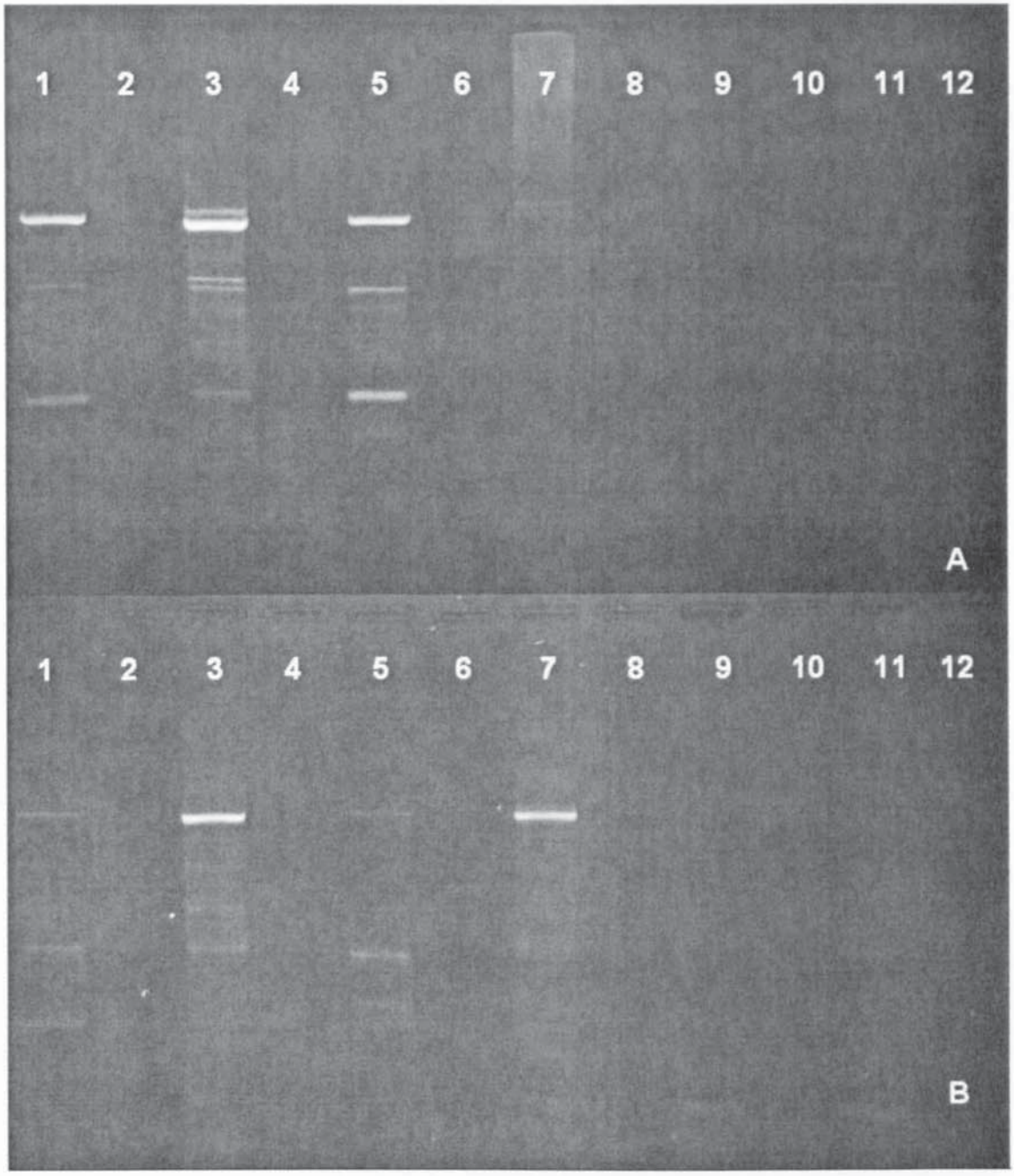


Figure 4.2.2 RAPD profiles of strain C05T1 (PFGE type C, type IV SCC*mec*) using the 12 buffers detailed in table 4.1. Lanes labelled according to buffer number. Gel A using primer 1254 and gel B using primer 797.

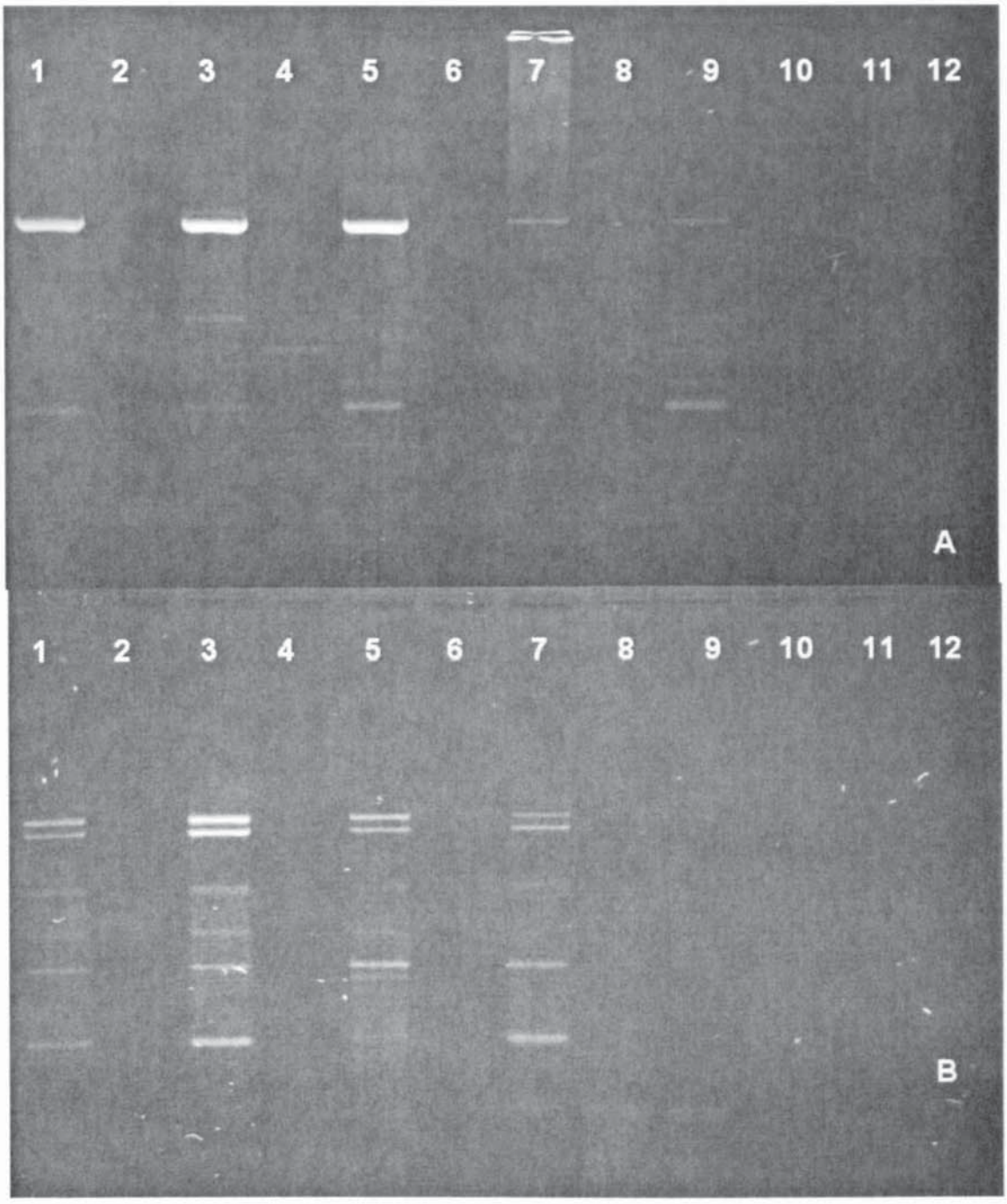


Figure 4.2.3 RAPD profiles of strain B07T2 (PFGE type F, SCC*mec* II) using the 12 buffers detailed in table 4.1. Lanes labelled according to buffer number. Gel A using primer 1254 and gel B using primer 797.

4.3.3 Effect of primer concentration

Two separate RAPD reactions were optimised using primers 1254 and 797 at stock concentrations of 50, 100, 200 and 300 μM with buffer 3. Profiles generated by RAPD analysis from the chromosomal DNA of three HA-MRSA strains (C05T1, B07T2 and A04T1) using varying concentrations of primers, 1254 and 797, are shown in figures 4.3.1, 4.3.2, and 4.3.3. The optimum stock concentration of primer for both RAPD reactions was 100 μM. The profiles obtained using this concentration were stable and the number of amplicons in each profile remained constant. Concentrations of primer above 200μM visibly reduced the number of amplicons.

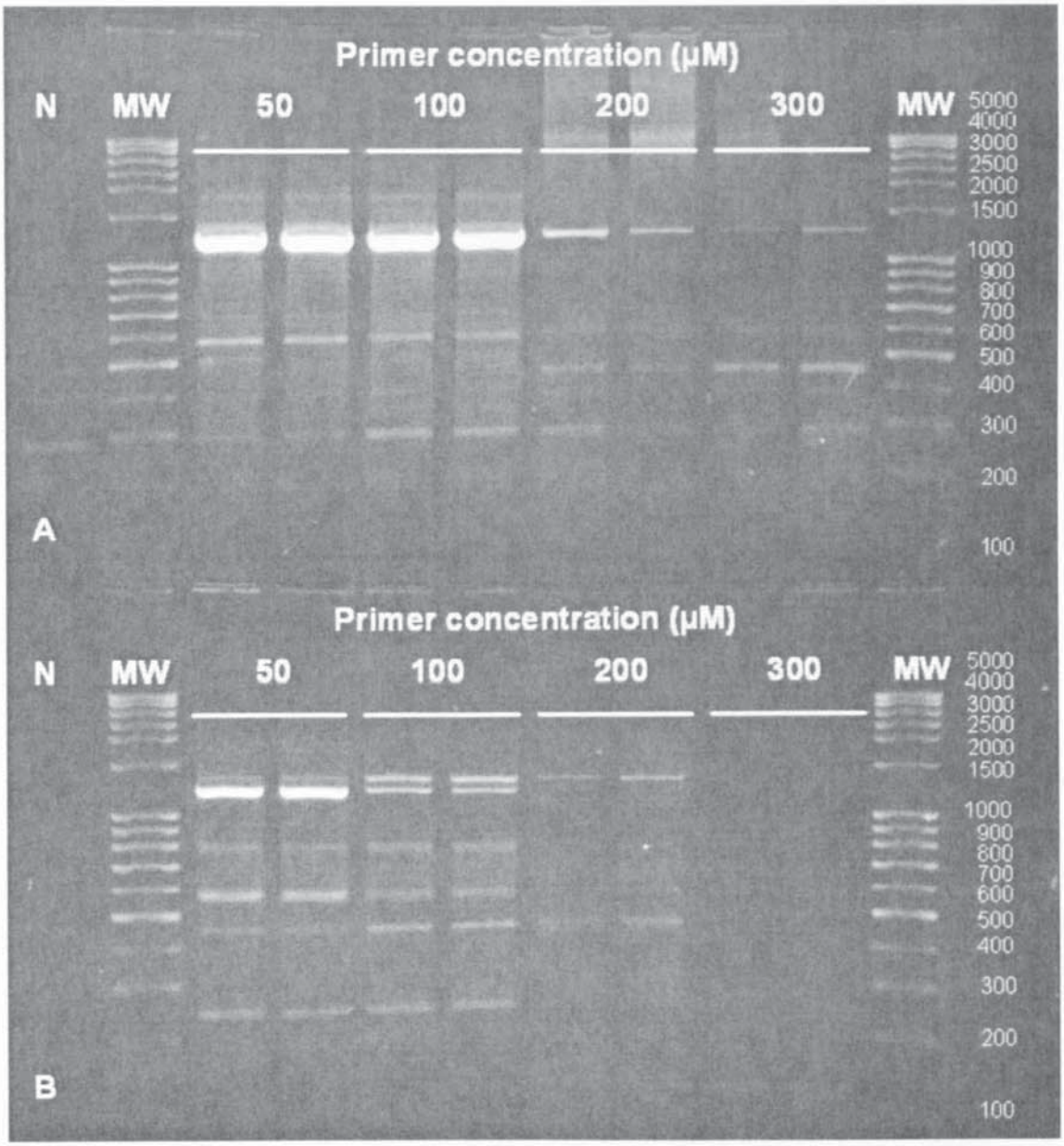


Figure 4.3.1 RAPD profiles of strain A04T1 (PFGE type G, type II SCC*mec*) using different stock concentrations of primer. Lanes labelled according to primer concentration. Gel A using primer 1254 and gel B using primer 797. N: negative control; MW: molecular weight ladder with size indicator (bp).

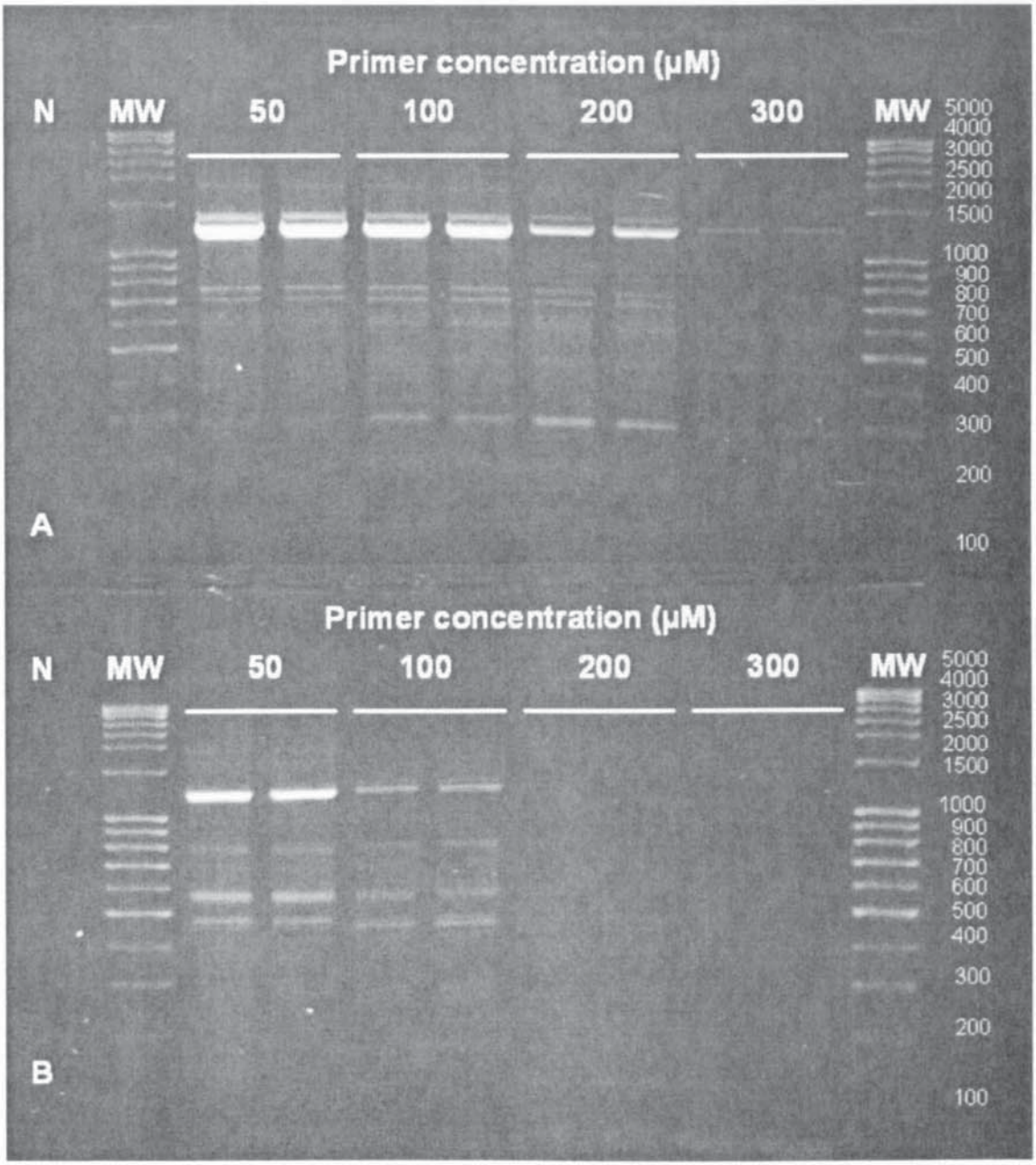


Figure 4.3.2 RAPD profiles of strain C05T1 (PFGE type C, type IV SCC*mec*) using different stock concentrations of primer. Lanes labelled according to primer concentration. Gel A using primer 1254 and gel B using primer 797. N: negative control; MW: molecular weight ladder with size indicator (bp).

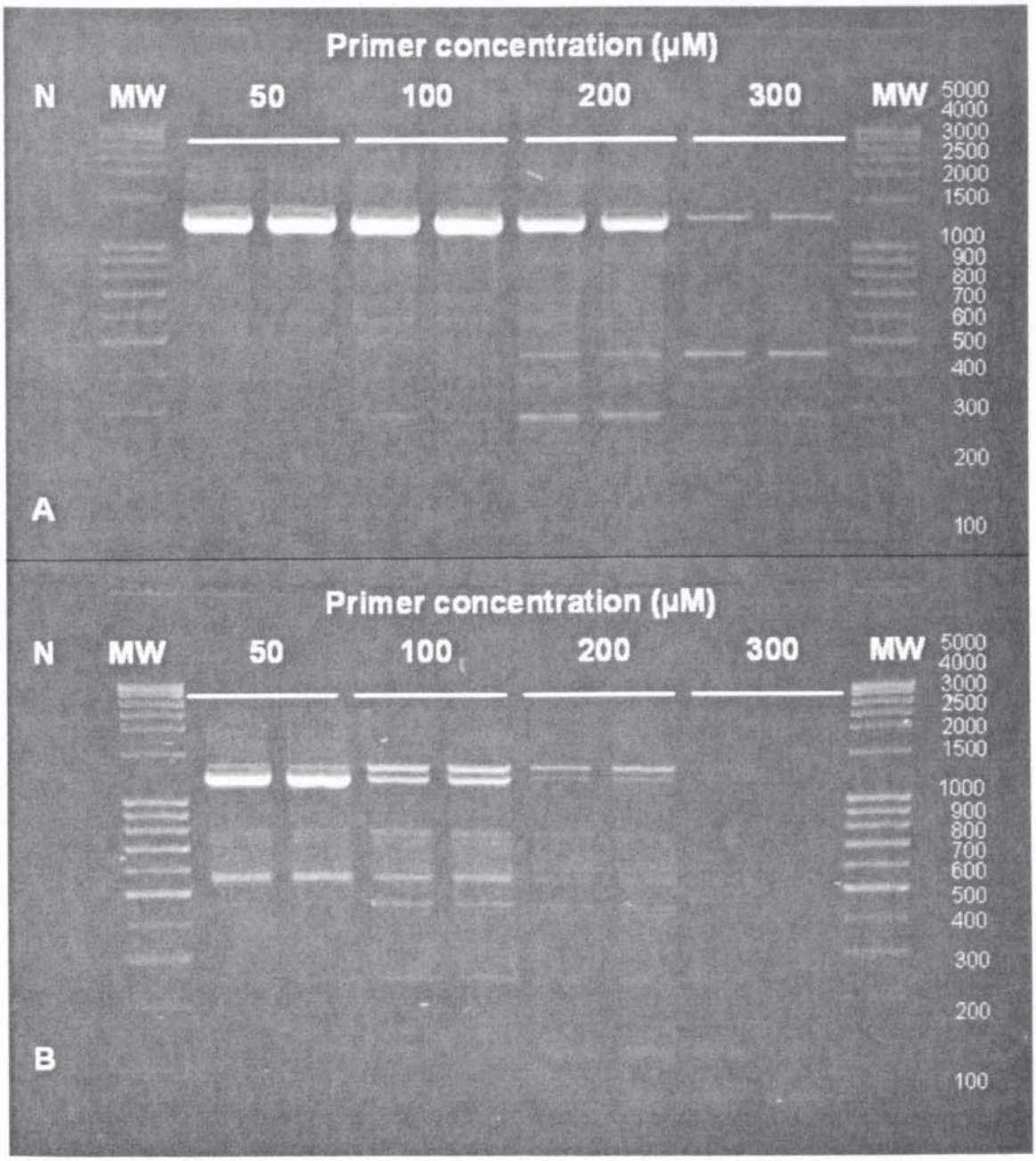


Figure 4.3.3 RAPD profiles of strain B07T2 (PFGE type F, SCC*mec* II) using different stock concentrations of primer. Lanes labelled according to primer concentration. Gel A using primer 1254 and gel B using primer 797. N: negative control; MW: molecular weight ladder with size indicator (bp).

4.3.4 Effect of dNTP concentration

Two separate RAPD reactions were optimised using primers 1254 and 797 at stock concentrations of 100 µM with buffer 3 and dNTPs at stock concentrations of 5, 10, 20 and 30mM. Profiles obtained by RAPD analysis from the chromosomal DNA of three HA-MRSA strains (C05T1, B07T2 and A04T1) using varying concentrations of

dNTPs are shown in figures 4.4.1, 4.4.2, and 4.4.3. The optimum stock concentration of dNTP for both RAPD reactions was 5mM. The profiles obtained using this concentration were stable and the number of amplicons in each profile remained constant. Concentrations of dNTP above 10mM visibly reduced the number of amplicons.

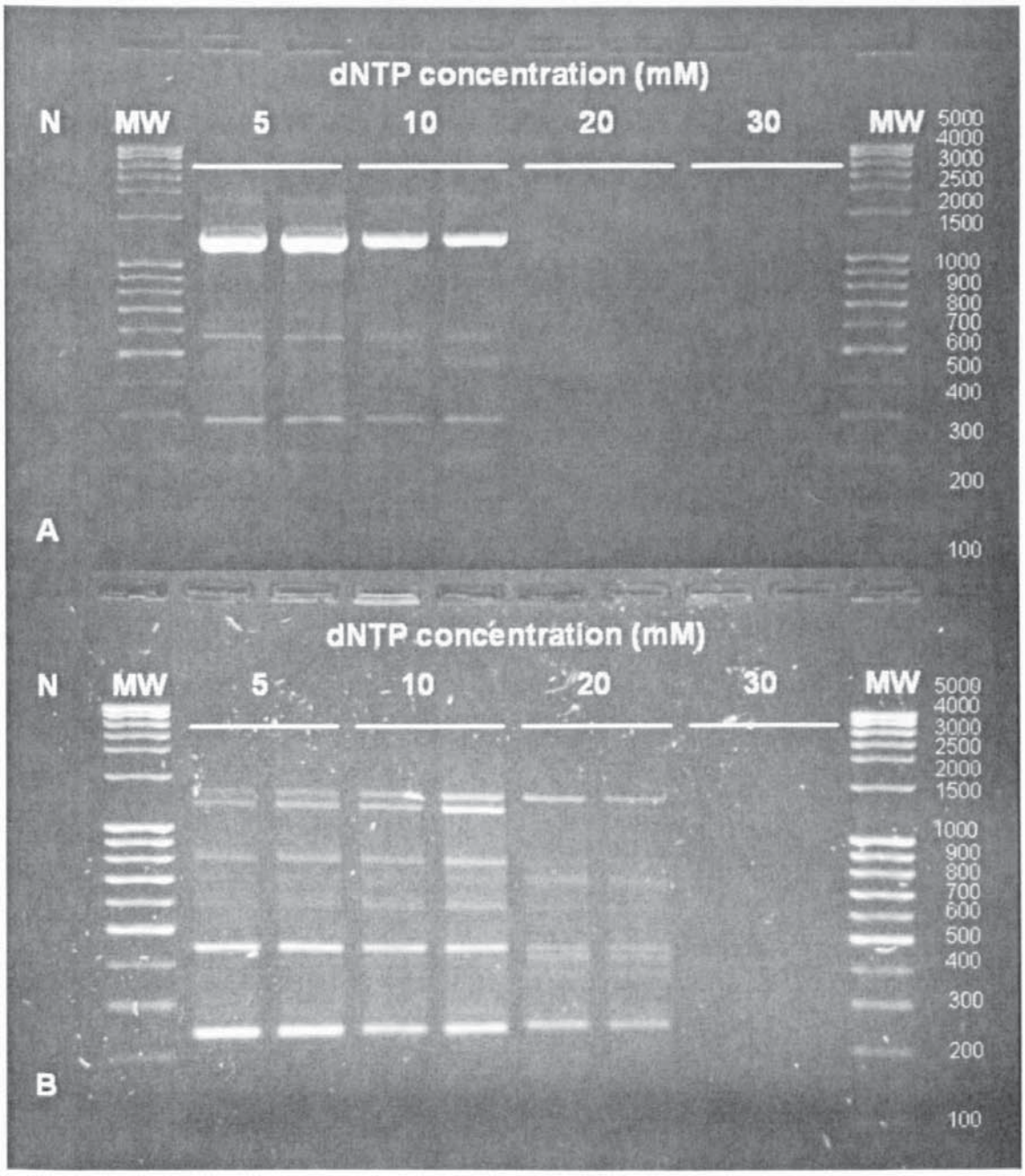


Figure 4.4.1 RAPD profiles of strain A04T1 (PFGE type G, type II SCC*mec*) using different stock concentrations of dNTP. Lanes labelled according to dNTP concentration. Gel A using primer 1254 and gel B using primer 797. N: negative control; MW: molecular weight ladder with size indicator (bp).

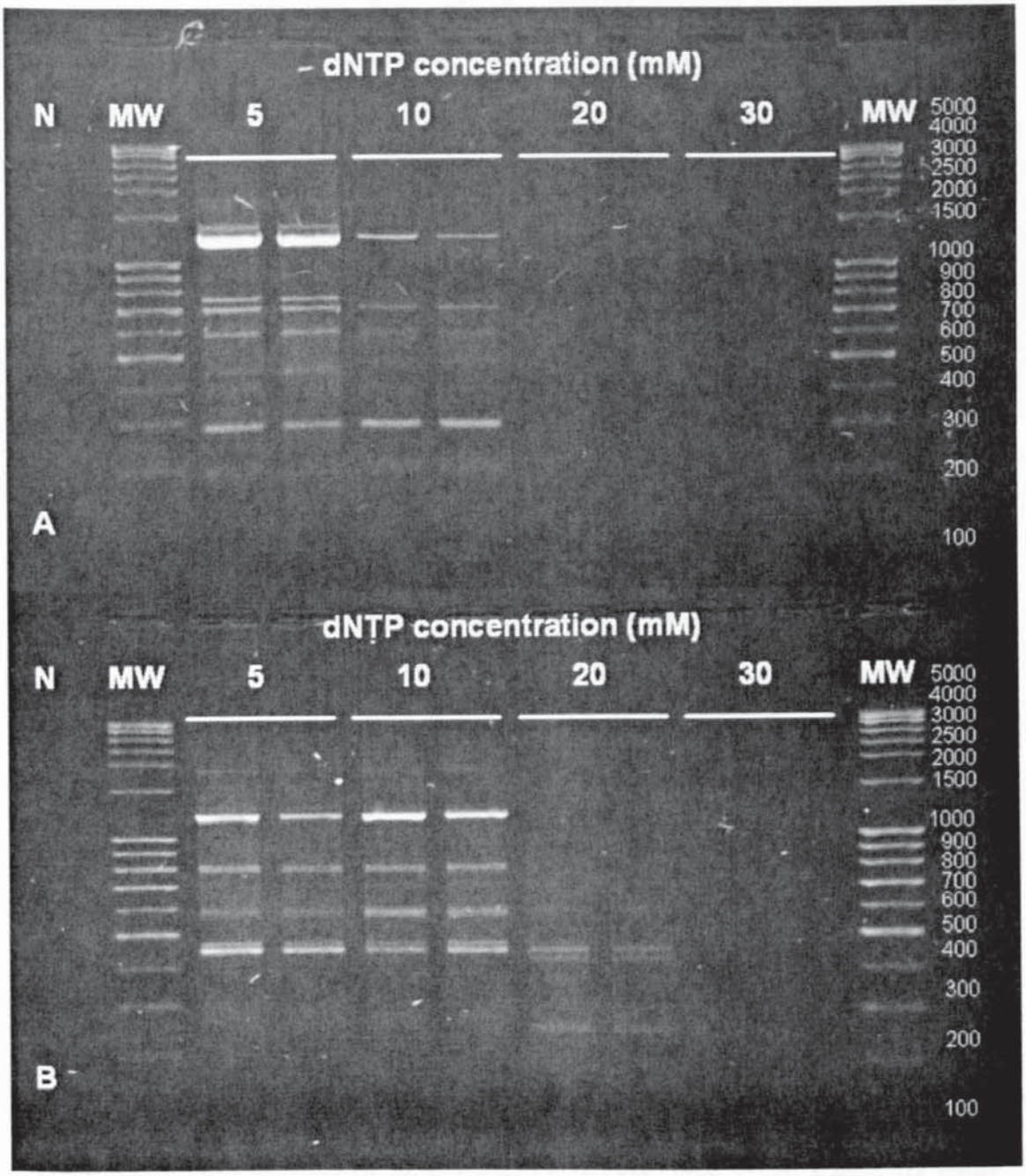


Figure 4.4.2 RAPD profiles of strain C05T1 (PFGE type C, type IV SCC*mec*) using different stock concentrations of dNTP. Lanes labelled according to dNTP concentration. Gel A using primer 1254 and gel B using primer 797. N: negative control; MW: molecular weight ladder with size indicator (bp).

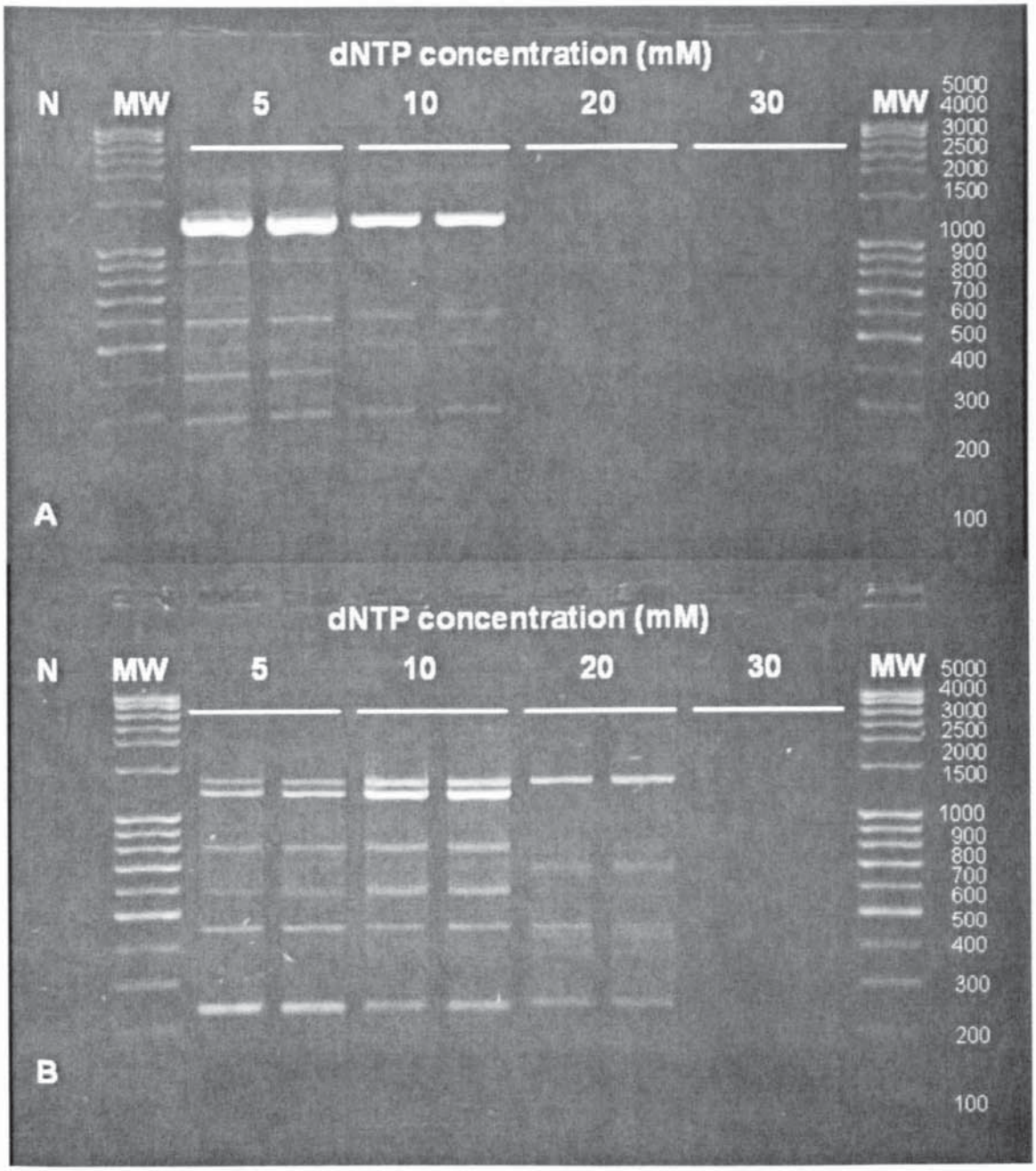


Figure 4.4.3 RAPD profiles of strain B07T2 (PFGE type F, SCC*mec* II) using different stock concentrations of dNTP. Lanes labelled according to dNTP concentration. Gel A using primer 1254 and gel B using primer 797. N: negative control; MW: molecular weight ladder with size indicator (bp).

4.3.4.1 Template DNA from boiled whole cells

Two separate optimised RAPD reactions (section 4.2.4) using primers 1254 and 797 at stock concentrations of 100μM and dNTPs at stock concentration of 5mM with buffer 3 were employed to determine the suitability of template DNA prepared using method 2 (section 4.2.3.3) in comparison to method 1 (section 4.2.3.1). Profiles generated by RAPD analysis from the chromosomal DNA of three HA-MRSA strains (C05T1, B07T2 and A04T1) using the two DNA extraction methods are shown in figure 4.5. The RAPD profiles obtained with template DNA prepared using method 2 were identical to those generated with template DNA prepared using method 1. The profiles obtained using method 2 were stable and the number of amplicons in each profile remained constant. Template DNA was prepared for using method 2 and strains were typed using the two optimised RAPD reactions.

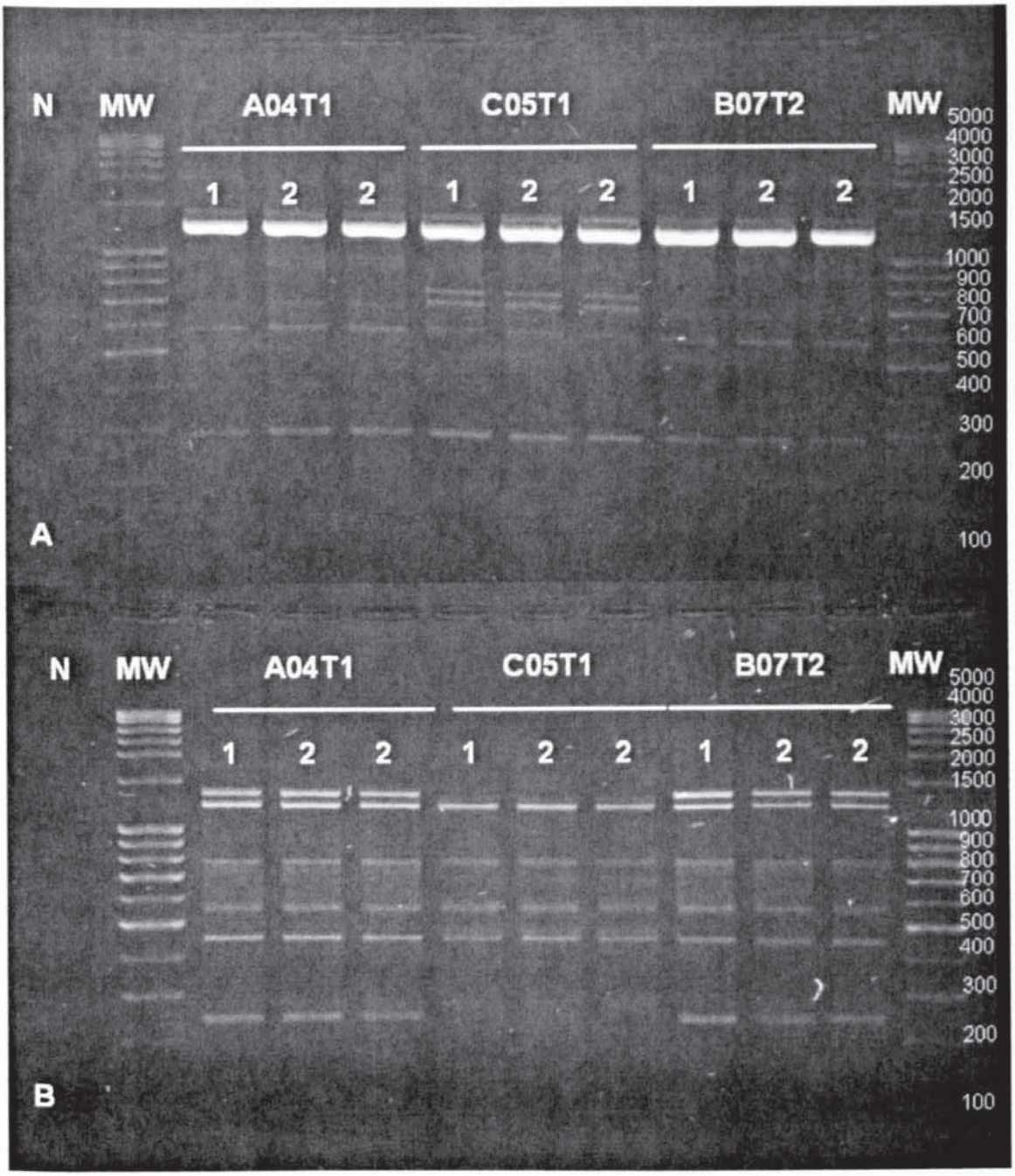


Figure 4.5 RAPD profiles of strains A04T1, C05T1 and B07T2 using template DNA prepared by two different methods. Lanes labelled according to position of strain. 1: method 1, phenol extraction and ethanol precipitation (section 4.2.3.1); 2: method 2, boiling of whole cells (section 4.2.3.3). Gel A using primer 1254 and gel B using primer 797. N: negative control; MW: molecular weight ladder with size indicator (bp).

4.3.5 RAPD intra- and inter-reproducibility

The strain A04T1 was used as a positive control to determine intra-reproducibility for every RAPD reaction. The RAPD reaction remained stable and reproducible for the duration of the study. Every month the strains C05T1, B07T2 and A04T1 underwent RAPD analysis in separate reactions to assess the stability of the profiles and to determine inter-reproducibility (figure 4.2.7).

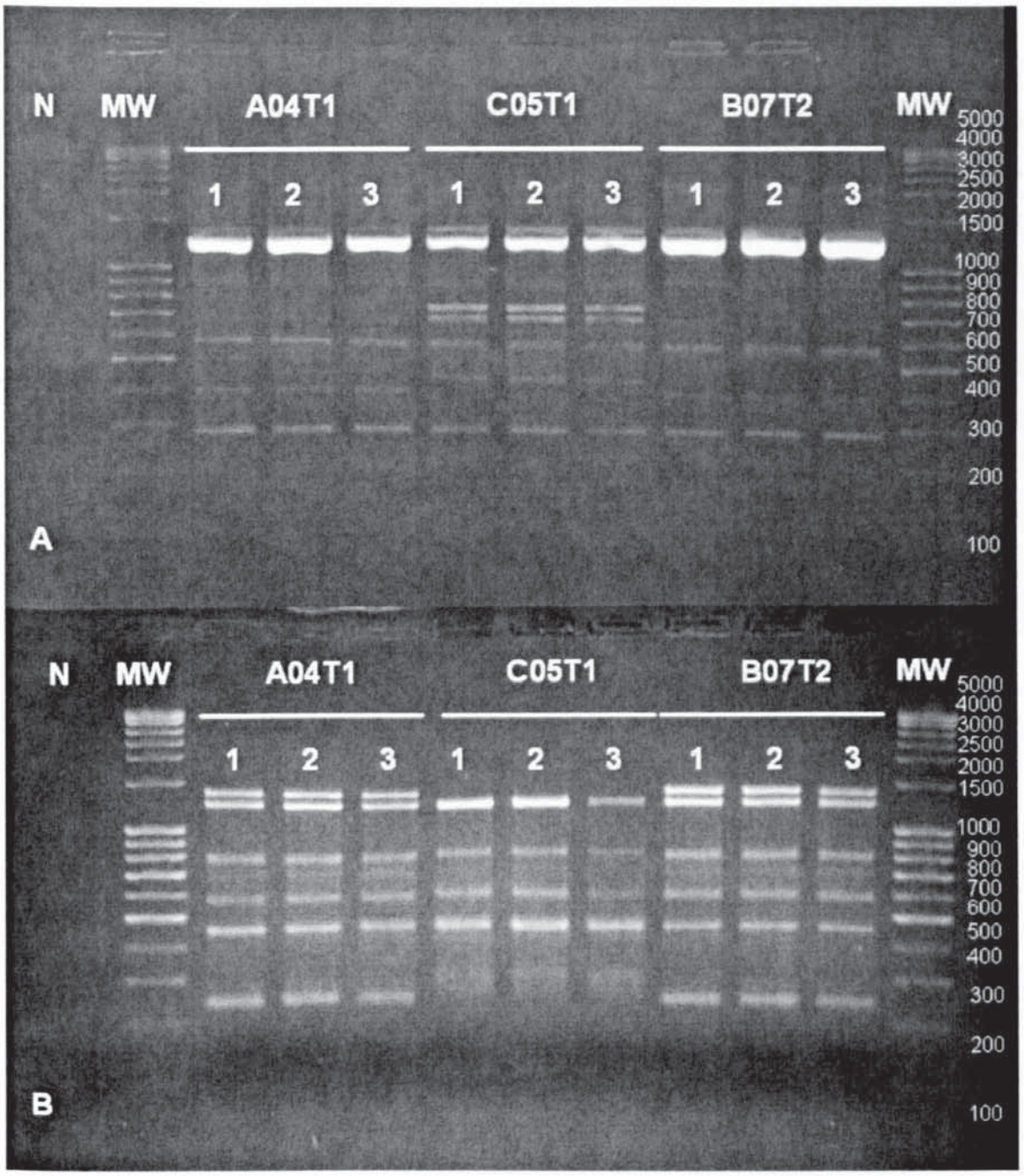
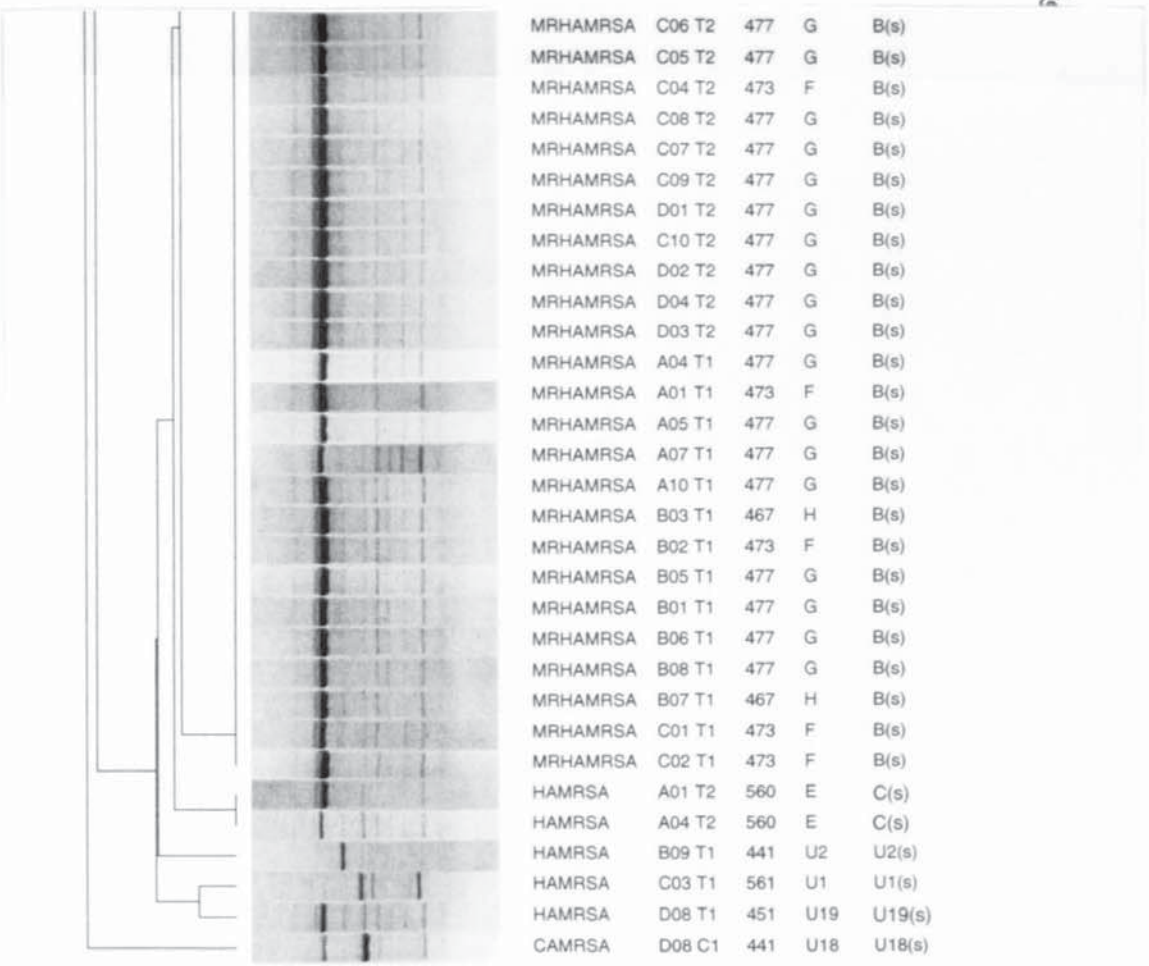


Figure 4.6 RAPD profiles of strains A04T1, C05T1 and B07T2. Lanes labelled according to the month RAPD analysis was performed. Gel A using primer 1254 and gel B using primer 797. N: negative control; MW: molecular weight ladder with size indicator (bp).

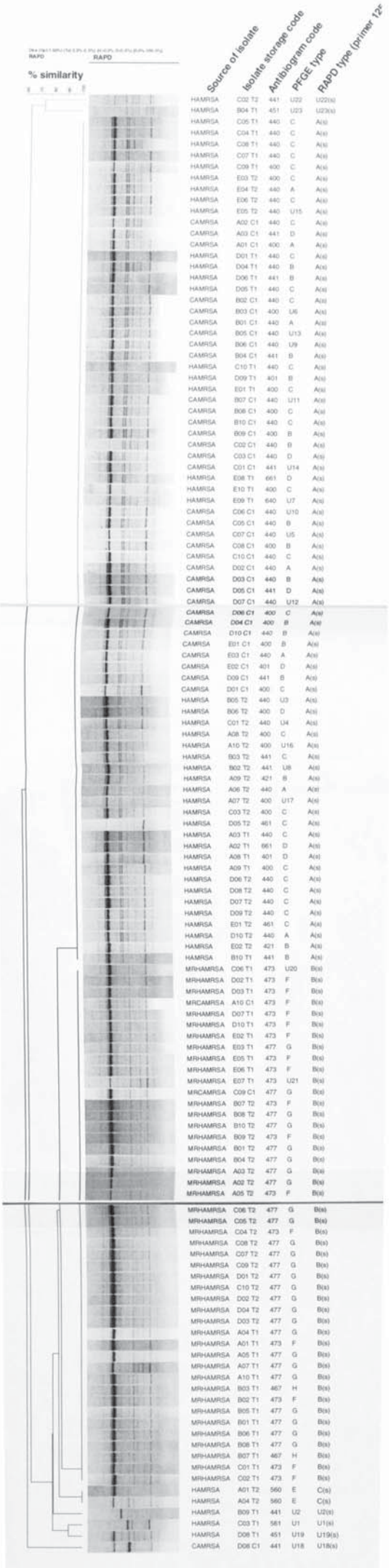
4.3.6 RAPD using primer 1254

Analysis of RAPD data revealed nine different RAPD profiles. Three RAPD clusters comprising of multiple isolates were designated types A(s)-C(s). Five HA-MRSA isolates, and one CA-MRSA isolate had unique profiles and were given the designation U(s) with the corresponding number allocated during PFGE analysis. The MR-HA-MRSA and MR-CA-MRSA isolates were separated into one cluster designated B(s). The NMR-HA-MRSA and NMR-CA-MRSA isolates were separated into two main clusters designated A and C with six unique types. A dendrogram of strains typed using RAPD employing primer 1254 is depicted in figure 4.7.



MRHAMRSA	C06 T2	477	G	B(s)
MRHAMRSA	C05 T2	477	G	B(s)
MRHAMRSA	C04 T2	473	F	B(s)
MRHAMRSA	C08 T2	477	G	B(s)
MRHAMRSA	C07 T2	477	G	B(s)
MRHAMRSA	C09 T2	477	G	B(s)
MRHAMRSA	D01 T2	477	G	B(s)
MRHAMRSA	C10 T2	477	G	B(s)
MRHAMRSA	D02 T2	477	G	B(s)
MRHAMRSA	D04 T2	477	G	B(s)
MRHAMRSA	D03 T2	477	G	B(s)
MRHAMRSA	A04 T1	477	G	B(s)
MRHAMRSA	A01 T1	473	F	B(s)
MRHAMRSA	A05 T1	477	G	B(s)
MRHAMRSA	A07 T1	477	G	B(s)
MRHAMRSA	A10 T1	477	G	B(s)
MRHAMRSA	B03 T1	467	H	B(s)
MRHAMRSA	B02 T1	473	F	B(s)
MRHAMRSA	B05 T1	477	G	B(s)
MRHAMRSA	B01 T1	477	G	B(s)
MRHAMRSA	B06 T1	477	G	B(s)
MRHAMRSA	B08 T1	477	G	B(s)
MRHAMRSA	B07 T1	467	H	B(s)
MRHAMRSA	C01 T1	473	F	B(s)
MRHAMRSA	C02 T1	473	F	B(s)
HAMRSA	A01 T2	560	E	C(s)
HAMRSA	A04 T2	560	E	C(s)
HAMRSA	B09 T1	441	U2	U2(s)
HAMRSA	C03 T1	561	U1	U1(s)
HAMRSA	D08 T1	451	U19	U19(s)
CAMRSA	D08 C1	441	U18	U18(s)

Figure 4.7 Dendrogram of 94 HA- and 36 CA-MRSA strains depicting RAPD profiles obtained using primer 1254. Similarity calculated by Dice coefficient (tolerance of 1% and optimisation of 0.5%) and represented by UPGMA clustering.



Source of isolate	Isolate storage code	Antibiogram code	PFGE type	RAPD type (primer 12)
HAMRSA	C02 T2	441	U22	U22(s)
HAMRSA	B04 T1	451	U23	U23(s)
HAMRSA	C05 T1	440	C	A(s)
HAMRSA	C04 T1	440	C	A(s)
HAMRSA	C08 T1	440	C	A(s)
HAMRSA	C07 T1	440	C	A(s)
HAMRSA	C09 T1	400	C	A(s)
HAMRSA	E03 T2	400	C	A(s)
HAMRSA	E04 T2	440	A	A(s)
HAMRSA	E06 T2	440	C	A(s)
HAMRSA	E05 T2	440	U15	A(s)
CAMRSA	A02 C1	440	C	A(s)
CAMRSA	A03 C1	441	D	A(s)
CAMRSA	A01 C1	400	A	A(s)
HAMRSA	D01 T1	440	C	A(s)
HAMRSA	D04 T1	440	B	A(s)
HAMRSA	D06 T1	441	B	A(s)
HAMRSA	D05 T1	440	C	A(s)
CAMRSA	B02 C1	440	C	A(s)
CAMRSA	B03 C1	400	U6	A(s)
CAMRSA	B01 C1	440	A	A(s)
CAMRSA	B05 C1	440	U13	A(s)
CAMRSA	B06 C1	440	U9	A(s)
CAMRSA	B04 C1	441	B	A(s)
HAMRSA	C10 T1	440	C	A(s)
HAMRSA	D09 T1	401	B	A(s)
HAMRSA	E01 T1	400	C	A(s)
CAMRSA	B07 C1	440	U11	A(s)
CAMRSA	B08 C1	400	C	A(s)
CAMRSA	B10 C1	440	C	A(s)
CAMRSA	B09 C1	400	B	A(s)
CAMRSA	C02 C1	440	B	A(s)
CAMRSA	C03 C1	440	D	A(s)
CAMRSA	C01 C1	441	U14	A(s)
HAMRSA	E08 T1	661	D	A(s)
HAMRSA	E10 T1	400	C	A(s)
HAMRSA	E09 T1	640	U7	A(s)
CAMRSA	C06 C1	440	U10	A(s)
CAMRSA	C05 C1	440	B	A(s)
CAMRSA	C07 C1	440	U5	A(s)
CAMRSA	C08 C1	400	B	A(s)
CAMRSA	C10 C1	440	C	A(s)
CAMRSA	D02 C1	440	A	A(s)
CAMRSA	**D03 C1**	**440**	**B**	**A(s)**
CAMRSA	**D05 C1**	**441**	**D**	**A(s)**
CAMRSA	D07 C1	440	U12	A(s)
CAMRSA	***D06 C1***	***400***	***C***	***A(s)***
CAMRSA	***D04 C1***	***400***	***B***	***A(s)***
CAMRSA	D10 C1	440	B	A(s)
CAMRSA	E01 C1	400	B	A(s)
CAMRSA	E03 C1	440	A	A(s)
CAMRSA	E02 C1	401	D	A(s)
CAMRSA	D09 C1	441	B	A(s)
CAMRSA	D01 C1	400	C	A(s)
HAMRSA	B05 T2	440	U3	A(s)
HAMRSA	B06 T2	400	D	A(s)
HAMRSA	C01 T2	440	U4	A(s)
HAMRSA	A08 T2	400	C	A(s)
HAMRSA	A10 T2	400	U16	A(s)
HAMRSA	B03 T2	441	C	A(s)
HAMRSA	B02 T2	441	U8	A(s)
HAMRSA	A09 T2	421	B	A(s)
HAMRSA	A06 T2	440	A	A(s)
HAMRSA	A07 T2	400	U17	A(s)
HAMRSA	C03 T2	400	C	A(s)
HAMRSA	D05 T2	461	C	A(s)
HAMRSA	A03 T1	440	C	A(s)
HAMRSA	A02 T1	661	D	A(s)
HAMRSA	A08 T1	401	D	A(s)
HAMRSA	A09 T1	400	C	A(s)
HAMRSA	D06 T2	440	C	A(s)
HAMRSA	D08 T2	440	C	A(s)
HAMRSA	D07 T2	440	C	A(s)
HAMRSA	D09 T2	440	C	A(s)
HAMRSA	E01 T2	461	C	A(s)
HAMRSA	D10 T2	440	A	A(s)
HAMRSA	E02 T2	421	B	A(s)
HAMRSA	B10 T1	441	B	A(s)
MRHAMRSA	C06 T1	473	U20	B(s)
MRHAMRSA	D02 T1	473	F	B(s)
MRHAMRSA	D03 T1	473	F	B(s)
MRCAMRSA	A10 C1	473	F	B(s)
MRHAMRSA	D07 T1	473	F	B(s)
MRHAMRSA	D10 T1	473	F	B(s)
MRHAMRSA	E02 T1	473	F	B(s)
MRHAMRSA	E03 T1	477	G	B(s)
MRHAMRSA	E05 T1	473	F	B(s)
MRHAMRSA	E06 T1	473	F	B(s)
MRHAMRSA	E07 T1	473	U21	B(s)
MRCAMRSA	C09 C1	477	G	B(s)
MRHAMRSA	B07 T2	473	F	B(s)
MRHAMRSA	B08 T2	477	G	B(s)
MRHAMRSA	B10 T2	477	G	B(s)
MRHAMRSA	B09 T2	473	F	B(s)
MRHAMRSA	B01 T2	477	G	B(s)
MRHAMRSA	B04 T2	477	G	B(s)
MRHAMRSA	A03 T2	477	G	B(s)
MRHAMRSA	**A02 T2**	**477**	**G**	**B(s)**
MRHAMRSA	**A05 T2**	**473**	**F**	**B(s)**
MRHAMRSA	**C06 T2**	**477**	**G**	**B(s)**
MRHAMRSA	**C05 T2**	**477**	**G**	**B(s)**
MRHAMRSA	C04 T2	473	F	B(s)
MRHAMRSA	C08 T2	477	G	B(s)
MRHAMRSA	C07 T2	477	G	B(s)
MRHAMRSA	C09 T2	477	G	B(s)
MRHAMRSA	D01 T2	477	G	B(s)
MRHAMRSA	C10 T2	477	G	B(s)
MRHAMRSA	D02 T2	477	G	B(s)
MRHAMRSA	D04 T2	477	G	B(s)
MRHAMRSA	D03 T2	477	G	B(s)
MRHAMRSA	A04 T1	477	G	B(s)
MRHAMRSA	A01 T1	473	F	B(s)
MRHAMRSA	A05 T1	477	G	B(s)
MRHAMRSA	A07 T1	477	G	B(s)
MRHAMRSA	A10 T1	477	G	B(s)
MRHAMRSA	B03 T1	467	H	B(s)
MRHAMRSA	B02 T1	473	F	B(s)
MRHAMRSA	B05 T1	477	G	B(s)
MRHAMRSA	B01 T1	477	G	B(s)
MRHAMRSA	B06 T1	477	G	B(s)
MRHAMRSA	B08 T1	477	G	B(s)
MRHAMRSA	B07 T1	467	H	B(s)
MRHAMRSA	C01 T1	473	F	B(s)
MRHAMRSA	C02 T1	473	F	B(s)
HAMRSA	A01 T2	560	E	C(s)
HAMRSA	A04 T2	560	E	C(s)
HAMRSA	B09 T1	441	U2	U2(s)
HAMRSA	C03 T1	561	U1	U1(s)
HAMRSA	D08 T1	451	U19	U19(s)
CAMRSA	D08 C1	441	U18	U18(s)

4.3.7 RAPD using primer 797

Analysis of RAPD data revealed 12 different RAPD profiles. Three RAPD clusters comprising of multiple isolates were designated types A(l)-C(l). One MR-HA-MRSA isolate, six HA-MRSA isolates, and two CA-MRSA isolates had unique profiles and were given the designation U-(l) with the corresponding number allocated during PFGE analysis. The MR-HA-MRSA and MR-CA-MRSA isolates were separated into one cluster designated B(l) with one unique type. The NMR-HA-MRSA and NMR-CA-MRSA isolates were separated into two main clusters designated A(l) and C(l) with 11 unique types. A dendrogram of strains typed using RAPD employing primer 797 is depicted in figure 4.8.

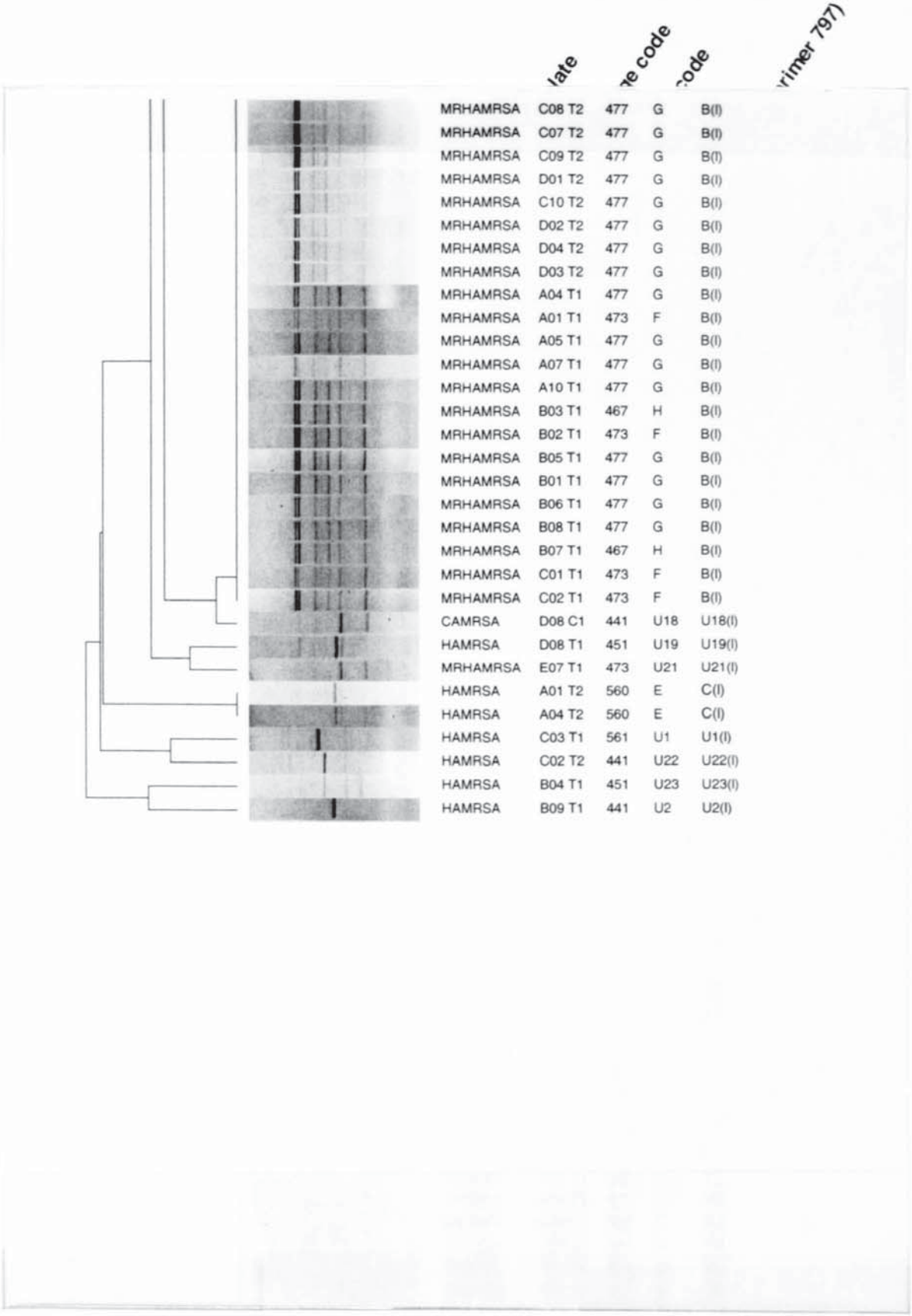
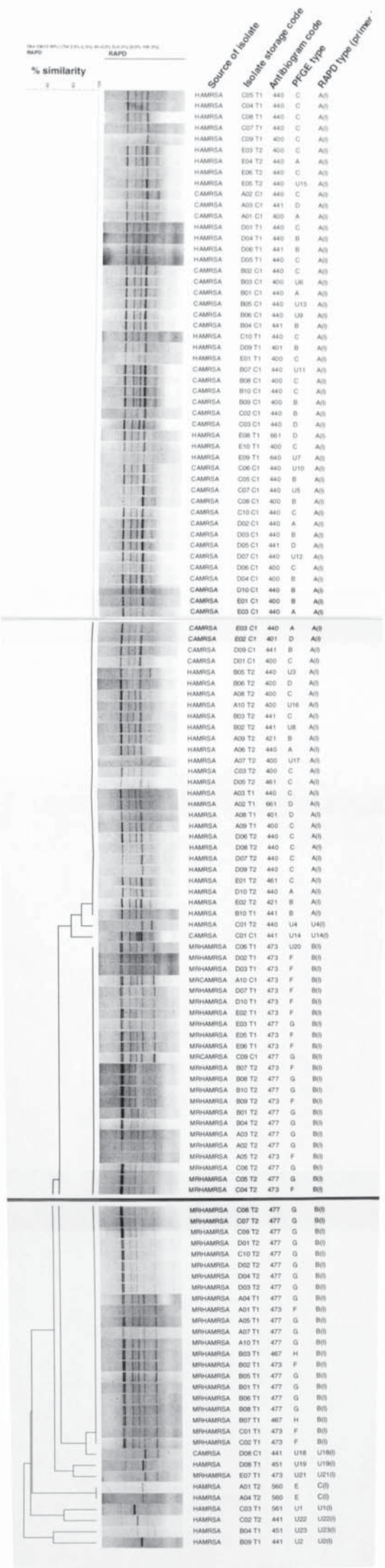


Figure 4.8 Dendrogram of 94 HA- and 36 CA-MRSA strains depicting RAPD profiles obtained using primer 797. Similarity calculated by Dice coefficient (tolerance of 1% and optimisation of 0.5%) and represented by UPGMA clustering.



Source of isolate	Isolate storage code	Antibiogram code	PFGE type	RAPD type (primer
HAMRSA	C05 T1	440	C	A(i)
HAMRSA	C04 T1	440	C	A(i)
HAMRSA	C08 T1	440	C	A(i)
HAMRSA	C07 T1	440	C	A(i)
HAMRSA	C09 T1	400	C	A(i)
HAMRSA	E03 T2	400	C	A(i)
HAMRSA	E04 T2	440	A	A(i)
HAMRSA	E06 T2	440	C	A(i)
HAMRSA	E05 T2	440	U15	A(i)
CAMRSA	A02 C1	440	C	A(i)
CAMRSA	A03 C1	441	D	A(i)
CAMRSA	A01 C1	400	A	A(i)
HAMRSA	D01 T1	440	C	A(i)
HAMRSA	D04 T1	440	B	A(i)
HAMRSA	D06 T1	441	B	A(i)
HAMRSA	D05 T1	440	C	A(i)
CAMRSA	B02 C1	440	C	A(i)
CAMRSA	B03 C1	400	U6	A(i)
CAMRSA	B01 C1	440	A	A(i)
CAMRSA	B05 C1	440	U13	A(i)
CAMRSA	B06 C1	440	U9	A(i)
CAMRSA	B04 C1	441	B	A(i)
HAMRSA	C10 T1	440	C	A(i)
HAMRSA	D09 T1	401	B	A(i)
HAMRSA	E01 T1	400	C	A(i)
CAMRSA	B07 C1	440	U11	A(i)
CAMRSA	B08 C1	400	C	A(i)
CAMRSA	B10 C1	440	C	A(i)
CAMRSA	B09 C1	400	B	A(i)
CAMRSA	C02 C1	440	B	A(i)
CAMRSA	C03 C1	440	D	A(i)
HAMRSA	E08 T1	661	D	A(i)
HAMRSA	E10 T1	400	C	A(i)
HAMRSA	E09 T1	640	U7	A(i)
CAMRSA	C06 C1	440	U10	A(i)
CAMRSA	C05 C1	440	B	A(i)
CAMRSA	C07 C1	440	U5	A(i)
CAMRSA	C08 C1	400	B	A(i)
CAMRSA	C10 C1	440	C	A(i)
CAMRSA	D02 C1	440	A	A(i)
CAMRSA	D03 C1	440	B	A(i)
CAMRSA	D05 C1	441	D	A(i)
CAMRSA	D07 C1	440	U12	A(i)
CAMRSA	D06 C1	400	C	A(i)
CAMRSA	D04 C1	400	B	A(i)
CAMRSA	**D10 C1**	**440**	**B**	**A(i)**
CAMRSA	**E01 C1**	**400**	**B**	**A(i)**
CAMRSA	**E03 C1**	**440**	**A**	**A(i)**
CAMRSA	***E03 C1***	***440***	***A***	***A(i)***
CAMRSA	***E02 C1***	***401***	***D***	***A(i)***
CAMRSA	D09 C1	441	B	A(i)
CAMRSA	D01 C1	400	C	A(i)
HAMRSA	B05 T2	440	U3	A(i)
HAMRSA	B06 T2	400	D	A(i)
HAMRSA	A08 T2	400	C	A(i)
HAMRSA	A10 T2	400	U16	A(i)
HAMRSA	B03 T2	441	C	A(i)
HAMRSA	B02 T2	441	U8	A(i)
HAMRSA	A09 T2	421	B	A(i)
HAMRSA	A06 T2	440	A	A(i)
HAMRSA	A07 T2	400	U17	A(i)
HAMRSA	C03 T2	400	C	A(i)
HAMRSA	D05 T2	461	C	A(i)
HAMRSA	A03 T1	440	C	A(i)
HAMRSA	A02 T1	661	D	A(i)
HAMRSA	A08 T1	401	D	A(i)
HAMRSA	A09 T1	400	C	A(i)
HAMRSA	D06 T2	440	C	A(i)
HAMRSA	D08 T2	440	C	A(i)
HAMRSA	D07 T2	440	C	A(i)
HAMRSA	D09 T2	440	C	A(i)
HAMRSA	E01 T2	461	C	A(i)
HAMRSA	D10 T2	440	A	A(i)
HAMRSA	E02 T2	421	B	A(i)
HAMRSA	B10 T1	441	B	A(i)
HAMRSA	C01 T2	440	U4	U4(i)
CAMRSA	C01 C1	441	U14	U14(i)
MRHAMRSA	C06 T1	473	U20	B(i)
MRHAMRSA	D02 T1	473	F	B(i)
MRHAMRSA	D03 T1	473	F	B(i)
MRCAMRSA	A10 C1	473	F	B(i)
MRHAMRSA	D07 T1	473	F	B(i)
MRHAMRSA	D10 T1	473	F	B(i)
MRHAMRSA	E02 T1	473	F	B(i)
MRHAMRSA	E03 T1	477	G	B(i)
MRHAMRSA	E05 T1	473	F	B(i)
MRHAMRSA	E06 T1	473	F	B(i)
MRCAMRSA	C09 C1	477	G	B(i)
MRHAMRSA	B07 T2	473	F	B(i)
MRHAMRSA	B08 T2	477	G	B(i)
MRHAMRSA	B10 T2	477	G	B(i)
MRHAMRSA	B09 T2	473	F	B(i)
MRHAMRSA	B01 T2	477	G	B(i)
MRHAMRSA	B04 T2	477	G	B(i)
MRHAMRSA	A03 T2	477	G	B(i)
MRHAMRSA	A02 T2	477	G	B(i)
MRHAMRSA	A05 T2	473	F	B(i)
MRHAMRSA	C06 T2	477	G	B(i)
MRHAMRSA	**C05 T2**	**477**	**G**	**B(i)**
MRHAMRSA	**C04 T2**	**473**	**F**	**B(i)**
MRHAMRSA	**C08 T2**	**477**	**G**	**B(i)**
MRHAMRSA	**C07 T2**	**477**	**G**	**B(i)**
MRHAMRSA	C09 T2	477	G	B(i)
MRHAMRSA	D01 T2	477	G	B(i)
MRHAMRSA	C10 T2	477	G	B(i)
MRHAMRSA	D02 T2	477	G	B(i)
MRHAMRSA	D04 T2	477	G	B(i)
MRHAMRSA	D03 T2	477	G	B(i)
MRHAMRSA	A04 T1	477	G	B(i)
MRHAMRSA	A01 T1	473	F	B(i)
MRHAMRSA	A05 T1	477	G	B(i)
MRHAMRSA	A07 T1	477	G	B(i)
MRHAMRSA	A10 T1	477	G	B(i)
MRHAMRSA	B03 T1	467	H	B(i)
MRHAMRSA	B02 T1	473	F	B(i)
MRHAMRSA	B05 T1	477	G	B(i)
MRHAMRSA	B01 T1	477	G	B(i)
MRHAMRSA	B06 T1	477	G	B(i)
MRHAMRSA	B08 T1	477	G	B(i)
MRHAMRSA	B07 T1	467	H	B(i)
MRHAMRSA	C01 T1	473	F	B(i)
MRHAMRSA	C02 T1	473	F	B(i)
CAMRSA	D08 C1	441	U18	U18(i)
HAMRSA	D08 T1	451	U19	U19(i)
MRHAMRSA	E07 T1	473	U21	U21(i)
HAMRSA	A01 T2	560	E	C(i)
HAMRSA	A04 T2	560	E	C(i)
HAMRSA	C03 T1	561	U1	U1(i)
HAMRSA	C02 T2	441	U22	U22(i)
HAMRSA	B04 T1	451	U23	U23(i)
HAMRSA	B09 T1	441	U2	U2(i)

4.3.8 Typeability and discriminatory power of PFGE and the optimised RAPD method

All of the isolates were typeable using PFGE and both RAPD reactions. The discriminatory power of PFGE and RAPD is compared in table 4.3.

Table 4.3 Discrimination indices of PFGE and RAPD calculated after typing 130 MRSA isolates

Typing method	Number of types	Type	[a]Number of strains		Discriminatory index (D=)
PFGE	31	A	7	(**3**, *4*)	0.88
		B	16	(**6**, *10*)	
		C	30	(**23**, *7*)	
		D	8	(**4**, *4*)	
		E	2	(**2**)	
		F	16	(**15**, *1*)	
		G	26	(**25**, *1*)	
		H	2	(**2**)	
		U	23	(**14**, *9*)	
RAPD (1254)	9	A(s)	76	(**43**, *33*)	0.54
		B(s)	46	(**44**, *2*)	
		C(s)	2	(**2**)	
		U(s)	6	(**5**, 1)	
RAPD (797)	12	A(l)	74	(**42**, *32*)	0.56
		B(l)	45	(**43**, *2*)	
		C(l)	2	(**2**)	
		U(l)	9	(**7**, *2*)	

[a]Total (Bold text indicates hospital acquired and italicised text indicates community-acquired isolates).

4.3.9 Summary of results

A summary of results is described in table 4.4. All MR-MRSA strains belonged to two antibiogram types that could be differentiated by PFGE into five types and by RAPD analysis into two types. All MR-MRSA with the exception of two strains were hospital acquired. Only the PFGE types belonging to the MR-MRSA strains correlated

well with the antibiogram types. All CA-MRSA with the exception of nine strains belonged to PFGE types also observed for the HA-MRSA.

Table 4.4 Summary of all antibiogram types, PFGE types and RAPD types using primers 1254 and 797 for 130 clinical MRSA strains.

[a]MR	[b]Code	PFGE type	RAPD type 1254	RAPD type 797	[c]Number. of isolates	
-	400	A	A(s)	A(l)	1	(*1*)
-	400	B	A(s)	A(l)	4	(*4*)
-	400	C	A(s)	A(l)	10	(**7**, *3*)
-	400	D	A(s)	A(l)	1	(**1**)
-	400	U6	A(s)	A(l)	1	(*1*)
-	400	U16	A(s)	A(l)	1	(**1**)
-	400	U17	A(s)	A(l)	1	(**1**)
-	401	B	A(s)	A(l)	1	(**1**)
-	401	D	A(s)	A(l)	2	(**1**, *1*)
-	421	B	A(s)	A(l)	2	(**2**)
-	440	U3	A(s)	A(l)	1	(**1**)
-	440	U4	A(s)	U4(l)	1	(**1**)
-	440	A	A(s)	A(l)	6	(**3**, *3*)
-	440	B	A(s)	A(l)	5	(**1**, *4*)
-	440	C	A(s)	A(l)	18	(**14**, *4*)
-	440	D	A(s)	A(l)	1	(*1*)
-	440	U5	A(s)	A(l)	1	(*1*)
-	440	U9	A(s)	A(l)	1	(*1*)
-	440	U10	A(s)	A(l)	1	(*1*)
-	440	U11	A(s)	A(l)	1	(*1*)
-	440	U12	A(s)	A(l)	1	(*1*)
-	440	U13	A(s)	A(l)	1	(*1*)
-	440	U15	A(s)	A(l)	1	(**1**)
-	441	U2	U2(s)	U2(l)	1	(**1**)
-	441	B	A(s)	A(l)	4	(**2**, *2*)
-	441	D	A(s)	A(l)	2	(**2**)
-	441	U8	A(s)	A(l)	1	(**1**)
-	441	U14	A(s)	U14(l)	1	(*1*)
-	441	U18	U18(s)	U18(l)	1	(*1*)
-	441	U22	U22(s)	U22(l)	1	(**1**)
-	451	U19	U19(s)	U19(l)	1	(**1**)
-	451	U23	U23(s)	U23(l)	1	(**1**)
-	461	C	A(s)	A(l)	2	(**2**)
-	560	E	C(s)	C(l)	2	(**2**)
-	561	U1	U1(s)	U1(l)	1	(**1**)
-	640	U7	A(s)	A(l)	1	(**1**)
-	661	D	A(s)	A(l)	2	(**2**)
MR	6473	U20	B(s)	B(l)	1	(**1**)
MR	6473	U21	B(s)	U21(l)	1	(**1**)
MR	6473	F	B(s)	B(l)	16	(**15**, *1*)
MR	6477	G	B(s)	B(l)	26	(**25**, *1*)
MR	6477	H	B(s)	B(l)	2	(**2**)

[a]MR designates multidrug-resistance. (Isolates resistant to 6 or more antibiotics).
[b]Antibiogram code see table 3.4.
[c]Total (Bold text indicates hospital acquired and italicised text indicates community-acquired isolates).

4.4 Discussion

The aim of this chapter was to characterise MRSA strains using PFGE, the gold standard typing technique, and to determine whether it was possible to distinguish MR-MRSA and CA-MRSA from other MRSA strains. In addition, two independent RAPD reactions were optimised and compared with PFGE to determine its ability to differentiate MR-MRSA and CA-MRSA from other MRSA strains and for its potential in the management infection control and directing therapy.

4.4.1 Interpretation of banding patterns

Guidelines for the interpretation of restriction patterns produced by PFGE have been published which detail the criteria that may be used to differentiate bacterial strains of the same species (Tenover *et al.*, 1995). Typically, these criteria are used to determine whether an isolate is related to an outbreak strain within a brief period of time from the onset of the outbreak i.e. one month, however, these guidelines are also suitable for the general discussion of strain relationships irrespective of time. To interpret and compare DNA fragment patterns generated by PFGE it is necessary to understand the random genetic events that may alter these patterns. A single band difference between two strain-types is likely to arise from one of two possible genetic events: i) loss or gain of a restriction site due to a point mutation; ii) insertion or deletion of DNA between two restriction sites. Multiple band differences between two strain-types are likely to arise from multiple genetic events and therefore the strains may be considered less closely related. Strains that were once genotypically indistinguishable are more likely to demonstrate a greater number of differences in their PFGE banding patterns after an extended period of time due to natural genetic divergence. During this discussion the following criteria, adapted from (Tenover *et al.*, 1995), will be used to interpret banding patterns generated by PFGE:

- Strains that have the same number of bands and corresponding bands of the same apparent size are considered indistinguishable.
- Strains that differ by one genetic difference, typically two or three band differences, may be considered closely related.

- Strains that differ by two genetic differences, typically four to six band differences, may be considered possibly related.
- Strains that differ by three or more genetic differences, typically seven or more band differences, may be considered unrelated.

4.4.2 Analysis of PFGE types

Molecular typing using PFGE revealed that there were 31 genotypes among 130 MRSA isolates from the UHB and South Birmingham area. It was not possible to distinguish CA-MRSA strains from HA-MRSA strains, however, it was possible to distinguish MR-MRSA strains from the other NMR-MRSA strains. All MR-MRSA strains belonged to one of five PFGE types that were considered closely related. All comparisons of strain relatedness based on percentage similarity are approximate and calculated using the "% similarity" measurement indicated in figure 4.1.

The majority of MR-MRSA strains belonged to two main PFGE types designated F (n=16) and G (n=26) that were 94% related. Two MR-MRSA strains were of a type designated H and two strains had unique profile types and were designated U20 and U21. All of the MR-MRSA strains were 92% related. All but two of the MR-MRSA strains were hospital acquired. It is possible, based on the observation that the MR-MRSA strains are all closely related and predominantly isolated within the UHB that they may be epidemiologically related and potentially adapted to the hospital environment. Further molecular analysis discussed during chapter 5 supports these observations.

The NMR-MRSA strains belonged to one of five main PFGE types designated A-E or were one of the 21 unique profile types, designated U1-U19, U22 and U23. The MRSA strains belonging to PFGE types A-D and U3-U17 were 80% related; profiles differed by 1-4 bands and were designated closely related or possibly related. The NMR-MRSA strains of PFGE types, A-D and U3-U17, were 50% related to the MR-MRSA strains of PFGE types, F-H, U20 and U21. The NMR-MRSA strains of PFGE types U1, U2, U18, U19, U22, U23 and E were the most diverse strains differing by 4-5 bands and were designated possibly related to the other two major groups of strains.

Comparison of the NMR-MRSA strains of PFGE types A-D and U3-U17 with the NMR-MRSA strains of PFGE types (U1 and U2) and U18 indicated a relationship of 52% and 70% respectively. Comparison of the MR-MRSA strains of PFGE types F-H, U20 and U21 with the NMR-MRSA strains of PFGE types (E and U19) and (U22 and U23) indicated a relationship of 65% and 60% respectively. All 130 strains were 50% related when typing was performed using PFGE. For the purposes of this discussion the PFGE types are designated to the following groups; group 1 (A-D and U3-U17); group 2 (F-H, U20 and U21); and group 3 (U1, U2, U18, U19, U22, U23 and E).

4.4.3 RAPD optimisation

One of the main criticisms of PFGE is that it is a time consuming technique. A number of groups have reported protocols that may reduce the time it takes to obtain genotypic results using PFGE (Matushek *et al.*, 1996; Leonard and Carroll, 1997), however, when these methods were examined it was only possible to reduce the time taken to prepare bacterial DNA for macrorestriction digest. It was therefore not possible to reduce the limiting factor of PFGE; the electrophoresis time of approximately 20 hours. One group have published a method for replacing band size determination by electrophoresis with high-sensitivity flow cytometry (Ferris *et al.*, 2004), however, this approach requires expensive peripheral equipment that is not widely available in clinical laboratories. The fact that PFGE is time consuming and it is not possible to significantly reduce the time the technique takes to perform ultimately lead to the optimisation of an RAPD typing system that may be suitable for managing infection control and directing appropriate therapy in a clinically relevant time scale.

The RAPD typing system was optimised to give reproducible and discriminatory profiles using a method described by (Hopkins and Hilton, 2001a). Several primers were tested with a series of buffers during optimisation to determine the quality and number of amplicons in the different reactions. Primers that possess different sequences may generate amplicons from different parts of the genome and therefore it is important to evaluate the performance of more than one primer for RAPD analysis (Hopkins and Hilton, 2001a). The primers tested were selected from the literature based on the success of RAPD typing (Akopyanz *et al.*, 1992; Tambic *et al.*, 1997;

Dautle *et al.*, 2002; Pereira *et al.*, 2002) and percentage G+C content and length as this has been demonstrated to affect RAPD (Caetano-Anolles *et al.*, 1992; Caetano-Anolles, 1993). Using more than one primer for RAPD analysis of clonal microorganisms, such as MRSA, may not offer additional discriminatory power (Hopkins and Hilton, 2001), however, other groups recommend the use of two independent primers (Louie *et al.*, 1996), therefore two primers, 1254 and 797, were chosen for further optimisation. This proved to be useful as primer 797 exceeded the discriminatory ability of primer 1254.

A series of buffers were tested during optimisation that varied in $MgCl_2$ concentration, KCl concentration and pH. Variations in $MgCl_2$ concentration have been reported to alter RAPD profiles (Ellsworth *et al.*, 1993), which was observed during optimisation. Varying $MgCl_2$ concentration is thought to affect the stability between primer-template interactions, to affect denaturation of template DNA and to affect the activity and fidelity of *Taq* DNA polymerase (Schoettlin *et al.*, 1994). Excessive free Mg^{2+} may bind with *Taq* DNA polymerase and dNTPs and may cause accumulation of non-specific amplicons (Saiki, 1989). In contrast insufficient Mg^{2+} may reduce amplicon yield due to unstable annealing between primers and template DNA. Other salts, such as KCl may also affect primer annealing and *Taq* DNA polymerase activity. These factors can greatly affect the quality and reproducibility of amplification (Saiki, 1989). Varying pH has been shown to affect RAPD and may also help stabilise primer and template DNA interactions (Schoettlin *et al.*, 1994). High $MgCl_2$ concentration (3.5mM), low KCl concentration (25mM) and low pH (pH 8.3) were found to support the generation of a satisfactory number of high-quality amplicons with both primers, therefore buffer 3 was selected for both independent RAPD reactions.

Primer and dNTP concentration and batch-to-batch variability coupled with DNA template concentration have been identified as factors that may affect RAPD reproducibility (Tyler *et al.*, 1997), therefore, during optimisation of the RAPD reaction a single batch of dNTPs and each primer were used. Primer concentration has been identified as a very sensitive factor for reproducibility. High concentrations may cause mispriming and frequently produce greater non-specific amplicons (du Toit *et al.*, 1993). Low concentrations may reduce the number and intensity of bands and may result in no amplification or low product yields (du Toit *et al.*, 1993). In addition, the

ratio of primer to template DNA concentration is considered critical (Hadrys *et al.*, 1992), therefore, during optimisation template DNA concentrations remained constant and primer concentrations were titrated. The RAPD profiles generated by doing this provided an optimal number of high intensity amplicons and therefore titration of template DNA concentration was not performed. A number of additional factors that may affect the reproducibility of RAPD are template DNA prepared using whole cell extracts and the presence of plasmid DNA (Tyler *et al.*, 1997). Using DNA template prepared from whole cell extracts did not affect reproducibility during optimisation and results were comparable with pure template DNA. Other groups have reported similar success (Mazurier *et al.*, 1992; Menard *et al.*, 1992; Mazurier and Wernars, 1992a; Lawrence *et al.*, 1993). In addition, template DNA from a whole cell extract may be prepared in less time than other methods providing the opportunity for the results of RAPD analysis to be obtained within several hours. The presence of plasmid DNA with chromosomal DNA template from whole cell extracts was not found to affect reproducibility of the RAPD, which has also been reported by other groups (Elaichouni *et al.*, 1994). The same brand of *Taq* DNA polymerase and thermocycler with the same cycle conditions were used during optimisation and screening as these are also factors that may affect RAPD reproducibility (Tyler *et al.*, 1997).

4.4.4 Analysis of RAPD types

No guidelines for the interpretation of RAPD profiles have been published to date and therefore similar criteria for the interpretation of PFGE profiles were employed. The RAPD analysis identifies loss and gain of annealing sites rather than the loss and gain of restriction sites or the insertion and deletion of DNA between restriction sites. The same principal regarding the number of band differences between strains may also be employed as strains that are more closely related will have RAPD profiles that are less divergent.

During this discussion RAPD types have been designated a letter code A-C that refers to the position of the groups of strains in the dendrogram in descending order. Unique strains identified using RAPD were allocated the letter U and a number corresponding to that of the PFGE type. In addition the RAPD type labels (s) and (l) refer to the independent RAPD reactions using primers 1254 and 797 respectively. As there are

two independent RAPD types for each strain they are separated using a forward slash. For example the strain D08T1 is PFGE type U19 and is RAPD type U19(s)/U19(l). All comparisons of strain relatedness based on percentage similarity are approximate and calculated using the mean "% similarity" measurement indicated in figures 4.7 and 4.8 for RAPD analysis using primer 1254 and 797 respectively.

The RAPD analysis for both independent reactions identified that the majority of strains were grouped into two main types designated A(s)/A(l) (n=76/74) and B(s)/B(l) (n=46/45). Using RAPD analysis it was not possible to differentiate CA-MRSA strains from HA-MRSA, however, it was possible to differentiate MR-MRSA strains from MRSA strains. All of the MR-MRSA strains belonged to the RAPD type B(s)/B(l) except one strain that was distinct when analysed using primer 797, which was designated RAPD type B(s)/U21(l). The majority of NMR-MRSA strains belonged to the RAPD type designated A(s)/A(l). Two NMR-MRSA strains belonged to the RAPD type C(s)/C(l) and the remaining NMR-MRSA strains belonged to one of six unique types when analysis was performed using primer 1254 or one of nine unique types when analysis was performed using primer 797. Both reactions distinguished the RAPD types U1(s)/U1(l), U2(s)/U2(l), U18(s)/U18(l), U19(s)/U19(l), U22(s)/U22(l) and U23(s)/U23(l). The additional RAPD types differentiated using primer 797 were A(s)/U4(l), A(s)/U14(l) and the MR-MRSA strain B(s)/U21(l). For the purposes of this discussion the RAPD types are designated to the following groups; group A (A(s)/A(l), A(s)/U4(l), and A(s)/U14(l)); group B (B(s)/B(l) and B(s)/U21(l)); and group C (C(s)/C(l), U1(s)/U1(l), U2(s)/U2(l), U18(s)/U18(l), U19(s)/U19(l), U22(s)/U22(l) and U23(s)/U23(l)). The strains of group A and group B were 82% related. All 130 MRSA strains were 55% related when typing was performed using RAPD analysis.

4.4.5 Comparison of PFGE with RAPD analysis

Typically, the ability of a new typing method is compared with existing techniques by evaluating the capacity of both systems to cluster and differentiate an appropriate selection and number of outbreak strains and non-outbreak strains (Riley, 2004). Based on the number of types defined by a method and the relative frequency of these types it is possible to calculate a single numerical index of discrimination for both

techniques and to directly compare their discriminatory power (Hunter and Gaston, 1988). The discriminatory power of a typing method is defined as its ability to distinguish between unrelated strains and for this purpose Simpson's index of diversity has been widely adopted (Hunter and Gaston, 1988).

The test population of strains typed using both PFGE and RAPD analysis during this study were clinical isolates and not chosen as an appropriate selection of known related and unrelated strains. For this reason it is not possible to accurately compare either technique with discriminatory powers reported by other groups. For the purposes of this study Simpsons's index of diversity as described by (Hunter and Gaston, 1988) was calculated to directly compare the discriminatory power of RAPD analysis with PFGE after typing the population of MRSA strains obtained from the UHB and South Birmingham area.

The results of Simpsons's index of diversity indicate that the discriminatory power of PFGE exceeds that of both independent RAPD reactions. This observation was expected as PFGE is typically more discriminatory than RAPD analysis (Saulnier *et al.*, 1993; van Belkum *et al.*, 1995; van Leeuwen *et al.*, 1996; Tambic *et al.*, 1997). During PFGE the whole chromosome of a microorganism is subjected to a restriction digest and all DNA fragments are subsequently resolved into a banding pattern, therefore the higher discriminatory ability of PFGE may be explained by the fact that approximately 90% of the chromosomal DNA is being analysed (Goering, 2000). In comparison, the banding patterns from RAPD analysis are related to the amplicons generated between primers and therefore may represent less than 10% of the whole genome (Goering, 2000). This means that during any RAPD reaction certain regions of the template are not amplified and therefore a smaller percentage of the chromosomal DNA is being analysed. Despite the differences in discriminatory power it was observed that the PFGE types of groups 1, 2 and 3 correlated well with the RAPD types of groups A, B and C respectively. Similar observations of highly correlated PFGE and RAPD results have also been reported by other groups (Saulnier *et al.*, 1993; Hojo *et al.*, 1995; van Belkum *et al.*, 1995; van Leeuwen *et al.*, 1996; Tambic *et al.*, 1997).

4.4.6 Conclusion

In summary, PFGE electrophoresis is more discriminatory than RAPD analysis and may be more suitable for long term epidemiological studies, however, the technique is time consuming and requires the use of expensive peripheral equipment that is not available in all clinical microbiology laboratories. The results of this study demonstrate that RAPD analysis was suitable for differentiating MR-MRSA strains from MRSA strains and that it could also identify a diverse group of unrelated strains. In addition, RAPD analysis is relatively inexpensive, simple to perform and if optimised may be highly reproducible. Direct use of colonies from an agar plate combined with a whole cell method of preparing template DNA for RAPD analysis may provide results within several hours, which may be in advance of the results from antibiotic sensitivity testing, It may therefore be possible for RAPD analysis to be used in the management of infection control and for directing appropriate therapy in a clinically relevant time scale.

Further molecular analysis by multiplex PCR for SCC*mec* type assignment may provide an indication of the genetic background of the MRSA strains from the UHB and South Birmingham area. In addition, investigation of the rates and type of plasmid DNA carried by MRSA strains, as determined by REAP, may further differentiate strains based on extrachromosomal elements.

CHAPTER 5 MOLECULAR ANALYSIS OF MRSA BY SCCMEC TYPE ASSIGNMENT AND REAP

5.1 Introduction

The mechanisms by which *S. aureus* acquires resistance and virulence genes may be classified into two main categories: chromosomal gene mutation and acquisition of genes as a result of conjugation, transduction and transformation. *Staphylococcus aureus* may acquire exogenous genes by horizontal transfer of genomic islands or phage-mediated transduction, or from mobile genetic elements such as plasmids and transposons.

In the case of acquisition of resistance genes possibly the most important resistance element acquired by *S. aureus* is SCC*mec*, a highly mobile genomic island that carries the *mecA* gene that confers methicillin-resistance to *S. aureus*. Five distinct types of SCC*mec* designated I-V and a few variants have been described to date; three (I-III) are associated with hospital-acquired MRSA (HA-MRSA) (Ito *et al.*, 2001) and two (IV and V) are associated with community-acquired MRSA (CA-MRSA) (Daum *et al.*, 2002; Ma *et al.*, 2002; Ito *et al.*, 2004). In addition, recent reports regarding CA-MRSA strains that carry the genes for PVL have raised concerns that a highly virulent pathogen potentially adapted to the hospital and community setting is emerging (Lina *et al.*, 1999). Such strains have been implicated with severe necrotising pneumonia and skin and soft-tissue infections that have ultimately led to patient deaths (Anonymous, 1999; Gillet *et al.*, 2002; Diep *et al.*, 2004).

Arguably the best understood mobile genetic elements are plasmids, which have been widely studied in *S. aureus* and MRSA since the 1970s (Meyers *et al.*, 1976; McGowan *et al.*, 1979; Locksley *et al.*, 1982; Coia *et al.*, 1988). The first plasmids identified in *S. aureus* often encoded genes for β-lactamases which conferred resistance to penicillin antibiotics. Currently, it is suggested that more than 90% of MRSA strains carry plasmids while numerous studies have supported the important role plasmids play in staphylococcal multidrug-resistance (Lyon *et al.*, 1983; Coia *et al.*, 1988; Morton *et al.*, 1995; Paulsen *et al.*, 1998; O'Brien *et al.*, 2002). It might

therefore be anticipated that a positive correlation would exist between the number and diversity of plasmid DNA and increasing multidrug-resistance phenotype.

In this chapter MRSA strains were typed using a multiplex PCR for SCC*mec* type assignment and REAP. In addition, strains were screened by PCR for the PVL genes and for β-lactamase production using a nitrocefin test and isoelectric focusing.

5.2 Materials and methods

5.2.1 Multiplex PCR for *mec* element type assignment

The multiplex PCR was performed using a GeneAmp® PCR system 9700 (Applied Biosystems, UK) with primers and cycle conditions as described by (Oliveira and de Lencastre, 2002). The multiplex PCR was carried out in a 25μL volume containing 17.7μL SDW, 2.5 μL 10 x PCR Buffer 3 (table 4.1), 2.5μL 10 x primer mix (table 5.1), 0.2μL 10mM dNTPs (Promega, UK), 0.1μL of 1.25 units/μL *Taq* DNA polymerase (Promega, UK) and 2μl of DNA template (section 4.2.3.3). The MRSA strains COL, PER34, N315, ANS46, HU25 and MW2, representing SCC*mec* types I, Ia, II, III, IIIa and IV respectively were tested to determine expected banding patterns (Oliveira and de Lencastre, 2002). The strain N315 was used as a positive control during each multiplex PCR.

Table 5.1 Stock primer concentrations used in the 10 x primer mix for multiplex PCR as described by (Oliveira and de Lencastre, 2002)

5.2.2 Screening for PVL

The PCR was performed in a 25µL reaction volume using a GeneAmp® PCR system 9700 (Applied Biosystems, UK) with primers and cycle conditions as described by Lina *et al.* (Lina *et al.*, 1999).The PCR reaction was carried out in a 25µL volume containing 19.4µL SDW, 2.5µL 10x PCR Buffer 3 (table 4.1), 0.6µL primer mix (table 5.2), 0.5µL 10 mM dNTPs (Promega, UK), 0.25µL of 1.25 units/µL *Taq* DNA polymerase (Promega, UK) and 2µl DNA template (section 4.2.3.3). The *S. aureus* strain ATCC 49775 was used as a positive control for PVL gene detection (Lina *et al.*, 1999).

Table 5.2 Primers used in PVL-PCR reaction as described by (Lina *et al.*, 1999).

Primer	Oligonucleotide sequence (5'-3')	Mix (μM)
luk-PV-1	ATC ATT AGG TAA AAT GTC TGG ACA TGA TCC A	50
luk-PV-2	GCA TCA AST GTA TTG GAT AGC AAA AGC	50

5.2.3 Preparation of plasmid DNA

Preparations of plasmid DNA were made using a modified protocol from the Sigma GenElute® plasmid miniprep kit (Sigma, UK). The isolate A02T1, identified as a carrier of plasmid DNA, was used as a positive control during each preparation. Test isolates of MRSA were recovered from frozen stocks by inoculating a single cryobead onto a BHI agar plate and incubating at 37°C for 16 hours. A single loop of 25 colonies was taken directly from the plate and washed in 0.5mL of TES buffer (50mM Tris-HCl, 5mM EDTA and 50mM NaCl, pH 8) and centrifuged at 5500 × *g* for 4 minutes in a bench-top microcentrifuge. The supernatant was removed and the pellet resuspended in 100μL of TESS buffer to which 20μL of lysostaphin (0.5units/μL) was added and briefly vortexed. Samples were incubated at 37°C for 20 minutes until clear and then at 80°C for 10 minutes. The resulting lysate was treated as described in the manufacturer's instructions, with two modifications. Instead of resuspending cells in 200μL of resuspension solution the lysate was mixed with 80μL of resuspension solution. The lysate was then treated with 200μL of lysis solution and mixed by inversion 6-8 times. Within 5 minutes of treatment with lysis solution the sample was treated with 350μL of neutralising/binding solution and mixed by gentle inversion 4-6 times and then centrifuged at 12000 x g for 10 minutes. The GenElute miniprep column was placed inside a microcentrifuge tube, treated with column preparation solution and centrifuged at 12000 x g for 1 minute. The flow-through was discarded and the supernatant of the sample loaded into the prepared column and centrifuged at 12000 x g for 1 minute. The flow-through was discarded and the column was washed with 750μL of wash solution and centrifuged at 12000 x g for 1 minute. The flow-through was discarded and the column was centrifuged for a further 1 minute at 12000 x g. The spin column was transferred to a fresh microcentrifuge tube and plasmid DNA was eluted with 100μL of SDW instead of 100μL of elution solution. The quantity and quality of extracted plasmid DNA was determined

spectrophotometrically as described in section 4.2.3.2. Only samples with A_{260}/A_{280} ratios between 1.7 and 1.9 were analysed. Plasmid DNA samples were adjusted to a concentration of 1µg/µL with SDW and stored at -20°C until required.

5.2.4 Restriction endonuclease analysis of plasmid DNA

Plasmid DNA was cleaved with restriction endonucleases *Hind*III, *Xba*I, *Eco*RI, *Hha*I, *Hae*III, *Pst*I or *Alu*I (Promega, UK) according to the manufacturer's instructions using the appropriate reaction buffer. The restriction digest was carried out in a 20µL volume containing 16.3µL SDW, 2µL of 10x RE Buffer, 0.2µL acetylated bovine serum albumin (10µg/µL), 1µL DNA (1µg/µL) and 0.5µL of restriction enzyme (10units/µL). Digested plasmid and corresponding uncut plasmid were resolved in a 2% agarose gel containing 1µg/mL ethidium bromide. Electrophoresis was performed in 1 x TAE buffer (40mM Tris-HCl, 1mM EDTA and 0.1% ($^{v}/_{v}$) glacial acetic acid) at 65 volts for 80 minutes. Gels were visualised under UV illumination and photographed using the GeneGenius Bio Imaging System (Syngene, UK). Images of profiles of the digested plasmid DNA were compared visually and by use of Gel Compar II software (Applied Maths, Kortrijk, Belgium). The similarity between the isolates was calculated by the Dice coefficient, and a dendrogram was constructed using UPGMA clustering. Profiles were numbered according to their position in the dendrogram.

5.2.5 Statistical analysis

The presence and absence of plasmid DNA in multidrug-resistant and non-multidrug-resistant MRSA isolates was compared by means of the Yates' corrected Chi-square statistic (Statistica 6.0, StatSoft, Tulsa, USA).

5.2.6 Preparation of MRSA strains for β-lactamase testing

Isolated enzyme extracts were prepared for 42 MRSA strains representing each of the molecular types as determined by PFGE and REAP (table 5.3). A culture of the required organism was grown in 10mL BHI broth supplemented with 0.1mM 6-aminopenicillanic acid (6-APA) for 16 hours at 37°C with shaking, to induce β-

lactamase production. The cells were harvested by centrifugation at 5500 x g for four minutes and washed in phosphate buffered saline pH 7 (PBS). The cells were subsequently resuspended in 1mL of PBS and transferred to a 1mL sterile plastic cuvette. The A_{600} was measured and adjusted to OD 1.7 with PBS. The cells were disrupted by freezing with liquid nitrogen followed by thawing at 37°C in a waterbath. The process was repeated 20 times. The disrupted cells were centrifuged at 3000 x g for 4 minutes to remove whole cells and cell debris.

5.2.7 Microplate nitrocefin assay

Disrupted cell preparations were used immediately by aliquoting 20µL of each preparation into separate wells of a 96 well plate and subsequently adding prewarmed (37°C) dilute nitrocefin solution (Oxoid, UK) and incubating for 10 minutes at 37°C. Nitrocefin solution is a lyophilised powder rehydrated in 1.9mL of 0.1M phosphate buffer pH 7 and further diluted 1:10 with PBS (50µg/mL). In the presence of β-lactamase, this chromogenic substrate is hydrolysed and changes colour from yellow to pink/red. After the initial reading the plate was incubated for a further 1 hour period. Preparations positive for β-lactamase activity were subjected to isoelectric focusing.

5.2.8 Isoelectric focusing

Isoelectric focusing (IEF) is an electrophoretic technique used to separate proteins according to their isoelectric point (pI) in a stable pH gradient. The technique exploits the fact that proteins possess a net charge that varies with the pH of its environment. The overall net charge represents the sum of positive and negative charges on the surface of the protein. When a protein passes from a low pH to a high pH the net charge continually alternates from positive to negative. At a certain defined pH, the net charge will be zero and the protein will no longer migrate. The position at which the protein stops is called the isoelectric point. Protein of the same species will become focussed at the same pI forming a distinct concentrated band.

Isoelectric focusing was performed using a LKB Bromma 2117 Multiphor II electrophoresis unit (LKB Bromma, Sweden), attached to a Bio-Rad Model 3000 XI

programmable power supply (Bio-Rad Laboratories Ltd, UK) and a LKB Bromma 2209 Multi Temp water bath and cooling plate (LKB Bromma, Sweden). Prepoured gels, 245x110x1mm Ampholine PAGplate 2.2% ($^{w}/_{v}$) pH 3.5-9.5 (Pharmacia Biotech, Sweden), were prepared and maintained at 4°C on the cooling plate. The position at which the cathode interfaced with the gel was prepared with a strip of filter paper soaked with 1M NaOH (sodium hydroxide) Similarly the anode-gel interface was prepared with a strip of filter paper soaked with 1M H_3PO_4 (phosphoric acid) A 100µL volume of each β-lactamase preparation was applied midway along the gel with approximately 2cm between each sample. When samples were dry, electrophoresis was carried out with a constant power of 50W for 3 hours. The presence of β-lactamase was detected using nitrocefin solution applied to the whole of the gel. Distinct bands of pink/red in the gel were indicative of focused β-lactamase.

5.3 Results

5.3.1 SCC*mec* type

Multiplex PCR for *mec* element assignment revealed that all of the isolates investigated were *mecA* positive and characteristic of eight types Ia, I, II, IIIa, III, IV and two new variants designated new 1 and new 2. All 44 MR-HA-MRSA isolates and 2 MR-CA-MRSA isolates were type II (n=46), 47 HA-MRSA isolates and 34 CA-MRSA isolates were type IV (n=81), four individual HA-MRSA isolates were designated type I, Ia, III, IIIa and three HA-MRSA isolates were characterised as belonging to two new variant types. A dendrogram of strains typed using a multiplex PCR for *mec* element type assignment is depicted in figure 5.1.

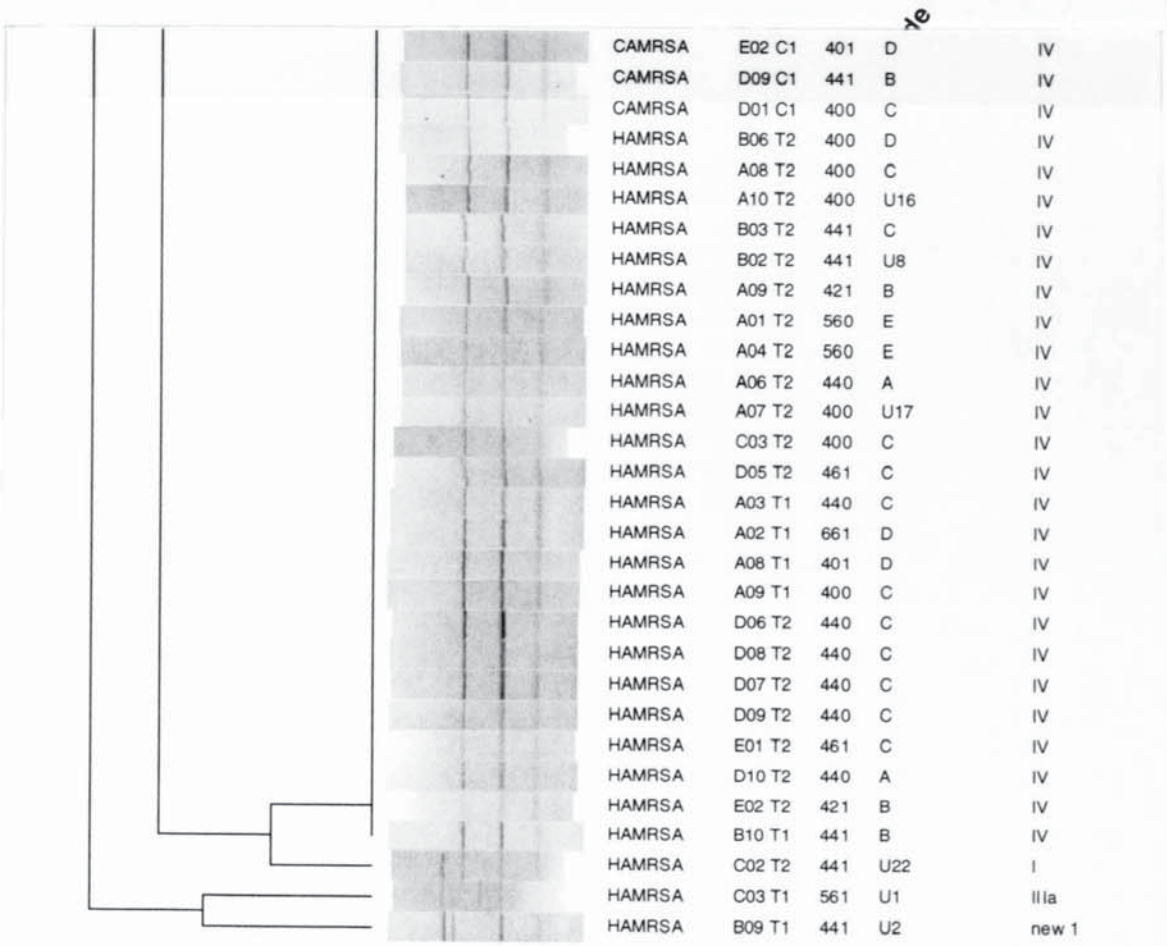
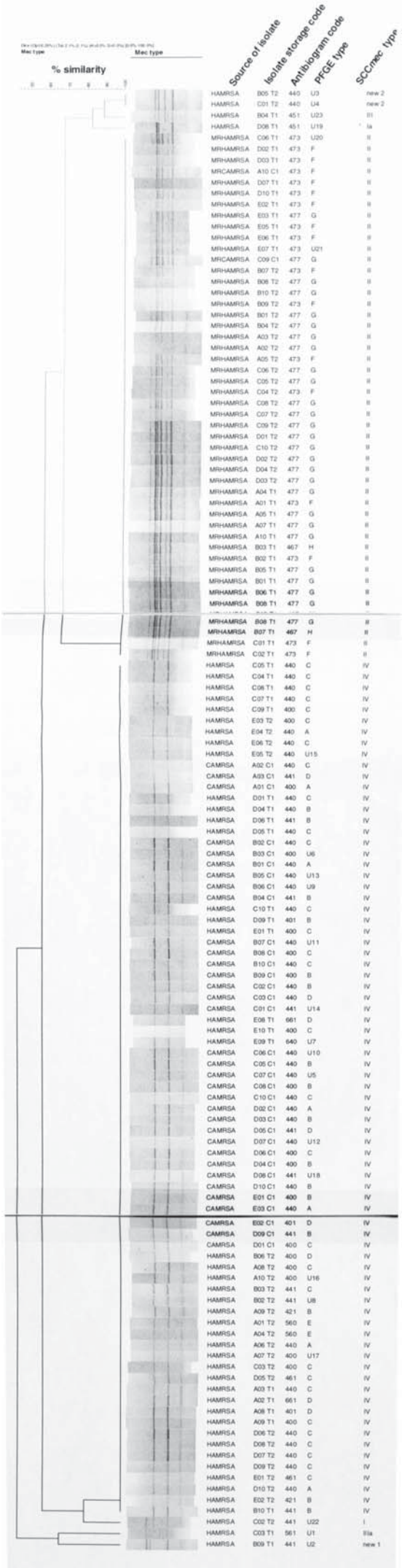


Figure 5.1 Dendrogram of 94 HA- and 36 CA-MRSA strains depicting SCC*mec* element type assignment. Similarity calculated by Dice coefficient and represented by UPGMA clustering.



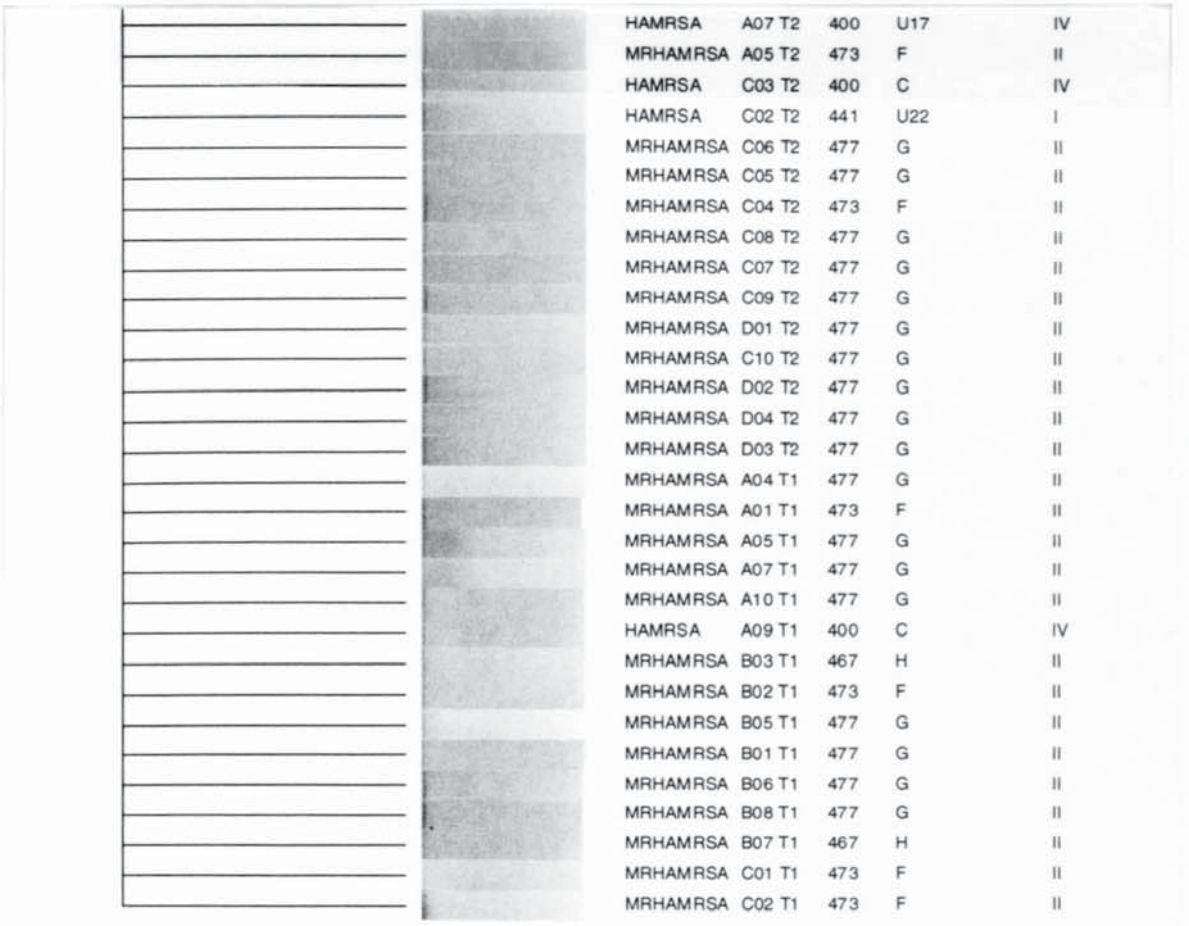
Source of isolate	Isolate storage code	Antibiogram code	PFGE type	SCCmec type
HAMRSA	B05 T2	440	U3	new 2
HAMRSA	C01 T2	440	U4	new 2
HAMRSA	B04 T1	451	U23	III
HAMRSA	D08 T1	451	U19	Ia
MRHAMRSA	C06 T1	473	U20	II
MRHAMRSA	D02 T1	473	F	II
MRHAMRSA	D03 T1	473	F	II
MRCAMRSA	A10 C1	473	F	II
MRHAMRSA	D07 T1	473	F	II
MRHAMRSA	D10 T1	473	F	II
MRHAMRSA	E02 T1	473	F	II
MRHAMRSA	E03 T1	477	G	II
MRHAMRSA	E05 T1	473	F	II
MRHAMRSA	E06 T1	473	F	II
MRHAMRSA	E07 T1	473	U21	II
MRCAMRSA	C09 C1	477	G	II
MRHAMRSA	B07 T2	473	F	II
MRHAMRSA	B08 T2	477	G	II
MRHAMRSA	B10 T2	477	G	II
MRHAMRSA	B09 T2	473	F	II
MRHAMRSA	B01 T2	477	G	II
MRHAMRSA	B04 T2	477	G	II
MRHAMRSA	A03 T2	477	G	II
MRHAMRSA	A02 T2	477	G	II
MRHAMRSA	A05 T2	473	F	II
MRHAMRSA	C06 T2	477	G	II
MRHAMRSA	C05 T2	477	G	II
MRHAMRSA	C04 T2	473	F	II
MRHAMRSA	C08 T2	477	G	II
MRHAMRSA	C07 T2	477	G	II
MRHAMRSA	C09 T2	477	G	II
MRHAMRSA	D01 T2	477	G	II
MRHAMRSA	C10 T2	477	G	II
MRHAMRSA	D02 T2	477	G	II
MRHAMRSA	D04 T2	477	G	II
MRHAMRSA	D03 T2	477	G	II
MRHAMRSA	A04 T1	477	G	II
MRHAMRSA	A01 T1	473	F	II
MRHAMRSA	A05 T1	477	G	II
MRHAMRSA	A07 T1	477	G	II
MRHAMRSA	A10 T1	477	G	II
MRHAMRSA	B03 T1	467	H	II
MRHAMRSA	B02 T1	473	F	II
MRHAMRSA	B05 T1	477	G	II
MRHAMRSA	B01 T1	477	G	II
MRHAMRSA	**B06 T1**	**477**	**G**	**II**
MRHAMRSA	**B08 T1**	**477**	**G**	**II**
MRHAMRSA	**B08 T1**	**477**	**G**	**II**
MRHAMRSA	**B07 T1**	**467**	**H**	**II**
MRHAMRSA	C01 T1	473	F	II
MRHAMRSA	C02 T1	473	F	II
HAMRSA	C05 T1	440	C	IV
HAMRSA	C04 T1	440	C	IV
HAMRSA	C08 T1	440	C	IV
HAMRSA	C07 T1	440	C	IV
HAMRSA	C09 T1	400	C	IV
HAMRSA	E03 T2	400	C	IV
HAMRSA	E04 T2	440	A	IV
HAMRSA	E06 T2	440	C	IV
HAMRSA	E05 T2	440	U15	IV
CAMRSA	A02 C1	440	C	IV
CAMRSA	A03 C1	441	D	IV
CAMRSA	A01 C1	400	A	IV
HAMRSA	D01 T1	440	C	IV
HAMRSA	D04 T1	440	B	IV
HAMRSA	D06 T1	441	B	IV
HAMRSA	D05 T1	440	C	IV
CAMRSA	B02 C1	440	C	IV
CAMRSA	B03 C1	400	U6	IV
CAMRSA	B01 C1	440	A	IV
CAMRSA	B05 C1	440	U13	IV
CAMRSA	B06 C1	440	U9	IV
CAMRSA	B04 C1	441	B	IV
HAMRSA	C10 T1	440	C	IV
HAMRSA	D09 T1	401	B	IV
HAMRSA	E01 T1	400	C	IV
CAMRSA	B07 C1	440	U11	IV
CAMRSA	B08 C1	400	C	IV
CAMRSA	B10 C1	440	C	IV
CAMRSA	B09 C1	400	B	IV
CAMRSA	C02 C1	440	B	IV
CAMRSA	C03 C1	440	D	IV
CAMRSA	C01 C1	441	U14	IV
HAMRSA	E08 T1	661	D	IV
HAMRSA	E10 T1	400	C	IV
HAMRSA	E09 T1	640	U7	IV
CAMRSA	C06 C1	440	U10	IV
CAMRSA	C05 C1	440	B	IV
CAMRSA	C07 C1	440	U5	IV
CAMRSA	C08 C1	400	B	IV
CAMRSA	C10 C1	440	C	IV
CAMRSA	D02 C1	440	A	IV
CAMRSA	D03 C1	440	B	IV
CAMRSA	D05 C1	441	D	IV
CAMRSA	D07 C1	440	U12	IV
CAMRSA	D06 C1	400	C	IV
CAMRSA	D04 C1	400	B	IV
CAMRSA	D08 C1	441	U18	IV
CAMRSA	D10 C1	440	B	IV
CAMRSA	**E01 C1**	**400**	**B**	**IV**
CAMRSA	**E03 C1**	**440**	**A**	**IV**
CAMRSA	**E02 C1**	**401**	**D**	**IV**
CAMRSA	**D09 C1**	**441**	**B**	**IV**
CAMRSA	D01 C1	400	C	IV
HAMRSA	B06 T2	400	D	IV
HAMRSA	A08 T2	400	C	IV
HAMRSA	A10 T2	400	U16	IV
HAMRSA	B03 T2	441	C	IV
HAMRSA	B02 T2	441	U8	IV
HAMRSA	A09 T2	421	B	IV
HAMRSA	A01 T2	560	E	IV
HAMRSA	A04 T2	560	E	IV
HAMRSA	A06 T2	440	A	IV
HAMRSA	A07 T2	400	U17	IV
HAMRSA	C03 T2	400	C	IV
HAMRSA	D05 T2	461	C	IV
HAMRSA	A03 T1	440	C	IV
HAMRSA	A02 T1	661	D	IV
HAMRSA	A08 T1	401	D	IV
HAMRSA	A09 T1	400	C	IV
HAMRSA	D06 T2	440	C	IV
HAMRSA	D08 T2	440	C	IV
HAMRSA	D07 T2	440	C	IV
HAMRSA	D09 T2	440	C	IV
HAMRSA	E01 T2	461	C	IV
HAMRSA	D10 T2	440	A	IV
HAMRSA	E02 T2	421	B	IV
HAMRSA	B10 T1	441	B	IV
HAMRSA	C02 T2	441	U22	I
HAMRSA	C03 T1	561	U1	IIa
HAMRSA	B09 T1	441	U2	new 1

5.3.2 PVL screening

Screening for PVL by PCR revealed that none of the 130 test strains and only the positive control strain ATCC 49775 carried the PVL gene.

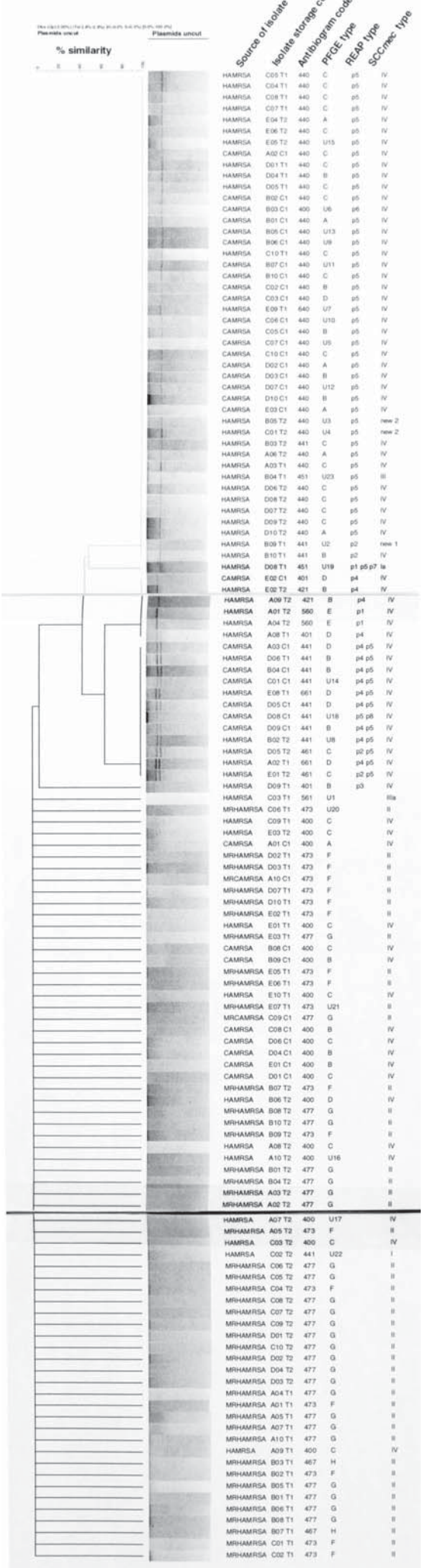
5.3.3 Plasmid DNA

Plasmid DNA was absent from all of the MR-HA-MRSA isolates with significance of p=<0.001. In addition, ten NMR-HA-MRSA isolates and five NMR-CA-MRSA isolates that were only resistant to flucloxacillin were also found not to carry plasmid DNA. One NMR isolate assigned SCC*mec* type I and another NMR isolate assigned type IIIa were also absent of plasmid DNA but were resistant to at least two additional antibiotics other than flucloxacillin. The remaining NMR isolates all carried at least one plasmid, seven of these carried two plasmids and one carried three plasmids. A dendrogram of strains typed using plasmid DNA is depicted in figure 5.2.



HAMRSA	A07 T2	400	U17	IV
MRHAMRSA	A05 T2	473	F	II
HAMRSA	C03 T2	400	C	IV
HAMRSA	C02 T2	441	U22	I
MRHAMRSA	C06 T2	477	G	II
MRHAMRSA	C05 T2	477	G	II
MRHAMRSA	C04 T2	473	F	II
MRHAMRSA	C08 T2	477	G	II
MRHAMRSA	C07 T2	477	G	II
MRHAMRSA	C09 T2	477	G	II
MRHAMRSA	D01 T2	477	G	II
MRHAMRSA	C10 T2	477	G	II
MRHAMRSA	D02 T2	477	G	II
MRHAMRSA	D04 T2	477	G	II
MRHAMRSA	D03 T2	477	G	II
MRHAMRSA	A04 T1	477	G	II
MRHAMRSA	A01 T1	473	F	II
MRHAMRSA	A05 T1	477	G	II
MRHAMRSA	A07 T1	477	G	II
MRHAMRSA	A10 T1	477	G	II
HAMRSA	A09 T1	400	C	IV
MRHAMRSA	B03 T1	467	H	II
MRHAMRSA	B02 T1	473	F	II
MRHAMRSA	B05 T1	477	G	II
MRHAMRSA	B01 T1	477	G	II
MRHAMRSA	B06 T1	477	G	II
MRHAMRSA	B08 T1	477	G	II
MRHAMRSA	B07 T1	467	H	II
MRHAMRSA	C01 T1	473	F	II
MRHAMRSA	C02 T1	473	F	II

Figure 5.2 Dendrogram depicting uncut plasmid DNA from 94 HA- and 36 CA-MRSA strains. Similarity calculated by Dice coefficient and represented by UPGMA clustering.



Source of isolate	Isolate storage code	Antibiogram code	PFGE type	REAP type	SCCmec type
HAMRSA	C05 T1	440	C	p5	IV
HAMRSA	C04 T1	440	C	p5	IV
HAMRSA	C08 T1	440	C	p5	IV
HAMRSA	C07 T1	440	C	p5	IV
HAMRSA	E04 T2	440	A	p5	IV
HAMRSA	E06 T2	440	C	p5	IV
HAMRSA	E05 T2	440	U15	p5	IV
CAMRSA	A02 C1	440	C	p5	IV
HAMRSA	D01 T1	440	C	p5	IV
HAMRSA	D04 T1	440	B	p5	IV
HAMRSA	D05 T1	440	C	p5	IV
CAMRSA	B02 C1	440	C	p5	IV
CAMRSA	B03 C1	400	U6	p6	IV
CAMRSA	B01 C1	440	A	p5	IV
CAMRSA	B05 C1	440	U13	p5	IV
CAMRSA	B06 C1	440	U9	p5	IV
HAMRSA	C10 T1	440	C	p5	IV
CAMRSA	B07 C1	440	U11	p5	IV
CAMRSA	B10 C1	440	C	p5	IV
CAMRSA	C02 C1	440	B	p5	IV
CAMRSA	C03 C1	440	D	p5	IV
HAMRSA	E09 T1	640	U7	p5	IV
CAMRSA	C06 C1	440	U10	p5	IV
CAMRSA	C05 C1	440	B	p5	IV
CAMRSA	C07 C1	440	U5	p5	IV
CAMRSA	C10 C1	440	C	p5	IV
CAMRSA	D02 C1	440	A	p5	IV
CAMRSA	D03 C1	440	B	p5	IV
CAMRSA	D07 C1	440	U12	p5	IV
CAMRSA	D10 C1	440	B	p5	IV
CAMRSA	E03 C1	440	A	p5	IV
HAMRSA	B05 T2	440	U3	p5	new 2
HAMRSA	C01 T2	440	U4	p5	new 2
HAMRSA	B03 T2	441	C	p5	IV
HAMRSA	A06 T2	440	A	p5	IV
HAMRSA	A03 T1	440	C	p5	IV
HAMRSA	B04 T1	451	U23	p5	III
HAMRSA	D06 T2	440	C	p5	IV
HAMRSA	D08 T2	440	C	p5	IV
HAMRSA	D07 T2	440	C	p5	IV
HAMRSA	D09 T2	440	C	p5	IV
HAMRSA	D10 T2	440	A	p5	IV
HAMRSA	B09 T1	441	U2	p2	new 1
HAMRSA	B10 T1	441	B	p2	IV
HAMRSA	**D08 T1**	**451**	**U19**	**p1 p5 p7**	**Ia**
CAMRSA	E02 C1	401	D	p4	IV
HAMRSA	E02 T2	421	B	p4	IV
HAMRSA	A09 T2	421	B	p4	IV
HAMRSA	**A01 T2**	**560**	**E**	**p1**	**IV**
HAMRSA	A04 T2	560	E	p1	IV
HAMRSA	A08 T1	401	D	p4	IV
CAMRSA	A03 C1	441	D	p4 p5	IV
HAMRSA	D06 T1	441	B	p4 p5	IV
CAMRSA	B04 C1	441	B	p4 p5	IV
CAMRSA	C01 C1	441	U14	p4 p5	IV
HAMRSA	E08 T1	661	D	p4 p5	IV
CAMRSA	D05 C1	441	D	p4 p5	IV
CAMRSA	D08 C1	441	U18	p5 p8	IV
CAMRSA	D09 C1	441	B	p4 p5	IV
HAMRSA	B02 T2	441	U8	p4 p5	IV
HAMRSA	D05 T2	461	C	p2 p5	IV
HAMRSA	A02 T1	661	D	p4 p5	IV
HAMRSA	E01 T2	461	C	p2 p5	IV
HAMRSA	D09 T1	401	B	p3	IV
HAMRSA	C03 T1	561	U1		IIIa
MRHAMRSA	C06 T1	473	U20		II
HAMRSA	C09 T1	400	C		IV
HAMRSA	E03 T2	400	C		IV
CAMRSA	A01 C1	400	A		IV
MRHAMRSA	D02 T1	473	F		II
MRHAMRSA	D03 T1	473	F		II
MRCAMRSA	A10 C1	473	F		II
MRHAMRSA	D07 T1	473	F		II
MRHAMRSA	D10 T1	473	F		II
MRHAMRSA	E02 T1	473	F		II
HAMRSA	E01 T1	400	C		IV
MRHAMRSA	E03 T1	477	G		II
CAMRSA	B08 C1	400	C		IV
CAMRSA	B09 C1	400	B		IV
MRHAMRSA	E05 T1	473	F		II
MRHAMRSA	E06 T1	473	F		II
HAMRSA	E10 T1	400	C		IV
MRHAMRSA	E07 T1	473	U21		II
MRCAMRSA	C09 C1	477	G		II
CAMRSA	C08 C1	400	B		IV
CAMRSA	D06 C1	400	C		IV
CAMRSA	D04 C1	400	B		IV
CAMRSA	E01 C1	400	B		IV
CAMRSA	D01 C1	400	C		IV
MRHAMRSA	B07 T2	473	F		II
HAMRSA	B06 T2	400	D		IV
MRHAMRSA	B08 T2	477	G		II
MRHAMRSA	B10 T2	477	G		II
MRHAMRSA	B09 T2	473	F		II
HAMRSA	A08 T2	400	C		IV
HAMRSA	A10 T2	400	U16		IV
MRHAMRSA	B01 T2	477	G		II
MRHAMRSA	B04 T2	477	G		II
MRHAMRSA	**A03 T2**	**477**	**G**		**II**
MRHAMRSA	**A02 T2**	**477**	**G**		**II**
HAMRSA	A07 T2	400	U17		IV
MRHAMRSA	**A05 T2**	**473**	**F**		**II**
HAMRSA	**C03 T2**	**400**	**C**		**IV**
HAMRSA	C02 T2	441	U22		I
MRHAMRSA	C06 T2	477	G		II
MRHAMRSA	C05 T2	477	G		II
MRHAMRSA	C04 T2	473	F		II
MRHAMRSA	C08 T2	477	G		II
MRHAMRSA	C07 T2	477	G		II
MRHAMRSA	C09 T2	477	G		II
MRHAMRSA	D01 T2	477	G		II
MRHAMRSA	C10 T2	477	G		II
MRHAMRSA	D02 T2	477	G		II
MRHAMRSA	D04 T2	477	G		II
MRHAMRSA	D03 T2	477	G		II
MRHAMRSA	A04 T1	477	G		II
MRHAMRSA	A01 T1	473	F		II
MRHAMRSA	A05 T1	477	G		II
MRHAMRSA	A07 T1	477	G		II
MRHAMRSA	A10 T1	477	G		II
HAMRSA	A09 T1	400	C		IV
MRHAMRSA	B03 T1	467	H		II
MRHAMRSA	B02 T1	473	F		II
MRHAMRSA	B05 T1	477	G		II
MRHAMRSA	B01 T1	477	G		II
MRHAMRSA	B06 T1	477	G		II
MRHAMRSA	B08 T1	477	G		II
MRHAMRSA	B07 T1	467	H		II
MRHAMRSA	C01 T1	473	F		II
MRHAMRSA	C02 T1	473	F		II

5.3.4 Restriction enzyme fragmentation patterns of plasmid DNA

The restriction endonucleases *Hind*III, *Xba*I, *Eco*RI, *Hha*I, *Hae*III, *Pst*I or *Alu*I were screened to determine their ability to cleave plasmid DNA. Of these enzymes only *Alu*I fully digested plasmid DNA and provided clearly resolved, discriminatory profiles. In total there were eight different *Alu*I plasmid types designated p1-p8, presence of multiple plasmids accounted for nine different plasmid profiles. A dendrogram of strains typed using REAP is depicted in figure 5.3.

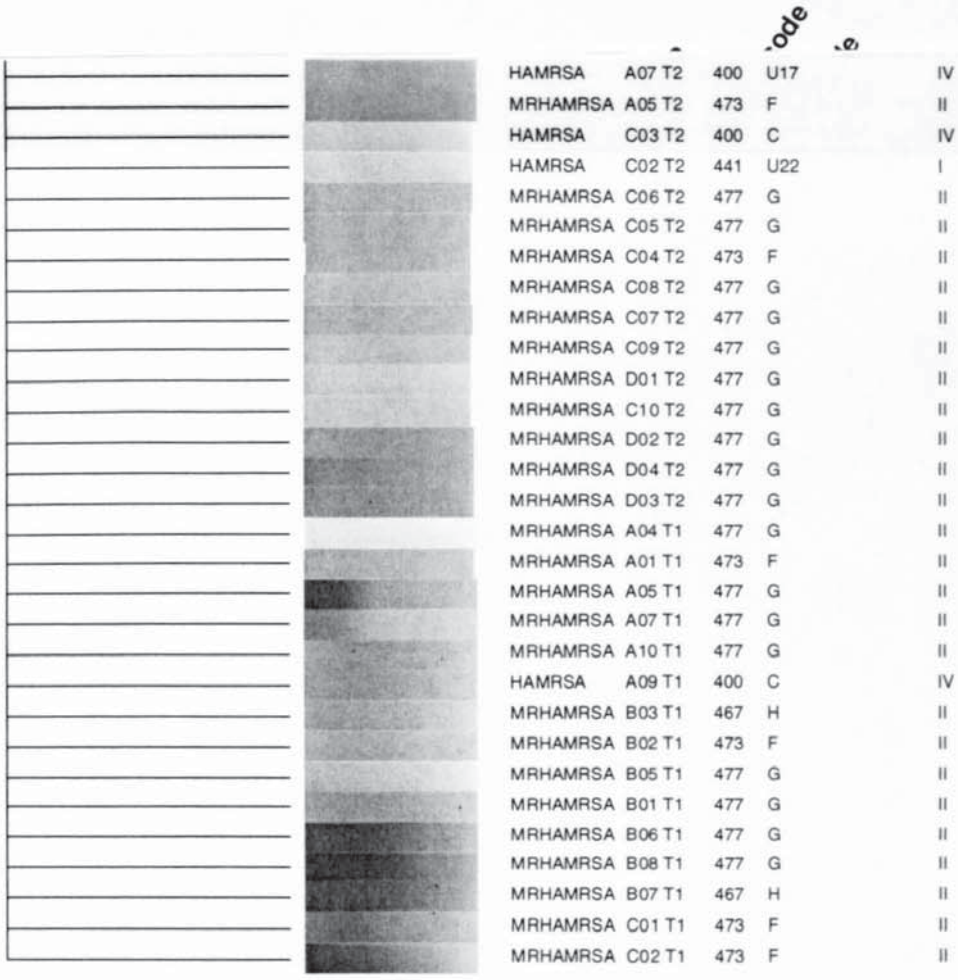
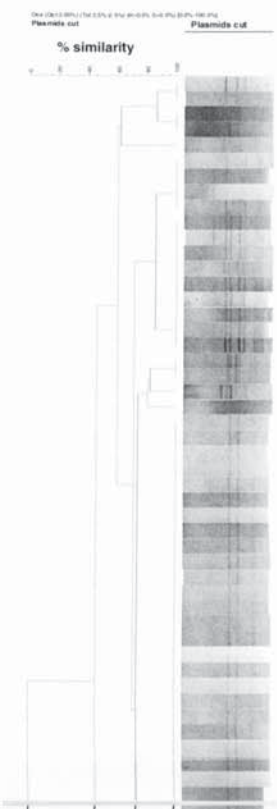


Figure 5.3 Dendrogram depicting REAP profiles obtained from 94 HA- and 36 CA-MRSA strains. Similarity calculated by Dice coefficient and represented by UPGMA clustering.



Source of isolate	Isolate storage code	Antibiogram code	PFGE type	REAP type	SCCmec type
HAMRSA	A01 T2	560	E	p1	IV
HAMRSA	A04 T2	560	E	p1	IV
HAMRSA	B09 T1	441	U2	p2	new 1
HAMRSA	B10 T1	441	B	p2	IV
HAMRSA	D09 T1	401	B	p3	IV
CAMRSA	E02 C1	401	D	p4	IV
HAMRSA	A09 T2	421	B	p4	IV
HAMRSA	A06 T1	401	D	p4	IV
HAMRSA	E02 T2	421	B	p4	IV
CAMRSA	A03 C1	441	D	p4 p5	IV
HAMRSA	D06 T1	441	B	p4 p5	IV
CAMRSA	B04 C1	441	B	p4 p5	IV
CAMRSA	C01 C1	441	U14	p4 p5	IV
HAMRSA	E08 T1	661	D	p4 p5	IV
CAMRSA	D05 C1	441	D	p4 p5	IV
CAMRSA	D09 C1	441	B	p4 p5	IV
HAMRSA	B02 T2	441	U8	p4 p5	IV
HAMRSA	A02 T1	661	D	p4 p5	IV
HAMRSA	D05 T2	461	C	p2 p5	IV
HAMRSA	E01 T2	461	C	p2 p5	IV
HAMRSA	D08 T1	451	U19	p1 p5 p7	Ia
CAMRSA	D08 C1	441	U18	p5 p8	IV
HAMRSA	C05 T1	440	C	p5	IV
HAMRSA	C04 T1	440	C	p5	IV
HAMRSA	C08 T1	440	C	p5	IV
HAMRSA	C07 T1	440	C	p5	IV
HAMRSA	E04 T2	440	A	p5	IV
HAMRSA	E06 T2	440	C	p5	IV
HAMRSA	E05 T2	440	U15	p5	IV
CAMRSA	A02 C1	440	C	p5	IV
HAMRSA	D01 T1	440	C	p5	IV
HAMRSA	D04 T1	440	B	p5	IV
HAMRSA	D05 T1	440	C	p5	IV
CAMRSA	B02 C1	440	C	p5	IV
CAMRSA	B01 C1	440	A	p5	IV
CAMRSA	B05 C1	440	U13	p5	IV
CAMRSA	B06 C1	440	U9	p5	IV
HAMRSA	C10 T1	440	C	p5	IV
CAMRSA	B07 C1	440	U11	p5	IV
CAMRSA	B10 C1	440	C	p5	IV
CAMRSA	C02 C1	440	B	p5	IV
CAMRSA	C03 C1	440	D	p5	IV
HAMRSA	E09 T1	640	U7	p5	IV
CAMRSA	C06 C1	440	U10	p5	IV
CAMRSA	**C05 C1**	**440**	**B**	**p5**	**IV**
CAMRSA	**C07 C1**	**440**	**U5**	**p5**	**IV**
CAMRSA	**C10 C1**	**440**	**C**	**p5**	**IV**
CAMRSA	**D02 C1**	**440**	**A**	**p5**	**IV**
CAMRSA	**D03 C1**	**440**	**B**	**p5**	**IV**
CAMRSA	D07 C1	440	U12	p5	IV
CAMRSA	D10 C1	440	B	p5	IV
CAMRSA	E03 C1	440	A	p5	IV
HAMRSA	B05 T2	440	U3	p5	new 2
HAMRSA	C01 T2	440	U4	p5	new 2
HAMRSA	B03 T2	441	C	p5	IV
HAMRSA	A06 T2	440	A	p5	IV
HAMRSA	A03 T1	440	C	p5	IV
HAMRSA	B04 T1	451	U23	p5	III
HAMRSA	D06 T2	440	C	p5	IV
HAMRSA	D08 T2	440	C	p5	IV
HAMRSA	D07 T2	440	C	p5	IV
HAMRSA	D09 T2	440	C	p5	IV
HAMRSA	D10 T2	440	A	p5	IV
CAMRSA	B03 C1	400	U6	p6	IV
HAMRSA	C03 T1	561	U1		IIIa
MRHAMRSA	C06 T1	473	U20		II
HAMRSA	C09 T1	400	C		IV
HAMRSA	E03 T2	400	C		IV
CAMRSA	A01 C1	400	A		IV
MRHAMRSA	D02 T1	473	F		II
MRHAMRSA	D03 T1	473	F		II
MRCAMRSA	A10 C1	473	F		II
MRHAMRSA	D07 T1	473	F		II
MRHAMRSA	D10 T1	473	F		II
MRHAMRSA	E02 T1	473	F		II
HAMRSA	E01 T1	400	C		IV
MRHAMRSA	E03 T1	477	G		II
CAMRSA	B08 C1	400	C		IV
CAMRSA	B09 C1	400	B		IV
MRHAMRSA	E05 T1	473	F		II
MRHAMRSA	E06 T1	473	F		II
HAMRSA	E10 T1	400	C		IV
MRHAMRSA	E07 T1	473	U21		II
MRCAMRSA	C09 C1	477	G		II
CAMRSA	C08 C1	400	B		IV
CAMRSA	D06 C1	400	C		IV
CAMRSA	D04 C1	400	B		IV
CAMRSA	E01 C1	400	B		IV
CAMRSA	D01 C1	400	C		IV
MRHAMRSA	B07 T2	473	F		II
HAMRSA	B06 T2	400	D		IV
MRHAMRSA	B08 T2	477	G		II
MRHAMRSA	B10 T2	477	G		II
MRHAMRSA	B09 T2	473	F		II
HAMRSA	A08 T2	400	C		IV
HAMRSA	A10 T2	400	U16		IV
MRHAMRSA	B01 T2	477	G		II
MRHAMRSA	B04 T2	477	G		II
MRHAMRSA	**A03 T2**	**477**	**G**		**II**
MRHAMRSA	A02 T2	477	G		II
HAMRSA	**A07 T2**	**400**	**U17**		**IV**
MRHAMRSA	**A05 T2**	**473**	**F**		**II**
HAMRSA	**C03 T2**	**400**	**C**		**IV**
HAMRSA	C02 T2	441	U22		I
MRHAMRSA	C06 T2	477	G		II
MRHAMRSA	C05 T2	477	G		II
MRHAMRSA	C04 T2	473	F		II
MRHAMRSA	C08 T2	477	G		II
MRHAMRSA	C07 T2	477	G		II
MRHAMRSA	C09 T2	477	G		II
MRHAMRSA	D01 T2	477	G		II
MRHAMRSA	C10 T2	477	G		II
MRHAMRSA	D02 T2	477	G		II
MRHAMRSA	D04 T2	477	G		II
MRHAMRSA	D03 T2	477	G		II
MRHAMRSA	A04 T1	477	G		II
MRHAMRSA	A01 T1	473	F		II
MRHAMRSA	A05 T1	477	G		II
MRHAMRSA	A07 T1	477	G		II
MRHAMRSA	A10 T1	477	G		II
HAMRSA	A09 T1	400	C		IV
MRHAMRSA	B03 T1	467	H		II
MRHAMRSA	B02 T1	473	F		II
MRHAMRSA	B05 T1	477	G		II
MRHAMRSA	B01 T1	477	G		II
MRHAMRSA	B06 T1	477	G		II
MRHAMRSA	B08 T1	477	G		II
MRHAMRSA	B07 T1	467	H		II
MRHAMRSA	C01 T1	473	F		II
MRHAMRSA	C02 T1	473	F		II

5.3.5 β-lactamase production

The strains D08T1, A01T2, E03 T1, B03T1, C06 T1, D02T1, E07T1 and C02T2 in wells 1, 2 and 4-9 respectively were positive for β-lactamase production after incubation with nitrocefin solution for 10 minutes at 37°C (figure 5.4). After a further 1 hour incubation at 37°C there were no additional β-lactamase positive strains observed. Results for the isoelectric focusing of β-lactamase from the positive strains were poorly resolved and inconclusive (figure 5.4). The strains selected for β-lactamase testing based on PFGE and REAP type are depicted in table 5.3.

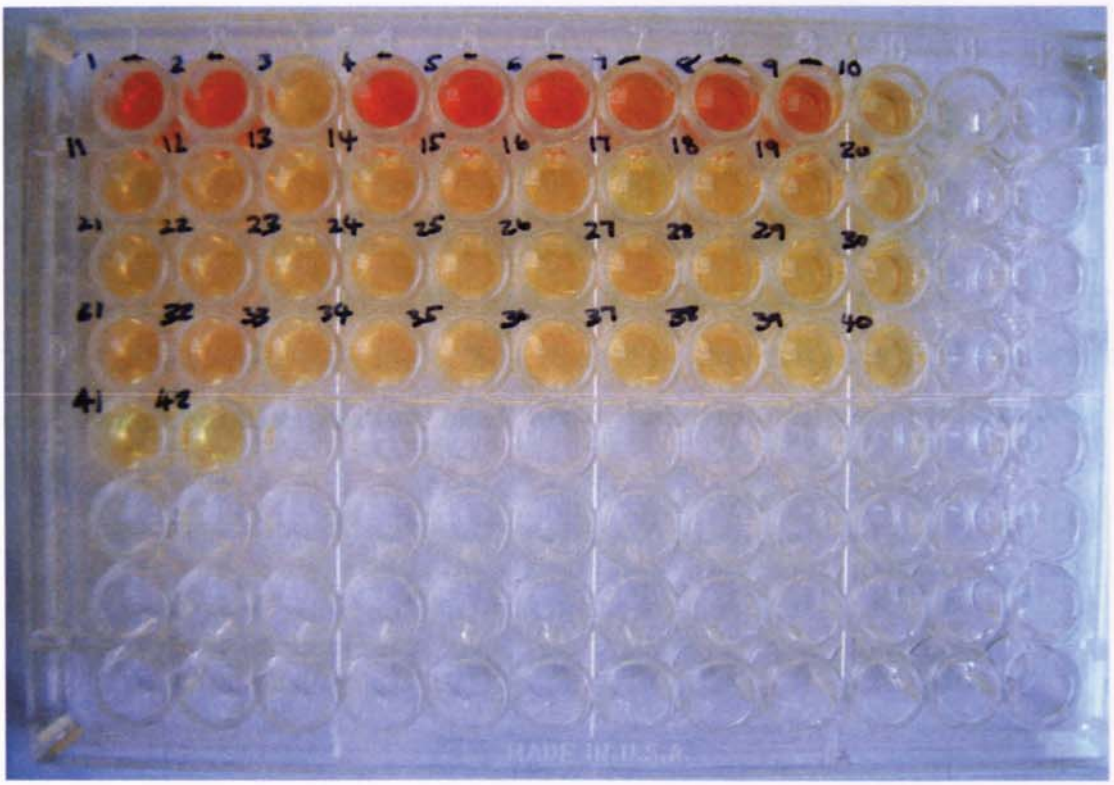


Figure 5.4 β-lactamase production of 42 MRSA strains representing each molecular type as determined by PFGE and REAP. Positive results were observed for strains D08T1, A01T2, E03 T1, B03T1, C06 T1, D02T1, E07T1 and C02T2 in wells 1, 2 and 4-9 respectively after 10 minutes incubation at 37°C. The results for strains tested using nitrocefin solution (pink/red indicates positive result; yellow indicates negative result) are summarised in table 5.4.

5.3.6 Isoelectric focusing

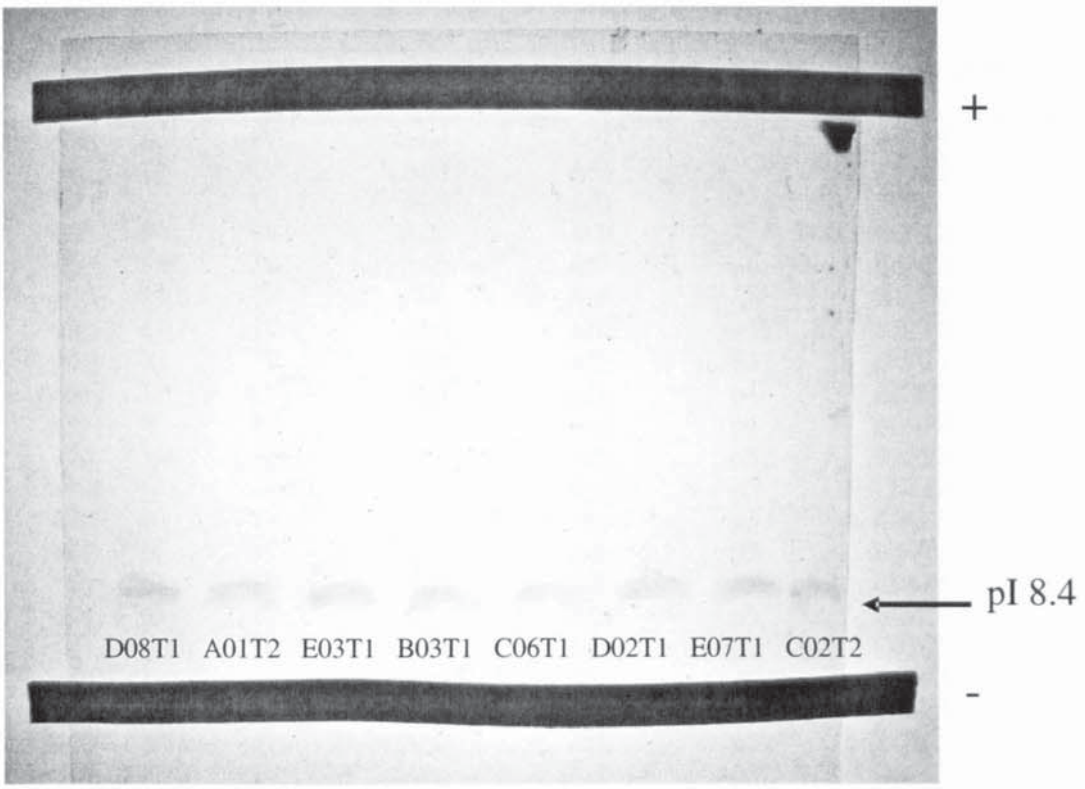


Figure 5.5 Isoelectric focusing of β-lactamase isolated from eight MRSA strains (strain codes indicate position, black arrow indicates position of β-lactamase at pI 8.4).

Table 5.3 Summary of the molecular typing data of 42 MRSA strains selected for testing of β-lactamase production.

[a]MR	[b]Code	PFGE type	SCC*mec* type	RAPD type 1254	RAPD type 797	REAP type	Strain code
-	400	A	IV	A(s)	A(l)	-	A01C1
-	400	B	IV	A(s)	A(l)	-	B09C1
-	400	C	IV	A(s)	A(l)	-	C09T1
-	400	D	IV	A(s)	A(l)	-	B06T2
-	400	U6	IV	A(s)	A(l)	p6	B03C1
-	400	U16	IV	A(s)	A(l)	-	A10T2
-	400	U17	IV	A(s)	A(l)	-	A07T2
-	401	B	IV	A(s)	A(l)	p3	D09T1
-	401	D	IV	A(s)	A(l)	p4	E02C1
-	421	B	IV	A(s)	A(l)	p4	A09T2
-	440	U3	new 2	A(s)	A(l)	p5	B05T2
-	440	U4	new 2	A(s)	U4(l)	p5	C01T2
-	440	A	IV	A(s)	A(l)	p5	E04T2
-	440	B	IV	A(s)	A(l)	p5	D04T1
-	440	C	IV	A(s)	A(l)	p5	C04T1
-	440	D	IV	A(s)	A(l)	p5	C03C1
-	440	U5	IV	A(s)	A(l)	p5	C07C1
-	440	U9	IV	A(s)	A(l)	p5	B06C1
-	440	U10	IV	A(s)	A(l)	p5	C06C1
-	440	U11	IV	A(s)	A(l)	p5	B07C1
-	440	U12	IV	A(s)	A(l)	p5	D07C1
-	440	U13	IV	A(s)	A(l)	p5	B05C1
-	440	U15	IV	A(s)	A(l)	p5	E05T2
-	441	U2	new 1	U2(s)	U2(l)	p2	B09T1
-	441	B	IV	A(s)	A(l)	p2	B10T1
-	441	B	IV	A(s)	A(l)	p4+p5	D06T1
-	441	D	IV	A(s)	A(l)	p4+p5	A03C1
-	441	U8	IV	A(s)	A(l)	p4+p5	B02T2
-	441	U14	IV	A(s)	U14(l)	p4+p5	C01C1
-	441	U18	IV	U18(s)	U18(l)	p5+p8	D08C1
-	441	U22	I	U22(s)	U22(l)	-	C02T2
-	451	U19	variant 1a	U19(s)	U19(l)	p1+p4+p7	D08T1
-	451	U23	III	U23(s)	U23(l)	p5	B04T1
-	461	C	IV	A(s)	A(l)	p2+p5	D05T2
-	560	E	IV	C(s)	C(l)	p1	A01T2
-	561	U1	variant IIIa	U1(s)	U1(l)	-	C03T1
-	640	U7	IV	A(s)	A(l)	p5	E09T1
MR	6473	U20	II	B(s)	B(l)	-	C06T1
MR	6473	U21	II	B(s)	U21(l)	-	E07T1
MR	6473	F	II	B(s)	B(l)	-	D02T1
MR	6477	G	II	B(s)	B(l)	-	E03T1
MR	6477	H	II	B(s)	B(l)	-	B03T1

[a]MR designates multidrug-resistance. (Isolates resistant to 6 or more antibiotics).
[b]Antibiogram code see table 3.4.

5.3.7 Summary of results

A summary of results is described in table 5.4. All MR-MRSA strains were SCC*mec* type II and belonged to two antibiogram types that could be differentiated by PFGE into five types and by RAPD analysis into two types. None of the MR-MRSA strains carried plasmid DNA and the MR-MRSA strains representing the five PFGE types selected for β-lactamase production tested positive for the enzyme. In addition, three unrelated HA-MRSA strains tested positive for β-lactamase production. All HA-MRSA and CA-MRSA strains that were resistant to more than one antibiotic in addition to flucloxacillin carried plasmid DNA with the exception of two HA-MRSA strains. One CA-MRSA strain carried a plasmid designated REAP type p6 that was not observed to confer any additional antibiotic resistance. The majority of HA-MRSA and CA-MRSA were SCC*mec* type IV. Single examples of HA-MRSA strains of SCC*mec* types I, Ia, III and IIIa were identified. In addition, two new SCC*mec* type variants designated new 1 and new 2 were observed in three HA-MRSA isolates.

Table 5.4 Summary of all antibiogram types, PFGE types, SCC*mec* element types, RAPD types using primers 1254 and 797, REAP types and β-lactamase production for 130 clinical MRSA strains.

[a]MR	[b]Code	PFGE type	SCC*mec* type	RAPD type 1254	RAPD type 797	REAP type	[c]β-lac	[d]Number. of isolates	
-	400	A	IV	A(s)	A(l)	-	-	1	(*1*)
-	400	B	IV	A(s)	A(l)	-	-	4	(*4*)
-	400	C	IV	A(s)	A(l)	-	-	10	(7, *3*)
-	400	D	IV	A(s)	A(l)	-	-	1	(1)
-	400	U6	IV	A(s)	A(l)	p6	-	1	(*1*)
-	400	U16	IV	A(s)	A(l)	-	-	1	(1)
-	400	U17	IV	A(s)	A(l)	-	-	1	(1)
-	401	B	IV	A(s)	A(l)	p3	-	1	(1)
-	401	D	IV	A(s)	A(l)	p4	-	2	(1, *1*)
-	421	B	IV	A(s)	A(l)	p4	-	2	(2)
-	440	U3	new 2	A(s)	A(l)	p5	-	1	(1)
-	440	U4	new 2	A(s)	U4(l)	p5	-	1	(1)
-	440	A	IV	A(s)	A(l)	p5	-	6	(3, *3*)
-	440	B	IV	A(s)	A(l)	p5	-	5	(1, *4*)
-	440	C	IV	A(s)	A(l)	p5	-	18	(14, *4*)
-	440	D	IV	A(s)	A(l)	p5	-	1	(*1*)
-	440	U5	IV	A(s)	A(l)	p5	-	1	(*1*)
-	440	U9	IV	A(s)	A(l)	p5	-	1	(*1*)
-	440	U10	IV	A(s)	A(l)	p5	-	1	(*1*)
-	440	U11	IV	A(s)	A(l)	p5	-	1	(*1*)
-	440	U12	IV	A(s)	A(l)	p5	-	1	(*1*)
-	440	U13	IV	A(s)	A(l)	p5	-	1	(*1*)
-	440	U15	IV	A(s)	A(l)	p5	-	1	(1)
-	441	U2	new 1	U2(s)	U2(l)	p2	-	1	(1)
-	441	B	IV	A(s)	A(l)	p2	-	1	(1)
-	441	B	IV	A(s)	A(l)	p4+p5	-	3	(1, 2)
-	441	D	IV	A(s)	A(l)	p4+p5	-	2	(2)
-	441	U8	IV	A(s)	A(l)	p4+p5	-	1	(1)
-	441	U14	IV	A(s)	U14(l)	p4+p5	-	1	(*1*)
-	441	U18	IV	U18(s)	U18(l)	p5+p8	-	1	(*1*)
-	441	U22	I	U22(s)	U22(l)	-	+	1	(1)
-	451	U19	variant 1a	U19(s)	U19(l)	p1+p4+p7	+	1	(1)
-	451	U23	III	U23(s)	U23(l)	p5	-	1	(1)
-	461	C	IV	A(s)	A(l)	p2+p5	-	2	(2)
-	560	E	IV	C(s)	C(l)	p1	+	2	(2)
-	561	U1	variant IIIa	U1(s)	U1(l)	-	-	1	(1)
-	640	U7	IV	A(s)	A(l)	p5	-	1	(1)
-	661	D	IV	A(s)	A(l)	p4+p5	-	2	(2)
MR	6473	U20	II	B(s)	B(l)	-	+	1	(1)
MR	6473	U21	II	B(s)	U21(l)	-	+	1	(1)
MR	6473	F	II	B(s)	B(l)	-	+	16	(15, *1*)
MR	6477	G	II	B(s)	B(l)	-	+	26	(25, *1*)
MR	6477	H	II	B(s)	B(l)	-	+	2	(2)

[a]MR designates multidrug-resistance. (Isolates resistant to 6 or more antibiotics).
[b]Antibiogram code see table 3.4.
[c]β-lac designates β-lactamase production.
[d]Total (Bold text indicates hospital acquired and italicised text indicates community-acquired isolates).

5.4 Discussion

The aim of this chapter was to characterise the MRSA isolates using SCC*mec* type assignment and REAP. In addition, MRSA strains were screened for the PVL gene, which is widely accepted as a marker for CA-MRSA strains when associated with SCC*mec* type IV (Lina *et al.*, 1999; Gillet *et al.*, 2002; Diep *et al.*, 2004; Holmes *et al.*, 2005). Screening of β-lactamase production was also performed on select strains to investigate whether the enzyme is produced by MRSA strains carrying the *mecA* gene.

5.4.1 MR-MRSA strains SCC*mec* type and plasmid DNA

It is widely accepted that plasmid DNA may confer antibiotic resistance (Lyon *et al.*, 1983; Morton *et al.*, 1995; Paulsen *et al.*, 1998; Hiramatsu *et al.*, 2001; O'Brien *et al.*, 2002) and therefore it may be expected that MR-MRSA strains may carry additional plasmids compared to MRSA strains with a less resistant phenotype. The results of this study however suggest that MR-MRSA strains were significantly associated ($p=<0.001$) with an absence of plasmid DNA. The results indicate that strains with the multidrug-resistance phenotype belong to one of five closely related PFGE profile types, possess the type II SCC*mec* element and do not carry plasmid DNA, therefore all resistance determinants in the MR-MRSA strains must be chromosomally encoded.

The type II SCC*mec* element, in addition to *mecA*, carries genes that confer resistance to non-β-lactam antibiotics (Ito *et al.*, 2001). In the case of type II SCC*mec* an integrated copy of plasmid pUB110 and transposon Tn*554* are carried in addition to the *mecA* gene conferring resistance to a number of antibiotics. The plasmid pUB110 encodes tobramycin and gentamicin resistance while transposon Tn*554* encodes erythromycin resistance (Hiramatsu *et al.*, 2001; Oliveira *et al.*, 2001a). Both erythromycin and gentamicin resistance were observed in the MR-MRSA strains from the UHB. It is possible that the type II SCC*mec* element in these MR-MRSA strains is similar to the type II SCC*mec* elements sequenced from the type strains N315 and Mu50. Both of these SCC*mec* elements encode resistance to β-lactams, macrolide-lincosamide-streptogramin B, and aminoglycosides (Hiramatsu *et al.*, 2001).

Additional point mutations and possible acquisition of resistance genes that are now chromosomally encoded may further explain the antibiotic resistance phenotype expressed by the MR-HA-MRSA. Two of the CA-MRSA isolates in this study were found to have the multidrug-resistance phenotype. Both possessed the type II SCC*mec* element and the same PFGE profiles as the MR-HA-MRSA strains; one shared the PFGE type F profile and the other shared the PFGE type G profile.

5.4.2 NMR-MRSA strains SCC*mec* type and plasmid DNA

Typically, it was observed that NMR-MRSA strains that were SCC*mec* type IV that carried one or more plasmids demonstrated a more resistant phenotype than strains that carried no plasmid DNA. Ten HA-MRSA strains and five CA-MRSA strains belonging to PFGE types A-D and SCC*mec* type IV did not carry plasmid DNA and were only resistant to flucloxacillin. The type IV SCC*mec* only carries the *mecA* gene (Okuma *et al.*, 2002; Vandenesch *et al.*, 2003) and therefore the absence of resistance to antibiotics other than flucloxacillin indicates that strains that are PFGE types A-D and that do not carry plasmid DNA may not possess any additional chromosomally encoded resistance genes. It was observed that HA-MRSA and CA-MRSA strains of PFGE type A-D that carried plasmid DNA were resistant to antibiotics other than flucloxacillin and that based on this observation it may be possible to predict antibiotic resistance genes encoded by different plasmid types. All MRSA strains that carried the REAP type p5 plasmid were observed to be erythromycin resistant. This may indicate that plasmid p5 carries the plasmid borne erythromycin resistance gene *ermC* (Projan *et al.*, 1987). Erythromycin resistance in MRSA is not uncommon but is typically observed in strains that carry the chromosomally encoded *ermA* gene (Westh *et al.*, 1995; Lina *et al.*, 1999). To investigate whether the REAP type p5 plasmid encodes a functional copy of the *ermC* gene it may be possible to cure carrier strains of the plasmid and then perform AST to confirm that sensitivity to erythromycin is restored (Sambrook *et al.*, 1989). Alternatively, a PCR reaction for the detection of the *ermC* gene as described by (Lina *et al.*, 1999; Martineau *et al.*, 2000a) may be carried out. The latter process would be less time consuming but would not confirm functional expression. It was also observed that trimethoprim resistance was associated with carriage of the REAP type p3 and p4 plasmids and resistance to both erythromycin and trimethoprim was associated with carriage of the REAP type p2

plasmid. Resistance to trimethoprim may be either chromosomally encoded (Dale *et al.*, 1995) or plasmid encoded (Burdeska *et al.*, 1990). The latter is associated with a type S1 trimethoprim-resistant DHFR encoded by the *dfrA* gene located on transposon Tn*4003* (Burdeska *et al.*, 1990). A plasmid encoded *dfrA* gene may be detected by curing As discussed previously (Sambrook *et al.*, 1989) or by PCR as described by (Dale *et al.*, 1995).

The PVL gene was not carried by any of the HA- or CA-MRSA strains but was detected in the positive control strain. This indicates that PVL carriage in clinical MRSA isolates may remain low and supports the findings of other groups (Holmes *et al.*, 2005). In addition highly virulent MRSA and MSSA strains that carry the PVL gene have been reported to be extremely invasive and in several cases fatal (Anonymous, 1999), therefore it may be necessary to maintain screening for the PVL gene for infection control management in both hospitals and the community (Holmes *et al.*, 2005).

5.4.3 Unique MRSA strains

A total of 14 NMR-MRSA strains were SCC*mec* type IV, which accounted for approximately 61% of all unique strains, as determined by PFGE. This may indicate that SCC*mec* type IV was associated with strains from the most diverse genetic backgrounds. Further analysis using MLST would give a stronger indication of the genetic backgrounds of MRSA strains (Enright *et al.*, 2000), however, these preliminary findings provide support for the acquisition of the SCC*mec* type IV element by multiple lineages of *S. aureus* and concur with reports by other groups (Coombs *et al.*, 2004). In addition to the type IV SCC*mec* element unique NMR-MRSA strains of type I, Ia, III, IIIa and two new types designated new 1 and new 2 were identified. A SCC*mec* type Ia and III strain were observed to be the only two NMR-MRSA strains resistant to gentamicin. The SCC*mec* type Ia carries a copy of pUB110 the detection of which is used to distinguish it from the SCC*mec* type I element (Oliveira and de Lencastre, 2002). This integrated plasmid possesses the same structure as that identified in the SCC*mec* type II element and was discussed previously. A SCC*mec* type III strain was identified as being resistant to gentamicin and sensitive to tetracycline. The SCC*mec* type III element does not encode genes that

confer resistance to aminoglycosides but does carry an integrated plasmid designated pT181 that encodes tetracycline resistance (Oliveira *et al.*, 2000; Hiramatsu *et al.*, 2001). It is possible that this strain carries a non-functional copy of pT181 and a chromosomal copy of transposon Tn*4001* which encodes the *aac(6')-Ie+aph(2'')* gene that confers gentamicin resistance (Ubukata *et al.*, 1984). In addition, a SCC*mec* type IIIa strain that did not carry plasmid DNA was identified as being resistant to tetracycline. The SCC*mec* type IIIa element is distinguished from the SCC*mec* type III element by the absence of pT181 (Oliveira and de Lencastre, 2002). Resistance may be due to *tet*(K), *tet*(L), *tet*(M) or *tet*(O) genes integrated elsewhere on the chromosome (Chopra and Roberts, 2001). Based on PFGE type the SCC*mec* type new 1 was most closely related to the SCC*mec* type IIIa strain (approximately 70%). The two SCC*mec* type new 2 strains were most closely related to the PFGE type A, SCC*mec* type IV strains (95%). A long range PCR followed by nucleotide sequencing as described by (Ito *et al.*, 1999) would be an appropriate approach for confirming the structural relationships of the new SCC*mec* types with previously reported SCC*mec* elements (Ito *et al.*, 1999; Ito *et al.*, 2001; Oliveira *et al.*, 2001a; Ma *et al.*, 2002; Ito *et al.*, 2004).

5.4.4 Molecular analysis summarised

Interestingly, most of the NMR-HA-MRSA were of the type IV SCC*mec* element assignment; an assignment commonly associated with CA-MRSA (Daum *et al.*, 2002; Ma *et al.*, 2002). Using PFGE, RAPD, SCC*mec* type assignment, antibiotic resistance phenotypes and restriction enzyme fragmentation patterns of plasmid DNA it was not possible to distinguish HA-MRSA from CA-MRSA. In addition, each REAP type profile observed in the HA-MRSA isolates was also observed in the CA-MRSA isolates, with two exceptions (plasmids p6 and p8). These observations indicate that MRSA strains possessing the type IV SCC*mec* element and carrying resistance plasmids may be better able to survive and spread in both the hospital and community environs (Coombs *et al.*, 2004). The fact that few MR-MRSA isolates were identified in the community may suggest that an MRSA population with a multidrug-resistance phenotype and possessing the type II SCC*mec* element is only truly sustainable in a hospital environment where antibiotic pressure is relatively high. In addition, the fact that the majority of HA-MRSA strains from the UHB are closely related to CA-

MRSA strains and that they are SCC*mec* type IV supports the observations of (Hiramatsu *et al.*, 2001); that SCC*mec* type IV represents a highly successful SCC*mec* element in both the hospital and community environs. Currently, it is only possible to speculate what biological cost antibiotic resistance comes at for MR-MRSA and MRSA in both hospitals and in the community (Andersson and Levin, 1999; Bjorkman and Andersson, 2000; Bjorkman *et al.*, 2000).

A discriminatory power of 0.88 was obtained when MRSA strains were typed using PFGE. The discriminatory power was increased to 0.92 when PFGE was combined with REAP, which was expected as PFGE characterises strains based on differences in chromosomal DNA restriction patterns and REAP characterises strains based on differences in plasmid DNA restriction patterns. Performing both techniques provides a high level of discrimination and allows for relationships between genotypic and phenotypic characteristics to be assessed however, combining both techniques remains time consuming and labour intensive. Characterising strains using RAPD analysis or SCC*mec* type assignment combined with REAP provided discriminatory powers of D=0.78 and D=0.77, respectively. Combining REAP with a PCR technique allows for the differentiation of MR-MRSA from NMR-MRSA strains and dramatically improves discriminatory power. In addition, results from all three techniques may still be obtained within a single working day.

5.4.5 Screening for β-lactamase production

The preliminary findings of the β-lactamase study indicated that approximately 38.5% (50 out of 130) of MRSA strains from the UHB produce β-lactamase. This estimate assumes that all the untested strains of the same PFGE type and REAP type also produce β-lactamase. Typically, in staphylococci the β-lactamase gene *blaZ* is plasmid encoded (Livermore, 1995), however, the results of this study indicated that β-lactamase production was associated with MR-MRSA strains and one unique MRSA strain (U22), which did not carry plasmid DNA. This suggests that *blaZ* may be chromosomally encoded in the MR-MRSA strains and MRSA strain. Chromosomally encoded *blaZ* has been reported in *S. aureus* (Voladri and Kernodle, 1998) and is typically associated with carriage on Tn*552*-related transposons (Lyon and Skurray, 1987), however, it is uncertain whether a wild-type MRSA strain carrying

chromosomally encoded *blaZ* has ever been reported. Two strains carrying plasmid DNA (D08T1 and A01T2) also tested positive for β-lactamase production. Both types were the only MRSA strains to carry the plasmid designated p1. Therefore, it may be speculated that in these two cases the *blaZ* operon is plasmid encoded. This could be confirmed by curing the strains of their plasmid DNA (Sambrook *et al.*, 1989). In addition, carriage of *blaZ* on chromosomal or plasmid DNA could be further assessed using PCR (Martineau *et al.*, 2000a; Sidhu *et al.*, 2002).

5.4.6 Isoelectric focusing

To determine the type of β-lactamases produced by positive MRSA strains from the UHB, IEF was employed. Isoelectric focusing has been successfully used to type β-lactamases from Gram-negative species, however, has been found to be unsatisfactory for those of staphylococci (Livermore, 1995). Previous studies have reported that staphylococcal β-lactamases smear on IEF gels and that this is probably due to poor insolubility around their isoelectric points (Zygmunt *et al.*, 1992). The results of this preliminary study concur with these findings and conclude that IEF is not suitable for typing β-lactamase from *S. aureus*. Measuring the relative activities of cell suspensions of *S. aureus* against cefazolin, cephaloridine and nitrocefin has been reported to be a suitable alternative (Kernodle *et al.*, 1990; Zygmunt *et al.*, 1992) and would certainly be considered for future studies.

5.4.7 Conclusion

In summary this study demonstrates that a multidrug-resistance phenotype in MRSA from the UHB is associated with one of five PFGE profile types, the type II SCC*mec* assignment and the absence of plasmid DNA and that the majority of NMR-HA-MRSA and CA-MRSA are type IV SCC*mec* and of a similar lineage. Combining a PCR technique such as RAPD or SCC*mec* type assignment with REAP provides a rapid and simple molecular typing system with a high discriminatory power that may be suitable for managing infection control and directing therapy. The combination of PFGE and REAP provided the highest discriminatory power but remains time consuming and expensive. Statistical analysis of banding patterns generated using these methods may identify discriminatory bands, suitable for a probe-mediated

typing system that is less time consuming and does not require expensive peripheral equipment.

CHAPTER 6 PRINCIPLE COMPONENTS ANALYSIS

6.1 Introduction

Pulsed-field gel electrophoresis is the most widely employed genotypic method used in reference and clinical laboratories for typing MRSA (Murchan *et al.*, 2003). The technique is highly reproducible when the same protocol is followed and criteria exist for the standardised interpretation of profiles (Tenover *et al.*, 1995).

To determine strain relationships from molecular typing techniques statistical packages that perform cluster analyses are typically employed. This approach organises strain profile types by similarity or difference indices with the objective of producing a hierarchical classification of isolates. The most commonly employed hierarchic clustering methods include nearest-neighbour, furthest-neighbour and unweighted pair-group method using arithmetic averages (UPGMA) cluster analyses (Clifford and Sokal, 1975); the most frequently used of which is UPGMA. When employing UPGMA to construct a dendrogram (tree diagram) an assumption is made during the calculation that each molecular strain-type is diverged equally from another type, when often this is not the case (Riley, 2004). This may lead to distinct strain profile types being organised in a dendrogram whereby types which are in fact unrelated are presented as closely related or *vice versa*.

In contrast, principal components analysis (PCA) can be employed to identify clustering within a group of types without arranging and presenting them in a hierarchical format. Principal components analysis is a multivariate analytical technique which can be applied to measurements that are continuous or binary and is therefore suitable for the analysis of data derived from many molecular strain-typing techniques including PFGE (Riley, 2004). Molecular strain-typing techniques that generate banding patterns may be coded into a binary format and compared using PCA (Baleiras Couto *et al.*, 1995). Principal components analysis is employed to reduce the dimension of the binary data by combining correlated features in the data into a set of new orthogonal (independent) variables called principle components (PCs). An advantage of using PCA to analyse complex multivariate data is that results

can be presented in a graph and therefore samples can be projected in the same score plot, revealing their similarity or dissimilarity to reference samples (Baleiras Couto *et al.*, 1995). Since the PCs are orthogonal, the similarity between types can be determined by simply measuring the Euclidian distance between their scores in the PCA score plot. Types which are similar will be closer to each other while dissimilar types will be farther apart when the relevant PCA coordinates are entered in a score plot. Data is thus presented based on actual type relationship and in a nonhierarchic format. Furthermore, data generated by PCA from binary profiles can be further analysed to identify key discriminatory bands within a genotypic profile which may be difficult when profiles are complex.

In this chapter PCA was employed as an alternative method to cluster analysis. The technique was used to represent the relationships between MRSA strains in a nonhierarchic format, and to identify statistically valid discriminatory PFGE bands within a group of molecular strain-types. In principal the bands could then be assessed for use as molecular probes or targets in conjunction with other molecular strain-typing techniques such as binary typing.

6.2 Materials and methods

6.2.1 PFGE and cluster analysis

The PFGE profiles and cluster analysis data is described previously in section 4.2.1.

6.2.2 SCC*mec* type

The SCC*mec* type assignment data is described previously in section 5.2.1.

6.2.3 Converting data for principal components analysis (PCA)

The molecular size of each band in each PFGE profile was determined using Gel Compar II, by comparison of individual profiles with the standard molecular size marker profile of NCTC 8325. Due to distortion in the gels small variations in the molecular size of comparable bands were observed. Based on visual examination of each profile, bands of corresponding molecular size were corrected by assigning the same molecular size to comparable bands in different gels. The molecular size of each band within a profile was transformed into a binary pattern, suitable for analysis by PCA, whereby the presence or absence of a band was represented by a one or a zero respectively. In this way a binary matrix table of the complete molecular size range of bands for each profile was built with each profile reserved to a column. Binary profiles for all 130 isolates were taken forward for analysis.

6.2.4 PCA data analysis

All data analysis was performed on the binary matrix table in the statistics package Statistica 6.0 (Statistica, StatSoft, Tulsa, USA). The number of principle components (PCs) extracted was determined using an eigenvalue plot (figure 6.2) and the 10% stopping rule, i.e., all PCs with an eigenvalue contributing $\geq 10\%$ to the total variance were included in the analysis (Armstrong *et al.*, 1996). The PCs explaining the greatest amount of variance and hence the most descriptive were presented graphically

(figure 6.3). The graphical markers depicting the position of a profile were given the same label assigned during the cluster analysis.

6.2.5 Correlation coefficient for identification of descriptive bands

The binary matrix table was transposed allowing each binary profile to be presented in rows. The unrotated factor loadings for each profile, generated during the PCA data analysis, were then incorporated into the table, positioned at the end of each isolate's corresponding binary profile. The nonparametric correlation statistic, Spearman's rank correlation coefficient (r_s), was performed comparing each profile with its respective factor loading. An output table was generated and results where $r_s = \geq 0.70$ and ≤ -0.70 were considered significant. Bands that were found to be significant and possessed by MR- MRSA strains were identified. Bands that were found to be highly significant and exclusive to MR-MRSA were considered suitable discriminatory probes for future work. A summary table of the significant results for PC1 only, PC2 only and PC1 and PC2 is presented in table 6.2.

6.3 Results

6.3.1 Cluster analysis of PFGE data

Analysis of the PFGE data revealed 31 different PFGE profiles (figure 6.1). Eight PFGE clusters comprising two or more isolates were designated types A-H. Two MR-HA-MRSA isolates, twelve HA-MRSA isolates and nine CA-MRSA isolates gave unique profiles and were given the designation U followed by a number relating to the position of the isolate in the dendrogram. The MR-HA-MRSA and MR-CA-MRSA isolates were separated into three clusters designated F-H with two unique types designated U20 and U21. The NMR-HA-MRSA and NMR-CA-MRSA isolates were separated into four main clusters designated A-E with 21 unique types.

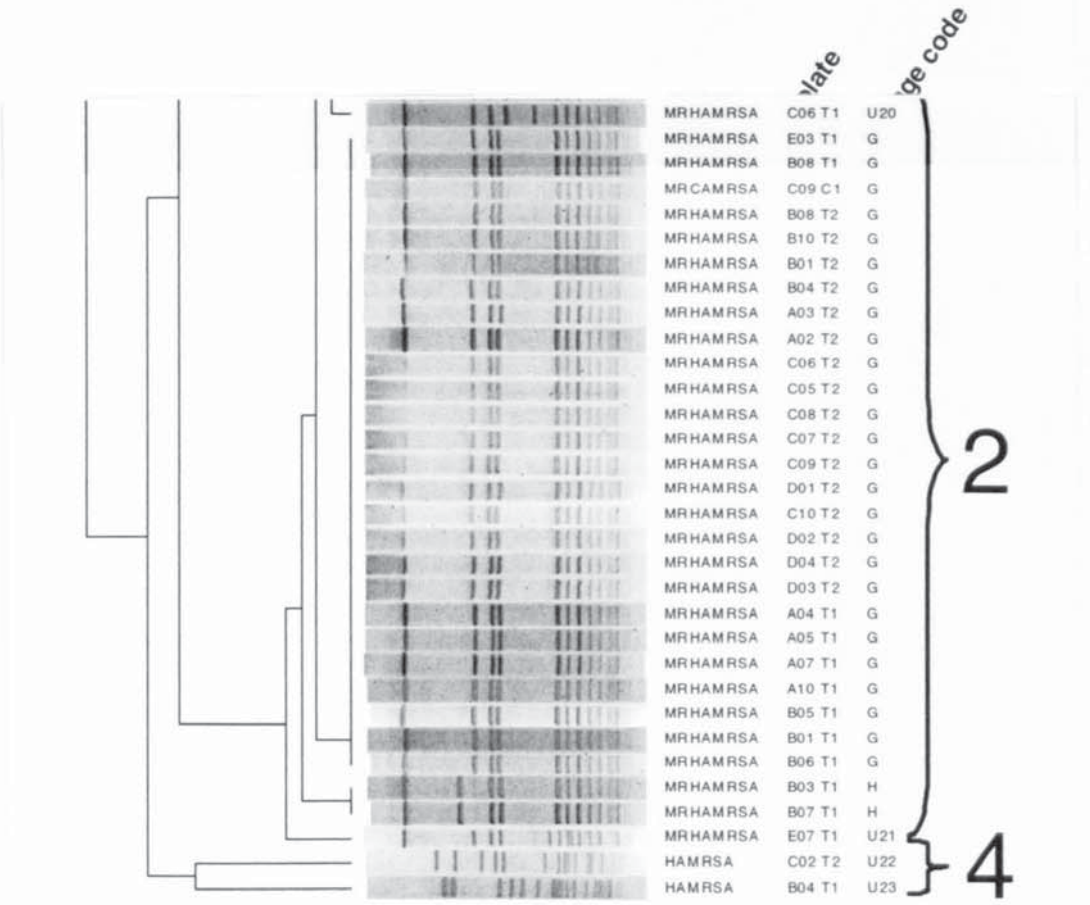
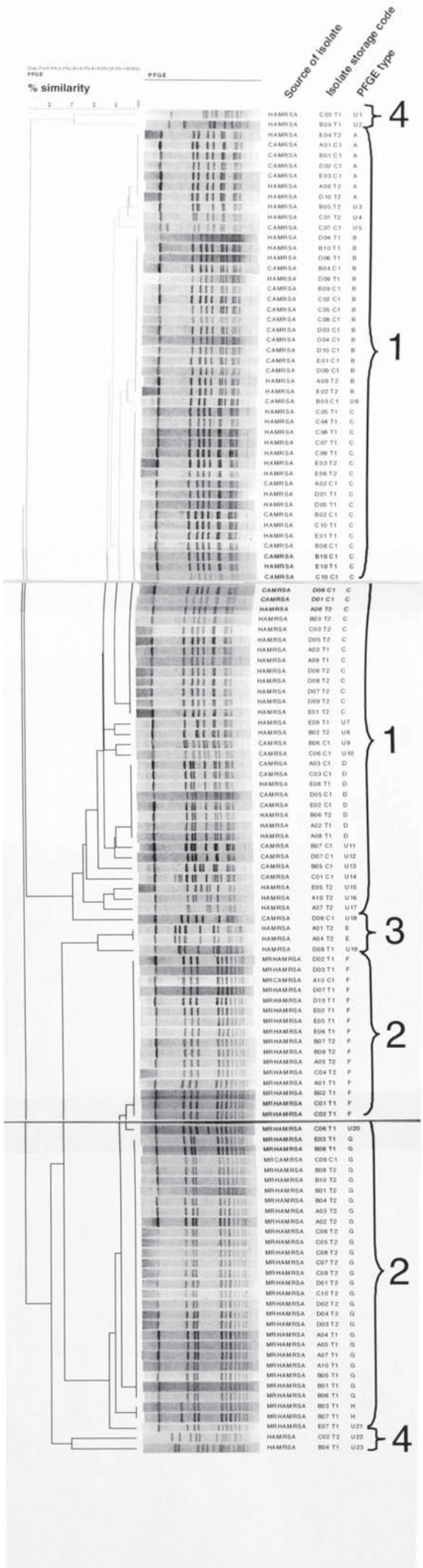


Figure 6.1 Dendrogram of all pulsed-field gel electrophoresis profiles of methicillin-resistant *S. aureus* isolates. The similarity between the isolates was calculated by the Dice coefficient, and a dendrogram was constructed using UPGMA clustering. Main clusters labelled A-H. Unique profiles are labelled U1 to U23 in descending order.


PFGE
% similarity
PFGE
Source of isolate
Isolate storage code
PFGE type
HAMRSA C03 T1 U1
HAMRSA B09 T1 U2
HAMRSA E04 T2 A
CAMRSA A01 C1 A
CAMRSA B01 C1 A
CAMRSA D02 C1 A
CAMRSA E03 C1 A
HAMRSA A06 T2 A
HAMRSA D10 T2 A
HAMRSA B05 T2 U3
HAMRSA C01 T2 U4
CAMRSA C07 C1 U5
HAMRSA D04 T1 B
HAMRSA B10 T1 B
HAMRSA D06 T1 B
CAMRSA B04 C1 B
HAMRSA D09 T1 B
CAMRSA B09 C1 B
CAMRSA C02 C1 B
CAMRSA C05 C1 B
CAMRSA C08 C1 B
CAMRSA D03 C1 B
CAMRSA D04 C1 B
CAMRSA D10 C1 B
CAMRSA E01 C1 B
CAMRSA D09 C1 B
HAMRSA A09 T2 B
HAMRSA E02 T2 B
CAMRSA B03 C1 U6
HAMRSA C05 T1 C
HAMRSA C04 T1 C
HAMRSA C06 T1 C
HAMRSA C07 T1 C
HAMRSA C09 T1 C
HAMRSA E03 T2 C
HAMRSA E06 T2 C
CAMRSA A02 C1 C
HAMRSA D01 T1 C
HAMRSA D05 T1 C
CAMRSA B02 C1 C
HAMRSA C10 T1 C
HAMRSA E01 T1 C
CAMRSA B08 C1 C
CAMRSA B10 C1 C
HAMRSA E10 T1 C
CAMRSA C10 C1 C
CAMRSA D06 C1 C
CAMRSA D01 C1 C
HAMRSA A08 T2 C
HAMRSA B03 T2 C
HAMRSA C03 T2 C
HAMRSA D05 T2 C
HAMRSA A03 T1 C
HAMRSA A09 T1 C
HAMRSA D06 T2 C
HAMRSA D08 T2 C
HAMRSA D07 T2 C
HAMRSA D09 T2 C
HAMRSA E01 T2 C
HAMRSA E09 T1 U7
HAMRSA B02 T2 U8
CAMRSA B06 C1 U9
CAMRSA C06 C1 U10
CAMRSA A03 C1 D
CAMRSA C03 C1 D
HAMRSA E08 T1 D
CAMRSA D05 C1 D
CAMRSA E02 C1 D
HAMRSA B06 T2 D
HAMRSA A02 T1 D
HAMRSA A08 T1 D
CAMRSA B07 C1 U11
CAMRSA D07 C1 U12
CAMRSA B05 C1 U13
CAMRSA C01 C1 U14
HAMRSA E05 T2 U15
HAMRSA A10 T2 U16
HAMRSA A07 T2 U17
CAMRSA D08 C1 U18
HAMRSA A01 T2 E
HAMRSA A04 T2 E
HAMRSA D08 T1 U19
MRHAMRSA D02 T1 F
MRHAMRSA D03 T1 F
MRCAMRSA A10 C1 F
MRHAMRSA D07 T1 F
MRHAMRSA D10 T1 F
MRHAMRSA E02 T1 F
MRHAMRSA E05 T1 F
MRHAMRSA E06 T1 F
MRHAMRSA B07 T2 F
MRHAMRSA B09 T2 F
MRHAMRSA A05 T2 F
MRHAMRSA C04 T2 F
MRHAMRSA A01 T1 F
MRHAMRSA B02 T1 F
MRHAMRSA C01 T1 F
MRHAMRSA C02 T1 F
MRHAMRSA C06 T1 U20
MRHAMRSA E03 T1 G
MRHAMRSA B08 T1 G
MRCAMRSA C09 C1 G
MRHAMRSA B09 T2 G
MRHAMRSA B10 T2 G
MRHAMRSA B01 T2 G
MRHAMRSA B04 T2 G
MRHAMRSA A03 T2 G
MRHAMRSA A02 T2 G
MRHAMRSA C06 T2 G
MRHAMRSA C05 T2 G
MRHAMRSA C08 T2 G
MRHAMRSA C07 T2 G
MRHAMRSA C09 T2 G
MRHAMRSA D01 T2 G
MRHAMRSA C10 T2 G
MRHAMRSA D02 T2 G
MRHAMRSA D04 T2 G
MRHAMRSA D03 T2 G
MRHAMRSA A04 T1 G
MRHAMRSA A05 T1 G
MRHAMRSA A07 T1 G
MRHAMRSA A10 T1 G
MRHAMRSA B05 T1 G
MRHAMRSA B01 T1 G
MRHAMRSA B06 T1 G
MRHAMRSA B03 T1 H
MRHAMRSA B07 T1 H
MRHAMRSA E07 T1 U21
HAMRSA C02 T2 U22
HAMRSA B04 T1 U23
4
1
1
3
2
2
4

6.3.2 Principal components analysis

Two principle components (PCs) were extracted from the data as determined by a scree plot (figure 6.2), PC1 accounted for 64% and PC2 23% of the total variance in the data. A plot of PC1 versus PC2 for the 130 PFGE profiles is shown in figure 6.3. All 31 profile types were represented graphically and clustered into one of four distinct groups labelled 1-4; group 1 containing the greatest number of isolates (n=76); group 2 containing the next greatest (n=46); group 3 (n=4) and 4 (n=4) containing the least. Groups 1, 3 and 4 were representative of the 26 NMR-MRSA types, while group 2 represented all 5 of the MR-MRSA types. Table 6.1 summarises the molecular typing data for each group.

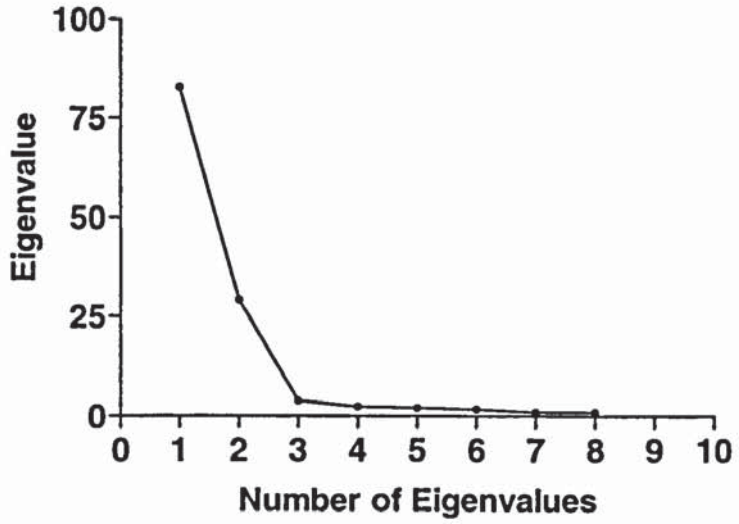


Figure 6.2 Scree plot of Eigenvalues for determining suitability of extracted principle components for analysis of the binary data set. Eigenvalues that contribute ≥ 10% of the total variance were included in the analysis.

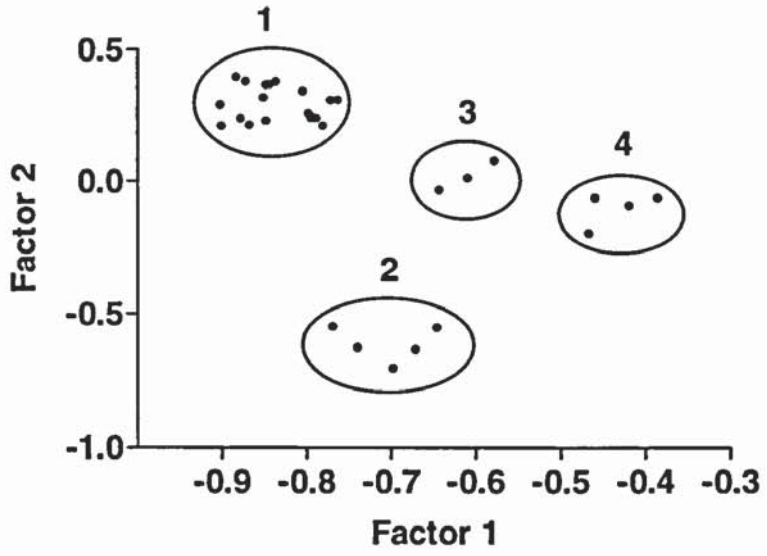


Figure 6.3 Plot of PC1 (factor 1) and PC2 (factor 2) of the binary profiles generated from 130 MRSA isolates after pulsed-field gel electrophoresis. Numbered clusters indicate comparable clustering observed with UPGMA cluster analysis (see corresponding numbered clusters in figure 6.1.1, 6.1.2 and 6.1.3). Group 1 comprises profile types A, B, C, D and U3-U17, group 2 comprises F, G, H, U20 and U21, group 3 comprises E, U18 and U19 and group 4 comprises U1, U2, U22 and U23.

Table 6.1 Summary of antibiotic sensitivity and molecular typing data for the PCA grouping of 130 MRSA isolates.

Group	Multidrug resistant	PFGE types	SCC*mec* types
1	No	A-C, U3-U17	new 2, IV
2	Yes	F-H, U20-U21	II
3	No	E, U18, U19	Ia, IV
4	No	U1, U2, U22, U23	new 1, I, III, IIIa

6.3.3 Spearman's r_s and descriptive bands

Analysis of binary profiles with Spearman's r_s identified a total of 15 highly significant discriminatory bands. Using the significance criteria, $r_s = \geq 0.70$ and ≤ -0.70, five and ten discriminatory bands were attributed to PC2 only and PC1 and PC2 combined respectively. No discriminatory bands were attributed to PC1 only. Four bands with molecular sizes of 290 kbp, 236kbp, 217 kbp and 102 kbp were identified as potential discriminatory markers for MR-MRSA (table 6.2 and figure 6.4). No bands were identified as potential markers for CA-MRSA.

Table 6.2 Summary of significant Spearman R correlation coefficients after comparison of binary representation of pulsed-field gel electrophoresis profiles of methicillin-resistant *S. aureus* with factor loadings from principal components analysis.

*Rank	Molecular size of highly discriminatory bands (kbp)	Significant correlation coefficient		
		PC1 only	PC2 only	PC1 and PC2
1	236†		0.835	
2	290†		0.814	
3	127		**0.808**	
4	102†		0.754	
5	217†		0.712	
1	44			**0.864** + 0.864
2	161			0.828 + **0.848**
3	71			0.774 + **0.833**
4	59			0.768 + **0.848**
5	48			**0.759** + 0.849
6	115			0.728 + **0.837**
7	68			0.726 + **0.845**
8	85			**0.726** + 0.845
9	50			**0.705** + 0.841
10	36			**0.701** + 0.839

* Ranked based on highest correlation coefficient (r value) of PC1 first and PC2 second.
† Indicates bands suitable for discriminating multi-drug resistant isolates.
Indicates positive value / indicates negative value.

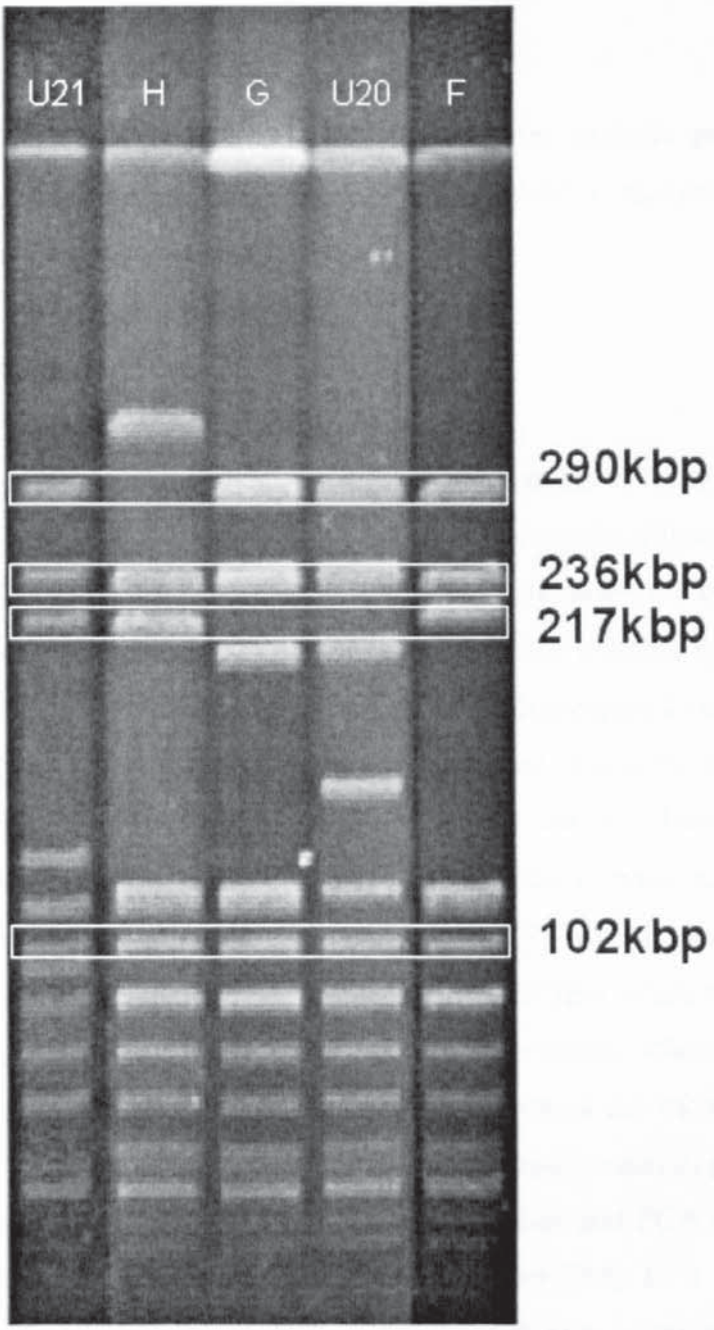


Figure 6.4 Position and molecular size of the four most discriminatory PFGE bands for MR-MRSA strains of PFGE types U21, H, G, U20 and F.

6.4 Discussion

The aim of this chapter was to perform PCA and cluster analysis and to compare strain clustering. In addition, the suitability of statistical analysis to identify discriminatory bands was assessed.

6.4.1 PCA and cluster analysis

The results of the PCA performed on the binary data obtained from the molecular strain-profiles are comparable with those of the cluster analysis, although there are a number of key differences. The most notable difference between the two methods is the way in which strains U1, U2, U22 and U23 are clustered together by PCA; cluster analysis places strains U1 and U2 at opposite ends of a dendrogram to strains U22 and U23 (figure 6.1.1, 6.1.2 and 6.1.3). Based on the visual similarity of the banding patterns for strains U1, U2, U22 and U23 it seems logical that PCA has clustered these strains together to form a separate group designated in this instance as group 4. The strains in group 4 appear most similar to strains in group 3 based on visual comparison of the PFGE banding patterns and are considerably different when compared with strains in group 1 and group 2. In support of this observation, when the Euclidean distances between samples in group 4 and the other groups are measured the data confirm strains in group 4 to be most closely related to those strains in group 3 (figure 6.3). The apparent differences between the cluster analysis and PCA results directly affect the interpretation of such findings and may contribute to a more accurate interpretation of strain relatedness; PCA is often employed to represent intra and interspecies relationships in taxonomical studies (Riley, 2004).

6.4.2 PCA score plot and discriminatory bands

The PCA score plot (figure 6.3) indicates that the variation between groups 1, 3 and 4 is best explained along the PC1 axis (factor 1), while variations between the MR-MRSA (group 2) and the NMR-MRSA (groups 1, 3 and 4) are best explained along the PC2 axis (factor 2). This divergence is supported by analysis using Spearman's r_s, whereby the most significant bands for MR-MRSA were found to be strongly

correlated with PC2 only. The information derived from Spearman's r_s allows for the statistical data generated from the PCA to be translated into meaningful information that refers directly to specific bands in a strain-type. During this study four bands were identified as highly descriptive markers for MR-MRSA strains. These had molecular sizes of 290 kbp, 236kbp 217 kbp and 102 kbp (table 6.2 and figure 6.4).

6.4.3 PCA and cluster analysis summary

Strain relatedness calculated by the Dice coefficient and dendrogram construction using UPGMA clustering are the most commonly used methods employed in the analysis of many molecular strain-typing techniques, including PFGE. However, this type of hierarchic phenetic clustering method may give an unrealistic representation of true epidemiologic relationships (Riley, 2004). In contrast, PCA is a nonhierarchic phenetic method (Riley, 2004) that attempts to display the pattern of variation between the molecular strain-types by plotting them in relation to axes which describe the greatest sources of variation in the data (Armstrong *et al.*, 1996). Hence, PCA does not assume that distinct groups of strain-profiles exist and is most useful in identifying important sources of variation between strain-profiles. Performing PCA in conjunction with cluster analysis is quick and simple to perform on molecular strain-types and provides a meaningful nonhierarchic representation of strain relatedness that is simple to interpret. Score plots readily illustrate strain groupings and the ability to measure Euclidean distances between individual points gives an improved perspective of strain relatedness. It is also possible to employ a statistically valid method to identify the most discriminatory bands. By using a statistical test such as Spearman's r_s it is possible to correlate factor loadings, generated by PCA, with the binary profile data and successfully identify descriptive bands from molecular strain-types. The most descriptive bands can be identified and used in conjunction with other techniques i.e. probes for a hybridisation method (van Leeuwen *et al.*, 1999) or primer targets for a PCR technique. Many current statistical software packages are able to perform PCA, making it readily available to most research groups. In addition, dedicated analysis software is available that will automatically perform PCA on genotypic profiles, however, they tend to provide a graphical output which is lacking in the numerical data which would be required for further analysis. To perform additional analysis on

PCA data it is necessary to be able to access the main statistical outputs, such as factor loadings.

6.4.4 Conclusion

In summary, this study provides a descriptive, detailed method for performing PCA using a database of PFGE profiles of MRSA isolates as a worked example. In comparison to the more typically employed hierarchic method of representing relationships between isolates using dendrograms, PCA, as a nonhierarchic technique, does not attempt to form relationships between profile types that may not readily exist. In this way PCA may represent more accurately true epidemiological relationships. A further benefit of PCA is the ability to perform post-analysis to identify the most descriptive or discriminatory bands within a database of complex profiles. Performing PCA may therefore provide valuable additional information from profile analysis to complement the epidemiological study of infectious diseases and may identify DNA probes suitable for a probe-mediated typing system.

CHAPTER 7 BINARY TYPING OF MRSA

7.1 Introduction

Multiple molecular typing techniques exist for differentiating MRSA strains, which vary in their speed, discriminatory ability and cost. For example PFGE is a highly discriminatory method and the gold standard for the typing of MRSA (Murchan *et al.*, 2003), however, the technique is time consuming and expensive; requiring peripheral electrophoresis equipment and an array of lytic and endonucleolytic enzymes. Alternative PCR based techniques such as RAPD have been investigated (van Belkum *et al.*, 1995; van Leeuwen *et al.*, 1996; Tambic *et al.*, 1997) and are typically rapid and inexpensive but may lack discriminatory ability and reproducibility (Saulnier *et al.*, 1993). Techniques such as MLST have combined PCR and sequencing to type MRSA (Spratt, 1999; Enright *et al.*, 2000) providing both discriminatory and reproducible results, however, the process is complex and too expensive for routine application. An alternative molecular typing technique, binary typing, has been recently developed and tested in a number of laboratories and reported to be highly reproducible, discriminatory, stable, relatively simple to perform and fast (van Leeuwen *et al.*, 2001; van Leeuwen *et al.*, 2002).

Binary typing is a probe-mediated method combined with reverse hybridisation (van Leeuwen *et al.*, 2001). The technique uses a set of *S. aureus* specific DNA probes, generated using RAPD, selected for their discriminatory ability (van Leeuwen *et al.*, 1996; van Leeuwen *et al.*, 1999) and employs the digoxygenin-universal linkage system (DIG-ULS®) for the labelling of genomic DNA.

The DIG-ULS® system uses digoxygenin, a nonradioactive steroid hapten, coupled with a compound designated ULS® that binds with the N7-position of guanine in a DNA molecule. During binary typing chromosomal DNA from MRSA strains is labelled with DIG-ULS® and hybridised with a series of strain-differentiating RAPD probes immobilised on a membrane. A polyclonal sheep anti-DIG, Fab fragment, conjugated to alkaline phosphatase is used to bind specifically to the DIG-ULS®-DNA complex. Subsequently the bound conjugated alkaline phosphatase may be detected

by a colourimetric substrate. A colour change is evident when labelled DNA has hybridised to a probe and results are recorded as a series of ones and zeros; one indicative of hybridisation; zero indicative of non-hybridisation. The method has been reported to be more discriminatory than PFGE, highly reproducible and to transfer successfully to other laboratories (van Leeuwen *et al.*, 1999; van Leeuwen *et al.*, 2002). In addition the binary codes have been shown to be stable within a strain, constant within an outbreak strain and unique for epidemiologically unrelated strains (van Belkum *et al.*, 1997; van Leeuwen *et al.*, 1998; van Leeuwen *et al.*, 1999; Zadoks *et al.*, 2000). This has demonstrated the technique to be technically reproducible, biologically reproducible and to have sufficient resolution as a typing method. In addition, binary codes can be stored in a computer database and exchanged between laboratories allowing strain relationships to be studied nationally and internationally (van Leeuwen *et al.*, 2002).

Binary typing appears to offer similar benefits associated with a number of molecular typing techniques without all of the disadvantages, however, recently the DIG-ULS® labelling system was discontinued and therefore to perform binary typing an alternative nucleotide labelling system is required. The original binary typing method employs DNA probes generated by RAPD to differentiate strains without the difficulties of interpreting complex banding patterns. The most discriminatory probes generated using a number of molecular typing techniques such as PFGE, RAPD, REAP and SCC*mec* type assignment could be selected using PCA and validated for a new binary typing protocol with even greater discriminatory ability.

In this chapter the preliminary validation and optimisation of a binary typing method employing an alternative DIG labelling system is described. In addition a series of PCR generated DNA probes specific for different SCC*mec* types were tested for their suitability as part of a new binary typing method

7.2 Materials and methods

7.2.1 Bacterial strains

The MRSA strains Witte 1-Witte 20 were used during method 1 section 7.2.3.1. The MRSA strains COL, PER34, N315, ANS46, HU25 and MW2 were used during method 2 section 7.2.3.2.

7.2.2 Preparation of chromosomal DNA

Three methods for preparing chromosomal DNA from MRSA strains were investigated. When DNA was prepared the integrity, quantity and quality was determined by agarose gel electrophoresis and spectrophotometrically as described in sections 4.2.3.2. Only samples with A_{260}/A_{280} ratios between 1.7 and 1.9 were used in subsequent stages. Chromosomal DNA samples were adjusted to a concentration of 0.5µg/µL with SDW and stored at -20°C until required.

7.2.2.1 Method 1 (purification of DNA by phenol extraction and ethanol precipitation)

Method 1 was performed as described in section 4.2.3.1.

7.2.2.2 Method 2 (Wizard® genomic DNA purification kit).

Chromosomal DNA was prepared using the Wizard® genomic DNA purification kit (Promega, UK) according to the manufacturer's instructions. An isolated colony was inoculated into 5mL BHI broth and incubated overnight, with shaking at 37°C. A 1mL aliquot of the over night culture was centrifuged at 12000 x g for 2 minutes and the supernatant discarded. The pellet was resuspended in 480µL of 50mM EDTA buffer (pH 8) to which 120µL of 50mM EDTA buffer containing lysozyme (10mg/mL) and lysostaphin (10mg/mL) was added. Samples were incubated for 60 minutes at 37°C and then centrifuged at 12000 x g for 2 minutes and the supernatant discarded. The pellet was resuspended in 600µL of nuclei lysis solution, mixed gently with a pipette

and incubated for 5 minutes at 80°C and then allowed to cool to room temperature. A 3μL aliquot of RNAse solution was added to the sample and gently mixed by inverting and incubated at 37°C for 15 minutes. A 200μL aliquot of protein precipitation solution was added to the sample and mixed by inverting 2-5 times. The samples were incubated on ice for 5 minutes and subsequently centrifuged at 12000 x g for 3 minutes. The supernatant was transferred to a fresh 1.5mL centrifuge tube containing 600μL of isopropanol, mixed by inversion until the DNA formed a visible mass and then centrifuged at 12000 x g for 2 minutes. The supernatant was removed, the pellet washed with 70% ethanol and then centrifuged at 12000 x g for 2 minutes. The supernatant was gently aspirated and the tube allowed to dry at room temperature for 15 minutes. The DNA was subsequently rehydrated in 100 μL of SDW.

7.2.2.3 Method 3 (QIAamp® DNA mini kit)

Preparations of chromosomal DNA were made using a modified protocol from the QIAamp® DNA mini kit (Qiagen, UK).Strains were inoculated onto BHI agar plates and incubated for 16 hours at 37°C. A single loop of 25 colonies was taken directly from the plate and washed in 0.5mL of TESS buffer (50mM Tris-HCl, 5mM EDTA, 50mM NaCl and 50mM Sucrose, pH 8) and centrifuged at 5500 × *g* for 4 minutes in a bench-top microfuge. The supernatant was removed and the pellet resuspended in 180μL of TE buffer (20mM Tris-HCl pH 8; 2mM EDTA; 1.2% Triton®) to which 20μL of lysostaphin (0.5 units/μL) was added and briefly vortexed. Samples were incubated at 37°C for 20 minutes until clear. The resulting lysate was treated with 20μL of proteinase K solution and 200μL buffer AL (both supplied by manufacturer) and mixed by vortexing. Samples were incubated for 30 minutes at 56°C and then for a further 15 minutes at 95°C. A 200μL aliquot of absolute ethanol was added to the samples, mixed by inverting and centrifuged by pulsing to collect droplets. The sample mixture was subsequently added to a QIAamp spin column, placed inside a 2mL collection tube (supplied by manufacturer) and centrifuged at 6000 x g for 1 minute. The collection tube containing the filtrate was discarded and the spin column was placed into a fresh collection tube. A 500μL aliquot of buffer AW1 was added to the spin column and centrifuged at 6000 x g for 1 minute. The collection tube and filtrate were discarded and the spin column was placed into a fresh collection tube. A 500μL aliquot of buffer AW2 was added to the spin column and centrifuged at 17500

x g for 3 minutes. The filtrate was discarded and the previous step was repeated. The spin column was placed into a fresh collection tube and chromosomal DNA was eluted with 200µL of SDW.

7.2.3 Preparation of binary typing DNA probes and nuclease gene

The original probes identified by (van Leeuwen *et al.*, 2001) were used for preliminary testing of the modified binary typing method. In subsequent stages DNA fragments from each of the SCC*mec* type assignments described by (Oliveira and de Lencastre, 2002) were used as probes.

7.2.3.1 Method 1 (binary typing probes from Dr. W. van Leeuwen)

The binary typing DNA probes were amplified from recombinant plasmid DNA using T7 and M13 primers (table 7.1) obtained from Dr. W. van Leeuwen and described in (van Leeuwen *et al.*, 1999). The PCR reaction was carried out in a 25µL volume containing 16.15µL SDW, 2.5µL 10 x PCR buffer B (Promega, UK), 3µL 25mM $MgCl_2$ (Promega, UK), 0.3µL 100µM primer T7 (forward), 0.3µL 100µM primer M13 (reverse), 0.5µL 10mM dNTPs (Promega, UK), 0.25µL of 1.25 units/µL *Taq* DNA polymerase (Promega, UK) and 2µl of 10ng/µL plasmid DNA template. Amplification was performed on a GeneAmp® PCR system 9700 (Applied Biosystems, UK) using the following conditions: four minutes 30 seconds at 94°C followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 25°C, one minute at 72°C and concluded with a final extension of five minutes at 72°C and storage at 4°C until analysis. Amplicons were separated by agarose gel electrophoresis as described in section 4.2.3.2. Amplification of the nuclease gene was performed in the same manner as described for the binary typing DNA probes with the following alterations: the primers T7 and M13 were substituted for the primers *nuc*1 and *nuc*2 (table 7.1); template DNA was extracted using method 3 (section 7.2.2.3) from the strain Witte 2 obtained from Dr. W. van Leeuwen as described in (van Leeuwen *et al.*, 2001); the annealing temperature during PCR was raised from 25°C to 55°C.

Table 7.1 Primers used for amplification of binary probes from recombinant plasmid DNA and *S. aureus* nuclease gene.

Primer	Sequence (5'-3')	Reference
T7	TAA TAC GAC TCA CTA TAG GG	(van Leeuwen *et al.*, 1996)
M13	AAC AGC TAT GAC CAT G	(van Leeuwen *et al.*, 1996)
*nuc*1	GCG ATT GAT GGT GAT ACG GTT	(Brakstad *et al.*, 1992)
*nuc*2	AGC CAA GCC TTG ACG AAC TAA AGC	(Brakstad *et al.*, 1992)

Table 7.2 Probe size and position on binary strip adapted from a personal communication from Dr. W. van Leeuwen.

Probe code	Probe size (bp)	Position on binary strip
AW-1	1031	1
AW-2	655	2
AW-3	756	3
AW-4	971	4
AW-5	1500	5
AW-6	567	6
AW-7	1182	7
AW-8	501	8
AW-9	1700	9
AW-11	1181	10
AW-14	881	11
AW15	3595	12
nuc	279	13 positive control
Fish sperm DNA	200	14 negative control (Roche Diagnostics, Germany)

7.2.3.2 Method 2 (binary probes from SCC*mec* type assignment)

The multiplex PCR strategy for SCC*mec* type assignment described in chapter 5 was separated into nine individual reactions using sets of primers for different loci listed in table 7.3. The template DNA for the PCR was prepared using method 2 as described in section 4.2.3.3 the strains used for each loci are listed in table 7.3. The PCR was performed using a GeneAmp® PCR system 9700 (Applied Biosystems, UK) with primers and cycle conditions as described by (Oliveira and de Lencastre, 2002). The PCR was carried out in a 25µL volume containing 19.6µL H_2O, 2.5 µL 10 x PCR Buffer 3 (table 4.1), 0.2µL 10mM dNTPs (Promega, UK), 0.1µL of 1.25 units/µL *Taq* DNA polymerase (Promega, UK), and 2µL of DNA template. Template DNA was

prepared from the strains listed in table 7.3 using method 2 section 4.2.2.3. The primer sets are listed in table 5.1 and 0.3μL of each primer for each locus at a concentration of 100μM was used in separate reactions. The appropriate hybridisation temperature was calculated for each probe using the following calculation:

T_m=49.82+0.41(%G+C)-(600/l), where T_m is the appropriate hybridisation and l is the length of the hybrid in base pairs (Sambrook *et al.*, 1989). The optimal hybridisation temperature (T_{opt}) when using DIG easy hyb solution (Roche Diagnostics, Germany) is 20-25°C below Tm (Anonymous, 2004). The optimum hybridisation temperature suitable for each probe was 39°C.

Table 7.3 Primer sets and strain template DNA for PCR of individual SCC*mec* fragments adapted from (Oliveira and de Lencastre, 2002).

7.2.4 Purification of PCR products

The binary typing DNA probes amplified using methods 1 and 2 (section 7.2.3.1 and section 7.2.3.2) and nuclease gene products of PCR were purified using the QIAquick® PCR purification kit (Qiagen, UK) following the manufacturer's instructions. A PCR sample of 25µL was mixed with 5 volumes (125µL) of buffer PB and subsequently added to a QIAquick spin column placed inside a 2mL collection tube (supplied by manufacturer) and centrifuged at 12000 x g for 1 minute. The filtrate was discarded and 750µL of buffer PE was added to the spin column and

centrifuged at 12000 x g for 1 minute. The filtrate was discarded and centrifugation repeated as previously described. The remaining filtrate was discarded and the spin column was placed inside a fresh 2 mL collection tube. A 50µL aliquot of SDW was added to the spin column and subsequently left to stand at room temperature for 1 minute. The spin column was subsequently centrifuged at 12000 x g for 1 minute to elute the PCR product. The integrity, quantity and quality of purified PCR products were determined by agarose gel electrophoresis and spectrophotometrically as described in sections 4.2.3.2. Only samples with A_{260}/A_{280} ratios between 1.7 and 1.9 were used in subsequent stages. Purified PCR samples were adjusted to a concentration of 20ng/µL with SDW and stored at -20°C until required.

7.2.5 Fragmentation of DNA

To study the effect of DNA fragment size on labelling efficiency and hybridisation dynamics two methods were employed to disrupt DNA integrity. During testing intact chromosomal DNA was used as a comparison.

7.2.5.1 Method 1 (restriction digest of purified chromosomal DNA)

Purified chromosomal DNA was cleaved with the restriction enzyme *Sau*3AI (Promega, UK) according to the manufacturer's instructions as described in section 5.2.4. The restricted DNA was purified as described in section 7.2.4.

7.2.5.2 Method 2 (sonication of chromosomal DNA)

Purified chromosomal DNA was sonicated 3 times for 30 seconds on amplitude 5 using a MSE Soniprep 150 (MSE Ltd., UK) with a 0.5cm diameter probe. Sonication was performed on ice. Fragment size ranged between 200 and 500 bp, confirmed by agarose gel electrophoresis as described in section 4.2.3.2.

7.2.6 DIG-Nick translation of sample DNA

The DIG-nick translation method is based on the ability of DNA polymerase I (DNase I) to introduce randomly distributed nicks into DNA at low enzyme concentrations in

the presence of $MgCl_2$. During the process of labelling DNA with this system, *Escherichia coli* DNase I synthesises DNA complementary to the intact strand in a 5' to 3' direction using the 3'-OH termini of the nick as a primer (Kelly *et al.*, 1970). The 5' to 3' exonucleolytic activity of DNase I simultaneously removes nucleotides in the direction of synthesis (Klett *et al.*, 1968). The polymerase activity sequentially replaces the removed nucleotides with a hapten-labelled dNTP, in this case DIG-11-dUTP (Rigby *et al.*, 1977). At low temperature (15°C), the unlabelled DNA in the reaction is thus replaced by newly synthesised labelled DNA.

Purified sample DNA was labelled using DIG-nick translation mix (Roche Diagnostics, Germany) following the manufacturer's instructions. A 2μL aliquot of 0.5ng/μL DNA sample (equal to 1μg of DNA) was made up to a total volume of 16μL in SDW to which an aliquot of 4μL of Dig-Nick translation mix was added and mixed. The sample was centrifuged at 5500g for 1 minute and subsequently incubated at 15°C for 90 minutes. The reaction was stopped with 1μL of 0.5M EDTA (pH 8) and heating to 65°C for 10 minutes.

7.2.7 Spotting of DNA probes

The DNA probes (table 7.2) were adjusted to a concentration of 10ng/μL each in denaturation buffer (0.25N NaOH and 1.5M NaCl). An aliquot of 1μL of each denatured probe was spotted sequentially (table 7.2) onto positively charged nylon membrane (Roche Diagnostics, Germany) strip 5 x 150mm and incubated at 37°C for 10 minutes. The membrane was soaked in denaturation buffer and incubated at room temperature for 10 minutes. Subsequently, strips were soaked with neutralisation buffer (0.5M Tris-HCl and 1.5M NaCl, pH7.4) and incubated for 10 minutes at room temperature. The strips were washed with 2x SSC (20x SSC, 3M NaCl and 300mM sodium citrate, pH 7) (Sambrook *et al.*, 1989) and the denatured DNA probes crosslinked on the strip with exposure to UV light at 280nm (Transilluminator, UVP inc., USA) for 30 seconds. The strips were allowed to dry at room temperature.

7.2.8 Hybridisation

Each strip was transferred to a 15mL polypropylene centrifuge tube (Fischer, UK) and 1mL of prewarmed (42°C) DIG Easy Hyb buffer (Roche Diagnostics, Germany) was added. Strips were pre-hybridised for 2 hours at 42°C in a rotation oven (Hybridiser HB-1D, Techne, UK). Digoxygenin labelled DNA was denatured for 5 minutes at 100°C in a heating block (Dri-block DB-2A, Techne, UK) followed by incubation on ice for 5 minutes. The probe was centrifuged at 5500 x g for 1 minute to collect condensate. An aliquot of 5µL of labelled DNA (equivalent of 200ng) was added to 1mL of fresh prewarmed (42°C) DIG Easy Hyb buffer. The buffer used during pre-hybridisation was discarded from the centrifuge tube and subsequently the labelled DNA and DIG Easy Hyb buffer mixture were added. The hybridisation mixture was incubated at 42°C for 16 hours in a rotation oven (Hybridiser HB-1D, Techne, UK). When SCC*mec* probes were employed the prewarming of DIG easy hyb solution and hybridisation temperatures were lowered to 39°C.

7.2.9 Detection of hybridised DNA

All incubations were at room temperature with shaking unless stated otherwise. Blocking reagent, anti-digoxygenin-alkaline phosphatase and CSPD are provided with the DIG luminescent detection kit (Roche Diagnostics, Germany).

After hybridisation, strips were washed twice in 50mL of 2x SSC/0.1% SDS for 5 minutes and twice in 0.5x SSC/0.1% SDS for 15 minutes at 60°C with shaking. The strips were washed with 50mL of washing buffer (0.1M maleic acid, 150mM NaCl, pH 7.5 and 0.3% $^{v}/_{v}$ Tween 20) for 5 minutes and subsequently blocked with 100mL of blocking solution (1% $^{w}/_{v}$ blocking reagent in 0.1M maleic acid, 150mM NaCl, pH 7.5) for 30 minutes. Strips were incubated in 20mL antibody solution (75mU/mL of anti-digoxygenin-alkaline phosphatase in blocking solution) for 30 minutes. Strips were washed twice with 100mL of washing buffer for 15 minutes and equilibrated with detection buffer (0.1M Tris-HCl, 0.1M NaCl, pH 9.5) for 5 minutes. Strips were placed DNA side up on a sheet of cling film and 2mL of diluted CSPD solution (CSPD diluted 1:100 in detection buffer) was applied to the strips. The diluted CSPD solution is a chemiluminescent substrate for alkaline phosphatase. A second sheet of

cling film was placed over the top, and the substrate was squeezed evenly and without air bubbles over the strips. The cling film bound strips were placed inside a development folder (Hypercassette, Amersham Life Science, UK) and incubated at 37°C for 15 minutes in darkness. In a dark room the strips were exposed to X-ray film (Hyperfilm, Amersham Life Science, UK) placed inside the development folder. The X-ray film was exposed for 15 minutes at room temperature. The film was developed in a dark room. The film was first treated with developer (Kodak Polymax RT developer/replenisher, Sigma, UK) for 2 minutes or until image was visually detectable under red light and then treated with fixer (Kodak Polymax RT fixer, Sigma, UK) for 2 minutes. The film was rinsed with tap water and allowed to dry at room temperature. The film images were photographed using the GeneGenius Bio Imaging System (Syngene, UK) with under lighting. Images were saved in the tagged image file format (TIFF).

7.3 Results

7.3.1 Preparation of chromosomal DNA

Preparation of chromosomal DNA using the QIAamp® DNA mini kit was identified as the quickest method to perform, providing consistently high yields of non-fragmented DNA, suitable for subsequent DIG-labelling. In comparison the Wizard® genomic DNA purification kit gave consistently low yields of DNA and preparation by phenol extraction with ethanol precipitation required significantly more time.

7.3.2 Fragmentation of chromosomal DNA

The best labelling and hybridisation results were obtained using non-fragmented chromosomal DNA. Labelling of sonicated DNA samples was possible however, hybridisation results were poor compared with non-fragmented DNA. The poorest hybridisation results were obtained when DNA samples were treated with the restriction endonuclease *Sau3*AI and subsequently labelled.

7.3.3 Application of the binary typing procedure

The results of the MRSA strains Witte 1-Witte 20 labelled using the DIG-Nick translation kit and hybridised with the binary probes described by (van Leeuwen *et al.*, 2001) are depicted in figure 7.1. The expected results reported for these strains are described in table 7.4. It was not possible to emulate the binary patterns reported by van Leeuwen *et al* with the new system.

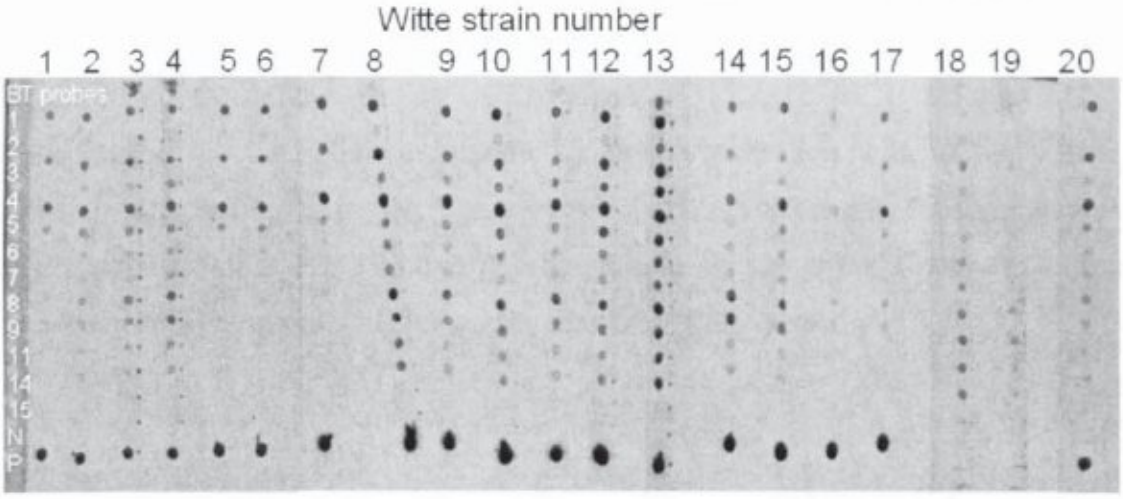


Figure 7.1 Binary typing results of the MRSA strains Witte 1-Witte 20 obtained after hybridisation on the strip-immobilised DNA probe panel (AW1-AW20). Probe positions are indicated on the left; N (fish sperm negative control); P (*nuc* gene positive control).

Table 7.4 Expected and observed binary patterns using the binary probes described by (van Leeuwen *et al.*, 2001).

7.3.4 Application of the modified binary typing system with SCC*mec* probes

The results of the MRSA strains COL, PER34, N315, ANS46, HU25 and MW2 labelled using the DIG-Nick translation kit and hybridisation with SCC*mec* type probes are depicted in figure 7.2. The expected and observed results for these strains are described in table 7.5. The hybridisation results do not entirely conform to the expected results but are of a higher quality than the results in section 7.3.3.

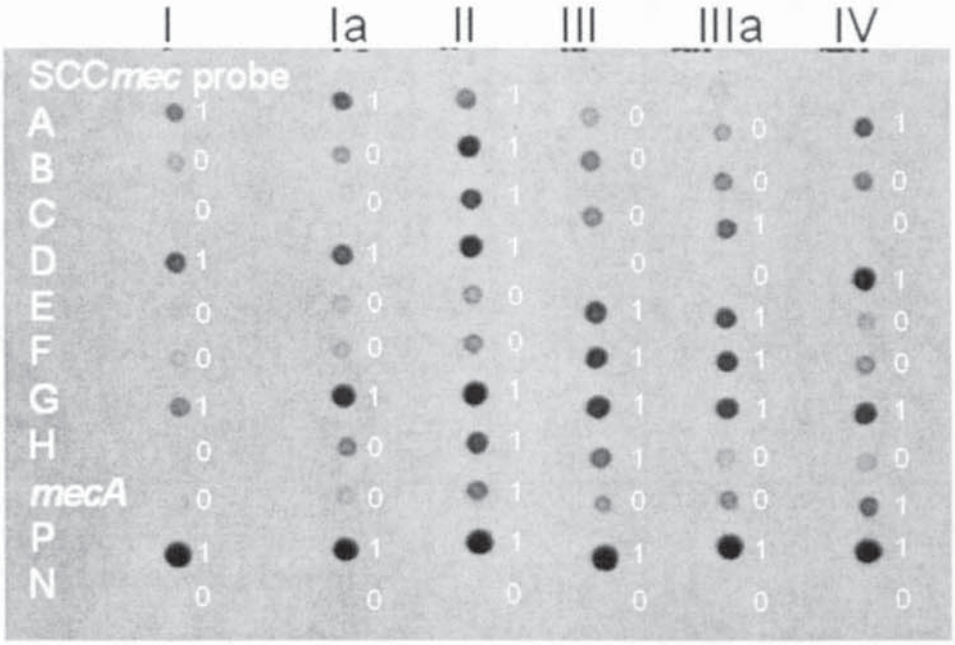


Figure 7.2 Binary typing results of the MRSA strains COL (I), PER34 (Ia), N315 (II), ANS46 (III), HU25 (IIIa) and MW2 (IV) after hybridisation with the SCC*mec* type specific DNA probes generated by PCR.

Table 7.5 Expected and observed binary patterns using SCC*mec* DNA fragment binary probes adapted from the original method described by (Oliveira and de Lencastre, 2002).

7.4 Discussion

The aim of this chapter was to evaluate an existing binary typing method and to begin optimisation of a modified system that synergistically combines the most discriminatory DNA probes from other molecular typing techniques.

7.4.1 Evaluation of binary typing

Prior to validation of the binary typing method it was discovered that production of the DIG-ULS® nucleotide labelling kit had been discontinued and therefore an alternative DNA labelling system was required. The DIG-Nick translation mix was selected as a replacement for the DIG-ULS® system, as it was suitable for labelling chromosomal DNA, can be used in filter hybridisation applications and detection is highly specific and sensitive (Anonymous, 2003). One major disadvantage associated with the DIG-ULS® labelling system was its ability to label RNA and proteins in addition to DNA, which necessitated highly purified DNA samples (van Leeuwen *et al.*, 2002). The DIG-Nick translation system utilises a DNA polymerase and therefore only DNA samples may be labelled. Labelling DNA with digoxgenin was preferential to using a radioactive isotope in terms of safety (Sambrook *et al.*, 1989) and for transfer of the technique to other laboratories. In addition, using a digoxygenin labelling system meant the original binary typing method could be emulated more stringently.

The efficiency of labelling using DIG-Nick translation depends on the time, temperature, molar ratio of DIG-11-dUTP to dTTP and concentration of chromosomal DNA (van Leeuwen *et al.*, 2001; Anonymous, 2003). All labelling was performed as recommended by the DIG-Nick translation mix literature; labelling of DNA was performed in a 20µL volume containing 1µg of DNA and 4µL of DIG-Nick translation mix incubated at 15°C for 90 minutes, and therefore the effect of changing any of these criteria was not investigated. Incubation at 15°C for longer than 90 minutes may be performed if signal strengths are weak (Anonymous, 2003), however, this was not required.

In the original binary typing method it was reported that the Wizard® genomic DNA purification kit yielded optimal results (van Leeuwen *et al.*, 2001), however, purification of chromosomal DNA using the QIAamp® DNA mini kit was identified as the best DNA preparation technique during this study. The method was the quickest and simplest to perform and yields of pure, non-fragmented chromosomal DNA were consistently high. Preparation of chromosomal DNA by the Wizard® genomic DNA purification kit and phenol extraction and ethanol precipitation were more time consuming and therefore excluded. During an intercentre study of binary typing it was reported that DNA preparation methods required standardising to yield good results (van Leeuwen *et al.*, 2002). The same problems were not observed during this study. As stated previously the DIG-ULS® labelling system requires highly purified DNA samples for efficient labelling, therefore, the use of a DIG-Nick translation system that labels DNA exclusively may reduce the problems observed during the intercentre study. Fragmentation of chromosomal DNA prior to labelling was not found to improve labelling efficiency, which has been previously reported (van Leeuwen *et al.*, 2001). It was possible to label sonicated chromosomal DNA and results were comparable with those using whole chromosomal DNA, however, the process was excluded as it was not required. The poorest results were observed with chromosomal DNA cleaved with *Sau3*AI. After chromosomal DNA was cleaved it was necessary to purify the DNA fragments from the components of the restriction digest. It is possible that excessive manipulation of the fragmented chromosomal DNA may have degraded the molecule (Sambrook *et al.*, 1989), directly affecting labelling with the DIG-Nick translation mix.

To emulate the binary typing method 1µL of 10ng/µL of each DNA probe was spotted onto each nylon membrane as described by (van Leeuwen *et al.*, 2001). In addition, 200ng of labelled chromosomal DNA was added during hybridisation (van Leeuwen *et al.*, 2001). Although the same concentrations of probe and labelled DNA were used throughout validation, different binary patterns were observed for the same strain when experiments were repeated. A series of hybridisation experiments similar to those described by (van Leeuwen *et al.*, 2001) using different amounts of labelled chromosomal DNA with varying concentrations of probe DNA may establish optimal hybridisation conditions. During an intercentre study it was demonstrated that adherence to the binary typing method was necessary to obtain reproducible results

(van Leeuwen *et al.*, 2002). Although the execution of the binary typing method was adhered to as closely as possible it is not known whether the use of the DIG-Nick translation labelling system, instead of the DIG-ULS® system, detrimentally affected results.

It was observed that amplification of DNA probes from the recombinant plasmids was poor. To collect adequate quantities of probe a number of PCR reactions were performed and the products were pooled and purified. Subsequent PCR of the amplicons yielded greater amounts of product, which may indicate that the PCR conditions were optimal and that the recombinant plasmid DNA may have degraded (Sambrook *et al.*, 1989). When the recombinant plasmids were subjected to agarose gel electrophoresis a number of the samples could not be visualised. Although it was possible to amplify probes from the degraded recombinant plasmids, over manipulation of DNA probes may in part explain the poor results of hybridisation. Based on this assumption DNA probes generated using separate PCR reactions directed at loci of different SCC*mec* type elements were used during subsequent validation experiments.

7.4.2 Binary typing using SCC*mec* probes

When binary typing was performed using the SCC*mec* probes, results were of higher quality than those obtained using the original probe set. The observed results obtained using the SCC*mec* probes correlated well with the expected results. In addition, it was observed that results were easier to interpret. This was due to high signal intensity for positive results and low signal intensity for negative results. All the MRSA strains tested during this study were positive for the *mecA* gene and therefore a positive signal for this locus would be expected, however, hybridisation to this probe was observed to be poor. During this study hybridisation was performed at the highest suitable temperature for all the probes in an attempt to increase stringency conditions (Sambrook *et al.*, 1989). The principle of high stringency hybridisation conditions is similar to those for primer annealing during PCR, discussed in chapter 4. To improve results it may be necessary to lower the hybridisation temperature. Lowering the hybridisation temperature to 38°C remains optimal for all of the DNA probes and may improve hybridisation of sample DNA to *mecA* without dramatically decreasing

stringency conditions (Sambrook *et al.*, 1989). Based on the high quality results obtained using the SCC*mec* probes it is highly probable that the binary typing system may be optimised using the DIG-Nick translation system, however, further optimisation is required.

7.4.3 Evaluating binary typing

Binary typing was originally developed to differentiate MRSA strains without producing complex banding patterns, which may be difficult to interpret, and to replace techniques that required expensive peripheral equipment (van Leeuwen *et al.*, 2001). Binary typing is less expensive than PFGE, however, the costs of labelling, detection and hybridisation reagents currently exceed that of PCR-based typing methods. A number of studies have reported PCR-based typing methods that are suitable for characterising MRSA strains, which provide results in a single working day and that are almost as discriminatory as PFGE (Louie *et al.*, 1996; van Leeuwen *et al.*, 1996; Martineau *et al.*, 2000a; Oliveira and de Lencastre, 2002; Stranden *et al.*, 2003). In addition, a number of these techniques provide results regarding genetic background (Oliveira and de Lencastre, 2002), antibiotic resistance (Martineau *et al.*, 2000a) and virulence factors (Lina *et al.*, 1999). Binary typing may yield results within a 24 hour period. In addition, the results do not require analysis with band matching computer software and therefore results may be read immediately and stored in a database (van Leeuwen *et al.*, 2001). Potentially extending the number of probes used in the binary typing system to detect resistance and virulence genes may improve the discriminatory power and cost effectiveness of the technique. In addition, it may be less labour intensive and time consuming than performing multiple PCR based methods (van Leeuwen *et al.*, 2001). Potentially a binary typing system that is more discriminatory than PFGE, highly reproducible and suitable for intercentre studies could be developed (van Leeuwen *et al.*, 2001; van Leeuwen *et al.*, 2002). A binary typing system that may be performed in 24 hours that is more discriminatory than PFGE, more cost effective and less time consuming than multiple PCR based methods may be a suitable typing technique for use in clinical microbiology laboratories. In addition, the potential for binary typing to be developed into a micro-array based system may be assessed (van Leeuwen *et al.*, 2001).

7.4.4 Conclusion

Although binary typing may be a highly adaptable discriminatory technique its suitability for replacing either PFGE or PCR based typing methods requires further evaluation and optimisation of the method. Future work may involve the evaluation of multiple PCR-based methods to compare typeability, reproducibility, discriminatory power, ease of interpretation and ease of use in comparison with both PFGE and an optimised binary typing system.

CHAPTER 8 GENERAL DISCUSSION

Methicillin-resistant *Staphylococcus aureus* is a significant cause of morbidity, mortality and health care costs, which is a worldwide problem that has been exacerbated by increasing reports of MR-MRSA strains and in recent years the emergence of highly virulent CA-MRSA strains. Increasingly, the reduced sensitivity of MRSA strains to many clinically available drugs has meant that only glycopeptides and investigational drugs are effective during treatment (Wenzel *et al.*, 1991; Aires de Sousa *et al.*, 1998). Vancomycin, was until recently the drug of choice for the treatment of MRSA, however, researchers in Japan, Europe and the USA have raised concerns regarding an emerging problem of clinically resistant strains, designated VISA and VRSA.

The rates of MRSA in the UK are estimated to be the highest in Europe, according to the European Antimicrobial Resistance Surveillance System. Reports from the National Audit Office estimate that every year there are 5000 deaths due to hospital-acquired infection, of which MRSA are the major cause. The cost to the NHS is estimated to be £1 billion. However, demonstrating the true impact and determining the real cost of treating MRSA infections is difficult due to confounding factors such as patient characteristics and the problem of determining cause and effect (Carbon, 1999). What is certain is that MRSA increases the length of time patients are hospitalised, rates of mortality and the cost of treatment in comparison to patients infected with MSSA (Boyce *et al.*, 1981; Holmberg *et al.*, 1987; Carbon, 1999). To aid infection control management multiple phenotypic and genotypic techniques have been employed to better understand the epidemiology of MRSA, however, a single technique that is universally applicable has not been identified and therefore a number of methods are typically employed in conjunction with each other. This thesis has sought to investigate phenotypic and genotypic characteristics of HA-MRSA, CA-MRSA and MR-MRSA strains isolated from bacteraemic patients in the hospital environment and patients with skin and soft tissue infections in the community with the purpose of identifying discriminatory probes that may be suitable as part of a modified binary typing system.

The phenotypic properties of clinical isolates of MRSA from both the hospital and community environments were investigated using antibiotic sensitivity testing and vancomycin E-tests. In addition, the growth and death kinetics of a select panel of HA-MRSA, CA-MRSA, MR-MRSA and *S. aureus* strains exposed to stressful conditions including, desiccation, high and low pH and increasing NaCl concentrations were investigated. Antibiotic sensitivity testing identified that a large proportion of MRSA strains in the hospital environment are multidrug resistant and largely remain sensitive to rifampicin, tetracycline and vancomycin and that the antibiogram types of non-multidrug resistant HA-MRSA and CA-MRSA are more diverse. Preliminary investigation of phenotypic stress responses have indicated that without the selective pressures of antibiotics the growth rates of MRSA strains remain comparable with those of MSSA strains. In addition, both MRSA and MSSA strains are highly tolerant to high and low pH, may survive desiccated conditions for over a month and are highly tolerant to low water activity conditions. These observations may have implications that affect strategies for eradicating MRSA strains that are isolated from the environment. Further studies investigating sigma B factors, related to stress responses in *S. aureus*, are required.

Typically, phenotypic methods such as antibiotic sensitivity testing are unstable due to the altered expression of resistance genes, therefore, a number of genotypic methods were assessed. Strains were characterised using PFGE, two independent optimised RAPD reactions, SCC*mec* type assignment by multiplex PCR and REAP. Molecular typing methods were assessed on their typeability, reproducibility, discriminatory power, ease of interpretation and ease of use (Maslow *et al.*, 1993; Tenover *et al.*, 1994). In addition, criteria such as cost and the speed of techniques were assessed. Using both PFGE and the optimised RAPD reactions it was possible to distinguish MR-MRSA from MRSA strains, however, it was not possible to distinguish HA-MRSA from CA-MRSA strains. The discriminatory power of PFGE was greater than RAPD, however, both methods clustered strains into similar groups and therefore results were comparable. The techniques SCC*mec* type assignment and REAP identified that all MR-MRSA strains were SCC*mec* type II and did not carry plasmid DNA. In addition, the majority of HA-MRSA and CA-MRSA strains were SCC*mec* type IV and did carry plasmid DNA. No other reports of SCC*mec* type II clones and the absence of plasmid DNA have been reported to date. In recent years the frequency

of studies reporting plasmid DNA typing has decreased due to the use of other more discriminatory and reproducible techniques. Typically, 90% of MRSA strains are considered to carry plasmids (van Leeuwen, 2003), which is contradictory to the observations of this study. The discriminatory power was dramatically increased when PCR-based typing methods were combined with REAP. Using REAP with a PCR-based typing method was also inexpensive, rapid, reproducible, easy to perform and results were easy to interpret. In addition, it may be possible to associate antibiotic sensitivity with specific MRSA types. This study indicates that PCR-based methods in combination with REAP may be suitable for routine clinical typing studies. Although, newer typing methods have been developed, such as MLST and *Spa* typing, and are routinely being assessed for their suitability for characterising strains in clinical microbiology laboratories, established typing techniques still appear to be suitable alternatives that are cheaper and faster to perform.

Analysis of molecular typing banding patterns is typically performed using a hierarchic format called cluster analysis. Profile similarity is generally calculated using the Dice coefficient and a dendrogram is constructed using UPGMA clustering. As an alternative method PCA was employed to represent the relationships between MRSA strain-profiles in a non-hierarchic format. As a non-hierarchic technique PCA, does not attempt to form relationships between profile types that may not readily exist and may represent more accurately true epidemiological relationships. In addition, post-analysis using Spearman's correlation coefficient was able to identify statistically valid discriminatory PFGE bands within a database of complex profiles. Performing PCA may therefore provide valuable additional information regarding epidemiology and may identify bands suitable for a probe-mediated typing system or a target for a PCR-based method. Few studies have examined different methods of statistical analysis that may be useful tools in the field of epidemiology, therefore it may be necessary to examine existing statistical methods for their use with molecular typing data.

Evaluation and optimisation of an existing binary typing method that potentially could synergistically combine the most discriminatory DNA probes from other molecular typing techniques was undertaken. The method may be developed to be more discriminatory than PFGE and PCR-based methods and performed in less than 24

hours. Few groups have reported the use of binary typing in clinical microbiology laboratories even though the method was the subject of an intercentre study (van Leeuwen *et al.*, 2002). Typically, DNA-based methods for the molecular typing of MRSA may be broadly separated into techniques that require restriction endonuclease digests of DNA and PCR-based methods. Both generally provide distinctive banding patterns for differentiating unrelated strains. Binary typing belongs to a third category designated, probe mediated techniques and requires the reverse hybridisation of labelled genomic DNA to discriminatory DNA probes. Binary typing does not require banding pattern interpretation or expensive peripheral equipment, however, during this study it was observed that essential reagents were expensive. It was also observed that the method is more complex than PFGE and PCR-based techniques and may not be suitable for most clinical microbiology laboratories.

The main advantages to PFGE are its discriminatory power and reproducibility, however, the technique is often criticised for being expensive and time consuming. Conversely, PCR-based methods are typically less discriminatory and may lack reproducibility, however they are less expensive and more rapid. Based on these observations, in a shorter time frame required to perform PFGE it may be possible to complete a series of multiplex PCR methods and combine the information to generate a more discriminatory typing system that remains inexpensive. For instance it may be possible to characterise strains based on SCC*mec* type assignment, antibiotic resistance genes and virulence genes. As stated previously, combining such an approach with REAP may be even more discriminatory. In addition, the gene targets for the PCR as well as discriminating between strains may provide clinically relevant information suitable for infection control management and directing therapy. Due to PCR being inexpensive the technique is also available to a wider range of laboratories. A detailed method for the analysis of complex banding patterns has been described during this thesis that may be used to identify discriminatory bands from many molecular typing techniques, therefore, sequenced bands may be used as suitable targets in future PCR-based typing methods. Instead of identifying one method to characterise MRSA strains a better approach may be to identify multiple techniques that provide a combined system that is rapid, inexpensive, discriminatory and yields clinically relevant data.

REFERENCES

Aboshkiwa, M., G. Rowland, *et al.* (1995). "Nucleotide sequence of the *Staphylococcus aureus* RNA polymerase rpoB gene and comparison of its predicted amino acid sequence with those of other bacteria." Biochim Biophys Acta **1262**(1): 73-8.

Adams, M. R. and M. O. Moss (2000). Food microbiology. Cambridge, The Royal Society of Chemistry.

Adhikari, R. P., G. M. Cook, *et al.* (2002). "Phenotypic and molecular characterization of community occurring, Western Samoan phage pattern methicillin-resistant *Staphylococcus aureus*." J Antimicrob Chemother **50**(6): 825-31.

Afif, W., P. Huor, *et al.* (2002). "Compliance with methicillin-resistant *Staphylococcus aureus* precautions in a teaching hospital." Am J Infect Control **30**(7): 430-3.

Aires de Sousa, M., I. S. Sanches, *et al.* (1998). "Intercontinental spread of a multidrug-resistant methicillin-resistant *Staphylococcus aureus* clone." J Clin Microbiol **36**(9): 2590-6.

Akopyanz, N., N. O. Bukanov, *et al.* (1992). "DNA diversity among clinical isolates of Helicobacter pylori detected by PCR-based RAPD fingerprinting." Nucleic Acids Res **20**(19): 5137-42.

Allignet, J., S. Aubert, *et al.* (1996). "Distribution of genes encoding resistance to streptogramin A and related compounds among staphylococci resistant to these antibiotics." Antimicrob Agents Chemother **40**(11): 2523-8.

Alt, V., T. Bechert, *et al.* (2004). "In vitro testing of antimicrobial activity of bone cement." Antimicrob Agents Chemother **48**(11): 4084-8.

Amin, U. S., T. D. Lash, *et al.* (1995). "Proline betaine is a highly effective osmoprotectant for Staphylococcus aureus." Arch Microbiol **163**(2): 138-42.

Andersson, D. I. and B. R. Levin (1999). "The biological cost of antibiotic resistance." Curr Opin Microbiol **2**(5): 489-93.

Andrews, J. M. (2001). "The development of the BSAC standardized method of disc diffusion testing." J Antimicrob Chemother **48 Suppl 1**: 29-42.

Anonymous (1998). "Revised guidelines for the control of methicillin-resistant *Staphylococcus aureus* infection in hospitals. British Society for Antimicrobial Chemotherapy, Hospital Infection Society and the Infection Control Nurses Association." J Hosp Infect **39**(4): 253-90.

Anonymous (1999). "Four pediatric deaths from community-acquired methicillin-resistant *Staphylococcus aureus*--Minnesota and North Dakota, 1997-1999." MMWR **48**: 707-710.

Anonymous (2000). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. National Committee for Clinical Laboratory Standard 5th edition. Villanova, Pa, NCCLS: Approved standard M7-A5.

Anonymous (2002). "Vancomycin-resistant *Staphylococcus aureus*. Pennsylvania, 2002." MMWR **51**(40): 902.

Anonymous (2002a). "*Staphylococcus aureus* resistant to vancomycin. United States, 2002." MMWR **51**(26): 565-7.

Anonymous (2003). "Dig-Nick translation mix." www.roche-applied-science.com/pack-insert/1745816a.pdf **4**: 1-3.

Anonymous (2004). "DIG easy hyb granules." www.roche-applied-science.com/pack-insert/11796895001a.pdf **1**: 1-3.

Anonymous (2004b). "Vancomycin-resistant *Staphylococcus aureus*. New York, 2004." MMWR **53**(15): 322-3.

Antonio, M., N. McFerran, *et al.* (2002). "Mutations affecting the Rossman fold of isoleucyl-tRNA synthetase are correlated with low-level mupirocin resistance in *Staphylococcus aureus*." Antimicrob Agents Chemother **46**(2): 438-42.

Arbeit, R. (1997). Laboratory procedures for the epidemiologic analysis. The Staphylococci in Human Disease. K. B. Crossley and G. L. Archer. New York, Churchill Livingstone: 253-286.

Archer, G. L. and J. L. Johnston (1983). "Self-transmissible plasmids in staphylococci that encode resistance to aminoglycosides." Antimicrob Agents Chemother **24**(1): 70-7.

Armstrong, R. A., L. Wood, *et al.* (1996). "The use of multivariate methods in the identification of subtypes of Alzheimer's disease: a comparison of principal components and cluster analysis." Dementia **7**(4): 215-20.

Aubry-Damon, H., C. J. Soussy, *et al.* (1998). "Characterization of mutations in the *rpoB* gene that confer rifampin resistance in *Staphylococcus aureus*." Antimicrob Agents Chemother **42**(10): 2590-4.

Baba, T., F. Takeuchi, *et al.* (2004). The *Staphylococcu aureus* genome. *Staphylococcus aureus* molecular and clinical aspects. K. Hiramatsu. Chichester, Horwood Publishing Limited: 66-153.

Baba, T., F. Takeuchi, *et al.* (2002). "Genome and virulence determinants of high virulence community-acquired MRSA." Lancet **359**(9320): 1819-27.

Baddour, L. M. (2000). "Cellulitis syndromes: an update." Int J Antimicrob Agents **14**(2): 113-6.

Baleiras Couto, M. M., J. T. Vogels, *et al.* (1995). "Random amplified polymorphic DNA and restriction enzyme analysis of PCR amplified rDNA in taxonomy: two identification techniques for food-borne yeasts." J Appl Bacteriol **79**(5): 525-35.

Bannerman, T. L., G. A. Hancock, *et al.* (1995). "Pulsed-field gel electrophoresis as a replacement for bacteriophage typing of *Staphylococcus aureus*." J Clin Microbiol **33**(3): 551-5.

Bar, A., D. Hantschke, *et al.* (2003). "Spectrum of bacterial isolates in HIV-positive patients with skin and soft tissue infections: emergence of methicillin-resistant Staphylococci." Aids **17**(8): 1253-6.

Barber, M. and M. Rozwadowska-Dowzenko (1948). "Infection by penicillin-resistant staphylococci." Lancet **252**: 641-644.

Barg, N. L. and T. Harris (1997). Toxin-Mediated Syndromes. The Staphylococci in Human Disease. K. B. Crossley and G. L. Archer. New York, Churchill Livingstone: 527-543.

Bassetti, S. and M. Battegay (2004). "*Staphylococcus aureus* infections in injection drug users: risk factors and prevention strategies." Infection **32**(3): 163-9.

Beard-Pegler, M. A., E. Stubbs, *et al.* (1988). "Observations on the resistance to drying of staphylococcal strains." J Med Microbiol **26**(4): 251-5.

Ben-David, D. and E. Rubinstein (2003). Treatment of MRSA infections. MRSA current perspectives. A. C. Fluit and F. J. Schmitz. Norfolk, Caister Academic Press: 275-315.

Bjorkman, J. and D. I. Andersson (2000). "The cost of antibiotic resistance from a bacterial perspective." Drug Resist Updat **3**(4): 237-245.

Bjorkman, J., I. Nagaev, *et al.* (2000). "Effects of environment on compensatory mutations to ameliorate costs of antibiotic resistance." Science **287**(5457): 1479-82.

Blumberg, H. M., D. Rimland, *et al.* (1992). "Epidemiologic typing of *Staphylococcus aureus* by DNA restriction fragment length polymorphisms of rRNA genes: elucidation of the clonal nature of a group of bacteriophage-nontypeable, ciprofloxacin-resistant, methicillin-susceptible *S. aureus* isolates." J Clin Microbiol **30**(2): 362-9.

Bone, R. C. (1994). "Gram-positive organisms and sepsis." Arch Intern Med **154**(1): 26-34.

Bouma, J. E. and R. E. Lenski (1988). "Evolution of a bacteria/plasmid association." Nature **335**(6188): 351-2.

Boyce, J. M., M. Landry, *et al.* (1981). "Epidemiologic studies of an outbreak of nosocomial methicillin-resistant Staphylococcus aureus infections." Infect Control **2**(2): 110-6.

Boyle-Vavra, S., H. Labischinski, *et al.* (2001). "A spectrum of changes occurs in peptidoglycan composition of glycopeptide-intermediate clinical *Staphylococcus aureus* isolates." Antimicrob Agents Chemother **45**(1): 280-7.

Brakstad, O. G., K. Aasbakk, *et al.* (1992). "Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the *nuc* gene." J Clin Microbiol **30**(7): 1654-60.

Bright, K. R., C. P. Gerba, *et al.* (2002). "Rapid reduction of *Staphylococcus aureus* populations on stainless steel surfaces by zeolite ceramic coatings containing silver and zinc ions." J Hosp Infect **52**(4): 307-9.

Brown, D. F. and P. E. Reynolds (1980). "Intrinsic resistance to beta-lactam antibiotics in *Staphylococcus aureus*." FEBS Lett **122**(2): 275-8.

Burdeska, A., M. Ott, *et al.* (1990). "Identical genes for trimethoprim-resistant dihydrofolate reductase from *Staphylococcus aureus* in Australia and central Europe." FEBS Lett **266**(1-2): 159-62.

Busch, F. H. (1990). "[Familial benign chronic neutropenia in a Danish family]." Ugeskr Laeger **152**(36): 2565-6.

Bush, K., G. A. Jacoby, *et al.* (1995). "A functional classification scheme for beta-lactamases and its correlation with molecular structure." Antimicrob Agents Chemother **39**(6): 1211-33.

Bushby, S. R. and G. H. Hitchings (1968). "Trimethoprim, a sulphonamide potentiator." Br J Pharmacol **33**(1): 72-90.

Caetano-Anolles, G. (1993). "Amplifying DNA with arbitrary oligonucleotide primers." PCR Methods Appl **3**(2): 85-94.

Caetano-Anolles, G., B. J. Bassam, *et al.* (1992). "Primer-template interactions during DNA amplification fingerprinting with single arbitrary oligonucleotides." Mol Gen Genet **235**(2-3): 157-65.

Carbon, C. (1999). "Costs of treating infections caused by methicillin-resistant staphylococci and vancomycin-resistant enterococci." J Antimicrob Chemother **44 Suppl A**: 31-6.

Casewell, M. W. and R. L. Hill (1986). "The carrier state: methicillin-resistant *Staphylococcus aureus*." J Antimicrob Chemother **18 Suppl A**: 1-12.

Casey, A. L., T. Worthington, *et al.* (2003). "A randomized, prospective clinical trial to assess the potential infection risk associated with the PosiFlow needleless connector." J Hosp Infect **54**(4): 288-93.

Chambers, H. F. (2004). Antimicrobial resistance and therapy of *Staphylococcus aureus* infections. *Staphylococcus aureus* molecular and clinical aspects. K. Hiramatsu. Chichester, Horwood Publishing Limited: 237-267.

Chambers, H. F., O. M. Korzeniowski, *et al.* (1983). "*Staphylococcus aureus* endocarditis: clinical manifestations in addicts and nonaddicts." Medicine (Baltimore) **62**(3): 170-7.

Chesneau, O., A. Morvan, *et al.* (2000). "Retrospective screening for heterogeneous vancomycin resistance in diverse *Staphylococcus aureus* clones disseminated in French hospitals." J Antimicrob Chemother **45**(6): 887-90.

Chiller, K., B. A. Selkin, *et al.* (2001). "Skin microflora and bacterial infections of the skin." J Investig Dermatol Symp Proc **6**(3): 170-4.

Chopra, I. (1976). "Mechanisms of resistance to fusidic acid in *Staphylococcus aureus*." J Gen Microbiol **96**(2): 229-38.

Chopra, I., P. M. Hawkey, *et al.* (1992). "Tetracyclines, molecular and clinical aspects." J Antimicrob Chemother **29**(3): 245-77.

Chopra, I. and M. Roberts (2001). "Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance." Microbiol Mol Biol Rev **65**(2): 232-60 ; second page, table of contents.

Chung, W. O., C. Werckenthin, *et al.* (1999). "Host range of the ermF rRNA methylase gene in bacteria of human and animal origin." J Antimicrob Chemother **43**(1): 5-14.

Clark, N. C., L. M. Weigel, *et al.* (2005). "Comparison of Tn1546-like elements in vancomycin-resistant Staphylococcus aureus isolates from Michigan and Pennsylvania." Antimicrob Agents Chemother **49**(1): 470-2.

Clifford, H. T. and R. R. Sokal (1975). An introduction to Numerical Classification. N. Y., Academic Press, New York.

Cohen, M. L. (1992). "Epidemiology of drug resistance: implications for a post-antimicrobial era." Science **257**(5073): 1050-5.

Coia, J. E., I. Noor-Hussain, *et al.* (1988). "Plasmid profiles and restriction enzyme fragmentation patterns of plasmids of methicillin-sensitive and methicillin-resistant isolates of *Staphylococcus aureus* from hospital and the community." J Med Microbiol **27**(4): 271-6.

Cookson, B., F. J. Schmitz, *et al.* (2003). Introduction. MRSA current perspectives. F. J. Schmitz. Norfolk, Caister Academic Press: 1-9.

Cookson, B. D., P. Aparicio, *et al.* (1996). "Inter-centre comparison of pulsed-field gel electrophoresis for the typing of methicillin-resistant Staphylococcus aureus." J Med Microbiol **44**(3): 179-84.

Coombs, G. W., G. R. Nimmo, *et al.* (2004). "Genetic diversity among community methicillin-resistant Staphylococcus aureus strains causing outpatient infections in Australia." J Clin Microbiol **42**(10): 4735-43.

Cooney, J., Z. Kienle, *et al.* (1993). "The gamma-hemolysin locus of *Staphylococcus aureus* comprises three linked genes, two of which are identical to the genes for the F and S components of leukocidin." Infect Immun **61**(2): 768-71.

Cosgrove, S. E., G. Sakoulas, *et al.* (2003). "Comparison of mortality associated with methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* bacteremia: a meta-analysis." Clin Infect Dis **36**(1): 53-9.

Cotter, P. D. and C. Hill (2003). "Surviving the acid test: responses of gram-positive bacteria to low pH." Microbiol Mol Biol Rev **67**(3): 429-53, table of contents.

Cucarella, C., C. Solano, *et al.* (2001). "Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation." J Bacteriol **183**(9): 2888-96.

Cui, L. and K. Hiramatsu (2003). Vancomycin-resistant *Staphylococcus aureus*. MRSA current perspectives. A. C. Fluit and F. J. Schmitz. Norfolk, Caister Academic Press: 187-212.

Cui, L., X. Ma, *et al.* (2003). "Cell wall thickening is a common feature of vancomycin resistance in *Staphylococcus aureus*." J Clin Microbiol **41**(1): 5-14.

Cunnion, K. M., J. C. Lee, *et al.* (2001). "Capsule production and growth phase influence binding of complement to *Staphylococcus aureus*." Infect Immun **69**(11): 6796-803.

Dale, G. E., C. Broger, *et al.* (1995). "Characterization of the gene for the chromosomal dihydrofolate reductase (DHFR) of *Staphylococcus epidermidis* ATCC 14990: the origin of the trimethoprim-resistant S1 DHFR from *Staphylococcus aureus*?" J Bacteriol **177**(11): 2965-70.

Dancer, S. J., R. Garratt, *et al.* (1990). "The epidermolytic toxins are serine proteases." FEBS Lett **268**(1): 129-32.

Daum, R. S., T. Ito, *et al.* (2002). "A novel methicillin-resistance cassette in community-acquired methicillin-resistant *Staphylococcus aureus* isolates of diverse genetic backgrounds." J Infect Dis **186**(9): 1344-7.

Dautle, M. P., R. L. Ulrich, *et al.* (2002). "Typing and subtyping of 83 clinical isolates purified from surgically implanted silicone feeding tubes by random amplified polymorphic DNA amplification." J Clin Microbiol **40**(2): 414-21.

Day, N. P., C. E. Moore, *et al.* (2001). "A link between virulence and ecological abundance in natural populations of *Staphylococcus aureus*." Science **292**(5514): 114-6.

Denis, O., C. Nonhoff, *et al.* (2002). "Emergence of vancomycin-intermediate Staphylococcus aureus in a Belgian hospital: microbiological and clinical features." J Antimicrob Chemother **50**(3): 383-91.

Diekema, D. J., M. A. Pfaller, *et al.* (2001). "Survey of infections due to Staphylococcus species: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY Antimicrobial Surveillance Program, 1997-1999." Clin Infect Dis **32 Suppl 2**: S114-32.

Diep, B. A., G. F. Sensabaugh, *et al.* (2004). "Widespread skin and soft-tissue infections due to two methicillin-resistant *Staphylococcus aureus* strains harboring the genes for Panton-Valentine leucocidin." J Clin Microbiol **42**(5): 2080-4.

Drlica, K. and X. Zhao (1997). "DNA gyrase, topoisomerase IV, and the 4-quinolones." Microbiol Mol Biol Rev **61**(3): 377-92.

du Toit, R., T. C. Victor, *et al.* (1993). "Empirical evaluation of conditions influencing the polymerase chain reaction: enterotoxigenic *Escherichia coli* as a test case." Eur J Clin Chem Clin Biochem **31**(4): 225-31.

Dufour, P., Y. Gillet, *et al.* (2002). "Community-acquired methicillin-resistant *Staphylococcus aureus* infections in France: emergence of a single clone that produces Panton-Valentine leukocidin." Clin Infect Dis **35**(7): 819-24.

Dyke, K. and P. Gregory (1997). Resistance to Beta-Lactam Antibiotics: Resistance mediated by Beta-Lactamase. The Staphylococci in human Disease. K. B. Crossley and G. L. Archer. New York, Churchill Publishing: 139-158.

Elaichouni, A., J. van Emmelo, *et al.* (1994). "Study of the influence of plasmids on the arbitrary primer polymerase chain reaction fingerprint of *Escherichia coli* strains." FEMS Microbiol Lett **115**(2-3): 335-9.

Elasri, M. O., J. R. Thomas, *et al.* (2002). "*Staphylococcus aureus* collagen adhesin contributes to the pathogenesis of osteomyelitis." Bone **30**(1): 275-80.
Ellsworth, D. L., K. D. Rittenhouse, *et al.* (1993). "Artifactual variation in randomly amplified polymorphic DNA banding patterns." Biotechniques **14**(2): 214-7.
Engemann, J. J., Y. Carmeli, *et al.* (2003). "Adverse clinical and economic outcomes attributable to methicillin resistance among patients with Staphylococcus aureus surgical site infection." Clin Infect Dis **36**(5): 592-8.
Enright, M. C., N. P. Day, *et al.* (2000). "Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*." J Clin Microbiol **38**(3): 1008-15.
Enright, M. C., D. A. Robinson, *et al.* (2002). "The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA)." Proc Natl Acad Sci U S A **99**(11): 7687-92.
Enright, M. C. and W. Witte (2004). Epidemiology of MRSA and MSSA. *Staphylococcus aureus* molecular and clinical aspects. A. A. Ala'Aldeen and K. Hiramatsu. Chichester, Horwood Publishing Limited**:** 31-65.
Espersen, F. and N. Frimodt-Moller (1986). "*Staphylococcus aureus* endocarditis. A review of 119 cases." Arch Intern Med **146**(6): 1118-21.
Farrell, A. M., D. Taylor, *et al.* (1995). "Cloning, nucleotide sequence determination and expression of the *Staphylococcus aureus* hyaluronate lyase gene." FEMS Microbiol Lett **130**(1): 81-5.
Ferraz, V., A. G. Duse, *et al.* (2000). "Vancomycin-resistant *Staphylococcus aureus* occurs in South Africa." S Afr Med J **90**(11): 1113.
Ferreras, M., F. Hoper, *et al.* (1998). "The interaction of *Staphylococcus aureus* bi-component gamma-hemolysins and leucocidins with cells and lipid membranes." Biochim Biophys Acta **1414**(1-2): 108-26.
Ferris, M. M., X. Yan, *et al.* (2004). "Performance assessment of DNA fragment sizing by high-sensitivity flow cytometry and pulsed-field gel electrophoresis." J Clin Microbiol **42**(5): 1965-76.
Finck-Barbancon, V., G. Prevost, *et al.* (1991). "Improved purification of leukocidin from *Staphylococcus aureus* and toxin distribution among hospital strains." Res Microbiol **142**(1): 75-85.
Foster, T. J. and M. Hook (1998). "Surface protein adhesins of *Staphylococcus aureus*." Trends Microbiol **6**(12): 484-8.
Fournier, J. M. (1990). Capsular polysaccharides of *Staphylococcus aureus*. Pathogenesis of wound and biomaterial-associated infections. T. Wadstrum, I. Eliason, I. Holder and A. Ljungh. London, Springer-Verlag**:** 533.
Francois, P., P. Vaudaux, *et al.* (1996). "Host-bacteria interactions in foreign body infections." Infect Control Hosp Epidemiol **17**(8): 514-20.
Fridkin, S. K., J. Hageman, *et al.* (2003). "Epidemiological and microbiological characterization of infections caused by *Staphylococcus aureus* with reduced susceptibility to vancomycin, United States, 1997-2001." Clin Infect Dis **36**(4): 429-39.
Fuller, A. T., G. Mellows, *et al.* (1971). "Pseudomonic acid: an antibiotic produced by *Pseudomonas fluorescens*." Nature **234**(5329): 416-7.
Garner, J. S., W. R. Jarvis, *et al.* (1988). "CDC definitions for nosocomial infections, 1988." Am J Infect Control **16**(3): 128-40.
Gillet, Y., B. Issartel, *et al.* (2002). "Association between *Staphylococcus aureus* strains carrying gene for Panton-Valentine leukocidin and highly lethal

necrotising pneumonia in young immunocompetent patients." Lancet **359**(9308): 753-9.
Godtfredsen, W., K. Roholt, *et al.* (1962). "Fucidin: a new orally active antibiotic." Lancet **1**: 928-31.
Goering, R. V. (2000). "Molecular strain typing for the clinical laboratory: current application and future direction." Clinical Microbiology Newsletter **22**(22): 169-173.
Gonzalez-Zorn, B. and P. Courvalin (2003). "VanA-mediated high level glycopeptide resistance in MRSA." Lancet Infect Dis **3**(2): 67-8.
Graham, J. E. and B. J. Wilkinson (1992). "Staphylococcus aureus osmoregulation: roles for choline, glycine betaine, proline, and taurine." J Bacteriol **174**(8): 2711-6.
Groom, A. V., D. H. Wolsey, *et al.* (2001). "Community-acquired methicillin-resistant *Staphylococcus aureus* in a rural American Indian community." Jama **286**(10): 1201-5.
Groves, P., M. S. Searle, *et al.* (1994). "The structure of an asymmetric dimer relevant to the mode of action of the glycopeptide antibiotics." Structure **2**(8): 747-54.
Guay, G. G., S. A. Khan, *et al.* (1993). "The *tet(K)* gene of plasmid pT181 of *Staphylococcus aureus* encodes an efflux protein that contains 14 transmembrane helices." Plasmid **30**(2): 163-6.
Gudmundsson, A., H. Erlendsdottir, *et al.* (1991). "Impact of pH and cationic supplementation on in vitro postantibiotic effect." Antimicrob Agents Chemother **35**(12): 2617-24.
Hadrys, H., M. Balick, *et al.* (1992). "Applications of random amplified polymorphic DNA (RAPD) in molecular ecology." Mol Ecol **1**(1): 55-63.
Hageman, J. C., D. A. Pegues, *et al.* (2001). "Vancomycin-intermediate *Staphylococcus aureus* in a home health-care patient." Emerg Infect Dis **7**(6): 1023-5.
Hanaki, H., K. Kuwahara-Arai, *et al.* (1998a). "Activated cell-wall synthesis is associated with vancomycin resistance in methicillin-resistant *Staphylococcus aureus* clinical strains Mu3 and Mu50." J Antimicrob Chemother **42**(2): 199-209.
Hanaki, H., H. Labischinski, *et al.* (1998b). "Increase in glutamine-non-amidated muropeptides in the peptidoglycan of vancomycin-resistant *Staphylococcus aureus* strain Mu50." J Antimicrob Chemother **42**(3): 315-20.
Hartman, B. J. and A. Tomasz (1984). "Low-affinity penicillin-binding protein associated with beta-lactam resistance in *Staphylococcus aureus*." J Bacteriol **158**(2): 513-6.
Hartstein, A. I., V. H. Morthland, *et al.* (1989). "Restriction enzyme analysis of plasmid DNA and bacteriophage typing of paired *Staphylococcus aureus* blood culture isolates." J Clin Microbiol **27**(8): 1874-9.
Hartstein, A. I., C. L. Phelps, *et al.* (1995). "In vivo stability and discriminatory power of methicillin-resistant *Staphylococcus aureus* typing by restriction endonuclease analysis of plasmid DNA compared with those of other molecular methods." J Clin Microbiol **33**(8): 2022-6.
Henkel, T. and J. Finlay (1999). "Emergence of resistance during mupirocin treatment: is it a problem in clinical practice?" J Chemother **11**(5): 331-7.
Hermans, K., P. De Herdt, *et al.* (2001a). "Sequence analysis of a RAPD band differentiating high and low virulence *Staphylococcus aureus* strains from rabbits." Vet Microbiol **82**(1): 61-7.

Hermans, K., P. De Herdt, *et al.* (2001). "Secreted antigens as virulence-associated markers in *Staphylococcus aureus* strains from rabbits." Vet Microbiol **81**(4): 345-52.

Hilton, A. C., J. G. Banks, *et al.* (1996). "Random amplification of polymorphic DNA (RAPD) of Salmonella: strain differentiation and characterization of amplified sequences." J Appl Bacteriol **81**(6): 575-84.

Hilton, A. C., J. G. Banks, *et al.* (1997). "Optimization of RAPD for fingerprinting Salmonella." Lett Appl Microbiol **24**(4): 243-8.

Hilton, A. C., D. Mortiboy, *et al.* (1997a). "RAPD analysis of environmental, food and clinical isolates of *Campylobacter spp.*" FEMS Immunol Med Microbiol **18**(2): 119-24.

Hiramatsu, K. (1995). "Molecular evolution of MRSA." Microbiol Immunol **39**(8): 531-43.

Hiramatsu, K. (1998). "Vancomycin resistance in staphylococci." Drug Resistance Update **1**: 135-150.

Hiramatsu, K., N. Aritaka, *et al.* (1997b). "Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin." Lancet **350**(9092): 1670-1673.

Hiramatsu, K., L. Cui, *et al.* (2001). "The emergence and evolution of methicillin-resistant *Staphylococcus aureus*." Trends Microbiol **9**(10): 486-93.

Hiramatsu, K., H. Hanaki, *et al.* (1997a). "Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility." J Antimicrob Chemother **40**(1): 135-136.

Hiramatsu, K., Y. Katayama, *et al.* (2002). "Molecular genetics of methicillin-resistant *Staphylococcus aureus*." Int J Med Microbiol **292**(2): 67-74.

Hodgson, J. E., S. P. Curnock, *et al.* (1994). "Molecular characterization of the gene encoding high-level mupirocin resistance in *Staphylococcus aureus* J2870." Antimicrob Agents Chemother **38**(5): 1205-8.

Hoeger, P. H., R. Ganschow, *et al.* (2000). "Staphylococcal septicemia in children with atopic dermatitis." Pediatr Dermatol **17**(2): 111-4.

Hojo, S., J. Fujita, *et al.* (1995). "[DNA fingerprinting by arbitrarily primed polymerase chain reaction (AP-PCR) for methicillin-resistant Staphylococcus aureus]." Kansenshogaku Zasshi **69**(5): 506-10.

Holden, M. T., E. J. Feil, *et al.* (2004). "Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance." Proc Natl Acad Sci U S A **101**(26): 9786-91.

Holmberg, S. D., S. L. Solomon, *et al.* (1987). "Health and economic impacts of antimicrobial resistance." Rev Infect Dis **9**(6): 1065-78.

Holmes, A., M. Ganner, *et al.* (2005). "*Staphylococcus aureus* isolates carrying Panton-Valentine leucocidin genes in England and Wales: frequency, characterization, and association with clinical disease." J Clin Microbiol **43**(5): 2384-90.

Hood, J., G. F. S. Edwards, *et al.* (2000). "Vancomycin-intermediate *Staphylococcus aureus* at a Scottish hospital." Journal of Infection **40**: A11.

Hopkins, K. L. and A. C. Hilton (2001). "Use of multiple primers in RAPD analysis of clonal organisms provides limited improvement in discrimination." Biotechniques **30**(6): 1262-4, 1266-7.

Hopkins, K. L. and A. C. Hilton (2001a). "Optimization of random amplification of polymorphic DNA analysis for molecular subtyping of Escherichia coli O157." Lett Appl Microbiol **32**(3): 126-30.

Hughes, J. and G. Mellows (1978). "On the mode of action of pseudomonic acid: inhibition of protein synthesis in *Staphylococcus aureus*." J Antibiot (Tokyo) **31**(4): 330-5.

Hunter, P. R. and M. A. Gaston (1988). "Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity." J Clin Microbiol **26**(11): 2465-6.

Ing, M. B., L. M. Baddour, *et al.* (1997). Bacteremia and infective endocarditis: Pathogenesis, diagnosis, and complications. The Staphylococci in Human Disease. K. B. Crossley and G. L. Archer. New York, Churchill Livingstone: 331-354.

Ito, T., Y. Katayama, *et al.* (2001). "Structural comparison of three types of staphylococcal cassette chromosome *mec* integrated in the chromosome in methicillin-resistant *Staphylococcus aureus*." Antimicrob Agents Chemother **45**(5): 1323-36.

Ito, T., Y. Katayama, *et al.* (1999). "Cloning and nucleotide sequence determination of the entire *mec* DNA of pre-methicillin-resistant *Staphylococcus aureus* N315." Antimicrob Agents Chemother **43**(6): 1449-58.

Ito, T., X. X. Ma, *et al.* (2004). "Novel type V staphylococcal cassette chromosome *mec* driven by a novel cassette chromosome recombinase, ccrC." Antimicrob Agents Chemother **48**(7): 2637-51.

Ito, T., K. Okuma, *et al.* (2003). "Insights on antibiotic resistance of *Staphylococcus aureus* from its whole genome: genomic island SCC." Drug Resist Updat **6**(1): 41-52.

Ito, Y., M. Funabashi Yoh, *et al.* (2002). "Staphylococcal scalded-skin syndrome in an adult due to methicillin-resistant *Staphylococcus aureus*." J Infect Chemother **8**(3): 256-61.

Jakob, H. G., M. Borneff-Lipp, *et al.* (2000). "The endogenous pathway is a major route for deep sternal wound infection." Eur J Cardiothorac Surg **17**(2): 154-60.

Jarraud, S., C. Mougel, *et al.* (2002). "Relationships between *Staphylococcus aureus* genetic background, virulence factors, agr groups (alleles), and human disease." Infect Immun **70**(2): 631-41.

Jernigan, J. A. and B. M. Farr (1993). "Short-course therapy of catheter-related *Staphylococcus aureus* bacteremia: a meta-analysis." Ann Intern Med **119**(4): 304-11.

Jevons, M. P. (1961). ""Celbenin" - resistant staphylococci." British Medical Journal **124**: 124-125.

Ji, G., R. C. Beavis, *et al.* (1995). "Cell density control of staphylococcal virulence mediated by an octapeptide pheromone." Proc Natl Acad Sci U S A **92**(26): 12055-9.

John, J. F. (2004). Staphylococcal infection: Emerging clinical syndromes. *Staphylococcus aureus* molecular and clinical aspects. K. Hiramatsu. Chichester, Horwood Publishing Limited: 1-30.

Karlsson, A., P. Saravia-Otten, *et al.* (2001). "Decreased amounts of cell wall-associated protein A and fibronectin-binding proteins in *Staphylococcus aureus* sarA mutants due to up-regulation of extracellular proteases." Infect Immun **69**(8): 4742-8.

Katayama, Y., T. Ito, *et al.* (2000). "A new class of genetic element, staphylococcus cassette chromosome *mec*, encodes methicillin resistance in *Staphylococcus aureus*." Antimicrob Agents Chemother **44**(6): 1549-55.

Kawabata, S., T. Morita, *et al.* (1985). "Enzymatic properties of staphylothrombin, an active molecular complex formed between staphylocoagulase and human prothrombin." J Biochem (Tokyo) **98**(6): 1603-14.

Kay, C. R. (1962). "Sepsis in the home." British Medical Journal **1**: 1048.

Kelly, R. B., N. R. Cozzarelli, *et al.* (1970). "Enzymatic synthesis of deoxyribonucleic acid. XXXII. Replication of duplex deoxyribonucleic acid by polymerase at a single strand break." J Biol Chem **245**(1): 39-45.

Kernodle, D. S. (2000). Mechanisms of resistance to ß-lactam antibiotics. Gram-positive pathogens. V. A. Fischetti, R. P. Novick, J. J. Ferretti, D. A. Portnoy and J. I. Rood. Washington, DC, USA, American Society of Microbiology**:** 609-620.

Kernodle, D. S., P. A. McGraw, *et al.* (1990). "Use of extracts versus whole-cell bacterial suspensions in the identification of *Staphylococcus aureus* beta-lactamase variants." Antimicrob Agents Chemother **34**(3): 420-5.

Khan, S. A. and R. P. Novick (1983). "Complete nucleotide sequence of pT181, a tetracycline-resistance plasmid from *Staphylococcus aureus*." Plasmid **10**(3): 251-9.

Kim, M. N., C. H. Pai, *et al.* (2000). "Vancomycin-intermediate *Staphylococcus aureus* in Korea." J Clin Microbiol **38**(10): 3879-81.

Kirst, H. A., D. G. Thompson, *et al.* (1998). "Historical yearly usage of vancomycin." Antimicrob Agents Chemother **42**(5): 1303-4.

Klett, R. P., A. Cerami, *et al.* (1968). "Exonuclease VI, a new nuclease activity associated with *E. coli* DNA polymerase." Proc Natl Acad Sci U S A **60**(3): 943-50.

Kluytmans, J., A. van Belkum, *et al.* (1997). "Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks." Clin Microbiol Rev **10**(3): 505-20.

Kluytmans, J. A., J. W. Mouton, *et al.* (1995). "Nasal carriage of *Staphylococcus aureus* as a major risk factor for wound infections after cardiac surgery." J Infect Dis **171**(1): 216-9.

Kniehl, E., A. Becker, *et al.* (2005). "Bed, bath and beyond: pitfalls in prompt eradication of methicillin-resistant Staphylococcus aureus carrier status in healthcare workers." J Hosp Infect **59**(3): 180-7.

Konig, B., G. Prevost, *et al.* (1997). "Composition of staphylococcal bi-component toxins determines pathophysiological reactions." J Med Microbiol **46**(6): 479-85.

Kotra, L. P., J. Haddad, *et al.* (2000). "Aminoglycosides: perspectives on mechanisms of action and resistance and strategies to counter resistance." Antimicrob Agents Chemother **44**(12): 3249-56.

Kreiswirth, B., J. Kornblum, *et al.* (1993). "Evidence for a clonal origin of methicillin resistance in *Staphylococcus aureus*." Science **259**(5092): 227-30.

Kullik, I., P. Giachino, *et al.* (1998). "Deletion of the alternative sigma factor sigmaB in Staphylococcus aureus reveals its function as a global regulator of virulence genes." J Bacteriol **180**(18): 4814-20.

Kuroda, M., T. Ohta, *et al.* (2001). "Whole genome sequencing of meticillin-resistant *Staphylococcus aureus*." Lancet **357**(9264): 1225-40.

Kusumaningrum, H. D., G. Riboldi, *et al.* (2003). "Survival of foodborne pathogens on stainless steel surfaces and cross-contamination to foods." Int J Food Microbiol **85**(3): 227-36.

Kuwahara-Arai, K., N. Kondo, *et al.* (1996). "Suppression of methicillin resistance in a *mecA*-containing pre-methicillin-resistant *Staphylococcus aureus* strain is caused by the mecI-mediated repression of PBP 2' production." Antimicrob Agents Chemother **40**(12): 2680-5.

Labischinski, H. (1992). "Consequences of the interaction of beta-lactam antibiotics with penicillin binding proteins from sensitive and resistant *Staphylococcus aureus* strains." Med Microbiol Immunol (Berl) **181**(5): 241-65.

Ladhani, S., C. L. Joannou, *et al.* (1999). "Clinical, microbial, and biochemical aspects of the exfoliative toxins causing staphylococcal scalded-skin syndrome." Clin Microbiol Rev **12**(2): 224-42.

Lang, S., M. A. Livesley, *et al.* (1999). "The genomic diversity of coagulase-negative staphylococci associated with nosocomial infections." J Hosp Infect **43**(3): 187-93.

Laube, S. and A. M. Farrell (2002). "Bacterial skin infections in the elderly: diagnosis and treatment." Drugs Aging **19**(5): 331-42.

Lawrence, L. M., J. Harvey, *et al.* (1993). "Development of a random amplification of polymorphic DNA typing method for *Listeria monocytogenes*." Appl Environ Microbiol **59**(9): 3117-9.

Lee, J. C. and G. A. Bohach (2004). Pathogenesis of disease. *Staphylococcus aureus* molecular and clinical aspects. A. A. Ala'Aldeen and K. Hiramatsu. Chichester, Horwood Publishing Limited: 176-236.

Leelaporn, A., N. Firth, *et al.* (1995). "Multidrug resistance plasmid pSK108 from coagulase-negative staphylococci; relationships to Staphylococcus aureus qacC plasmids." Plasmid **34**(1): 62-7.

Leonard, R. B. and K. C. Carroll (1997). "Rapid lysis of gram-positive cocci for pulsed-field gel electrophoresis using achromopeptidase." Diagn Mol Pathol **6**(5): 288-91.

Leonard, R. B., J. Mayer, *et al.* (1995). "Comparison of MIDI Sherlock system and pulsed-field gel electrophoresis in characterizing strains of methicillin-resistant Staphylococcus aureus from a recent hospital outbreak." J Clin Microbiol **33**(10): 2723-7.

Lewis, K. and A. M. Klibanov (2005). "Surpassing nature: rational design of sterile-surface materials." Trends Biotechnol **23**(7): 343-8.

Libman, H. and R. D. Arbeit (1984). "Complications associated with *Staphylococcus aureus* bacteremia." Arch Intern Med **144**(3): 541-5.

Lina, G., Y. Piemont, *et al.* (1999). "Involvement of Panton-Valentine leukocidin-producing *Staphylococcus aureus* in primary skin infections and pneumonia." Clin Infect Dis **29**(5): 1128-32.

Lina, G., A. Quaglia, *et al.* (1999). "Distribution of genes encoding resistance to macrolides, lincosamides, and streptogramins among staphylococci." Antimicrob Agents Chemother **43**(5): 1062-6.

Lindsay, J. A. and M. T. Holden (2004). "*Staphylococcus aureus*: superbug, super genome?" Trends Microbiol **12**(8): 378-85.

Liu, P. Y., Z. Y. Shi, *et al.* (1996). "Use of restriction endonuclease analysis of plasmids and pulsed-field gel electrophoresis to investigate outbreaks of methicillin-resistant Staphylococcus aureus infection." Clin Infect Dis **22**(1): 86-90.

Livermore, D. M. (1995). "Beta-Lactamases in laboratory and clinical resistance." Clin Microbiol Rev **8**(4): 557-84.

Locksley, R. M., M. L. Cohen, *et al.* (1982). "Multiply antibiotic-resistant *Staphylococcus aureus*: introduction, transmission, and evolution of nosocomial infection." Ann Intern Med **97**(3): 317-24.

Louie, M., P. Jayaratne, *et al.* (1996). "Comparison of ribotyping, arbitrarily primed PCR, and pulsed-field gel electrophoresis for molecular typing of Listeria monocytogenes." J Clin Microbiol **34**(1): 15-9.

Lowy, F. D. (1998). "*Staphylococcus aureus* infections." N Engl J Med **339**(8): 520-32.

Lowy, F. D. (2003). "Antimicrobial resistance: the example of *Staphylococcus aureus*." J Clin Invest **111**(9): 1265-73.

Lowy, F. D. and M. Miller (2002). "New methods to investigate infectious disease transmission and pathogenesis--Staphylococcus aureus disease in drug users." Lancet Infect Dis **2**(10): 605-12.

Lyon, B. R., J. W. May, *et al.* (1983). "Analysis of plasmids in nosocomial strains of multiple-antibiotic-resistant *Staphylococcus aureus*." Antimicrob Agents Chemother **23**(6): 817-26.

Lyon, B. R. and R. Skurray (1987). "Antimicrobial resistance of *Staphylococcus aureus*: genetic basis." Microbiol Rev **51**(1): 88-134.

Ma, X. X., T. Ito, *et al.* (2002). "Novel type of staphylococcal cassette chromosome *mec* identified in community-acquired methicillin-resistant *Staphylococcus aureus* strains." Antimicrob Agents Chemother **46**(4): 1147-52.

Mackay, J. P., U. Gerhard, *et al.* (1994). "Dissection of the contributions toward dimerization of glycopeptide antibiotics." The Journal of the American Chemical Society **116**: 4573-4580.

Maguire, G. P., A. D. Arthur, *et al.* (1998). "Clinical experience and outcomes of community-acquired and nosocomial methicillin-resistant *Staphylococcus aureus* in a northern Australian hospital." J Hosp Infect **38**(4): 273-81.

Maiden, M. C., J. A. Bygraves, *et al.* (1998). "Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms." Proc Natl Acad Sci U S A **95**(6): 3140-5.

Marchese, A., G. Balistreri, *et al.* (2000). "Heterogeneous vancomycin resistance in methicillin-resistant *Staphylococcus aureus* strains isolated in a large Italian hospital." J Clin Microbiol **38**(2): 866-9.

Marshall, M. J., G. A. Bohach, *et al.* (2000). "Characterization of *Staphylococcus aureus* beta-toxin induced leukotoxicity." J Nat Toxins **9**(2): 125-38.

Martineau, F., F. J. Picard, *et al.* (2000a). "Multiplex PCR assays for the detection of clinically relevant antibiotic resistance genes in staphylococci isolated from patients infected after cardiac surgery. The ESPRIT Trial." J Antimicrob Chemother **46**(4): 527-34.

Martineau, F., F. J. Picard, *et al.* (2000). "Correlation between the resistance genotype determined by multiplex PCR assays and the antibiotic susceptibility patterns of *Staphylococcus aureus* and *Staphylococcus epidermidis*." Antimicrob Agents Chemother **44**(2): 231-8.

Maslow, J. N., M. E. Mulligan, *et al.* (1993). "Molecular epidemiology: application of contemporary techniques to the typing of microorganisms." Clin Infect Dis **17**(2): 153-62; quiz 163-4.

Matsuda, Y., H. Kato, *et al.* (2003). "Early and definitive diagnosis of toxic shock syndrome by detection of marked expansion of T-cell-receptor VBeta2-positive T cells." Emerg Infect Dis **9**(3): 387-9.

Matsuoka, M., M. Inoue, *et al.* (2002). "New erm gene in *Staphylococcus aureus* clinical isolates." Antimicrob Agents Chemother **46**(1): 211-5.
Matushek, M. G., M. J. Bonten, *et al.* (1996). "Rapid preparation of bacterial DNA for pulsed-field gel electrophoresis." J Clin Microbiol **34**(10): 2598-600.
Mayer, S., M. Boos, *et al.* (2001). "Distribution of the antiseptic resistance genes qacA, qacB and qacC in 497 methicillin-resistant and -susceptible European isolates of Staphylococcus aureus." J Antimicrob Chemother **47**(6): 896-7.
Mazmanian, S. K., G. Liu, *et al.* (1999). "*Staphylococcus aureus* sortase, an enzyme that anchors surface proteins to the cell wall." Science **285**(5428): 760-3.
Mazurier, S., A. van de Giessen, *et al.* (1992). "RAPD analysis of *Campylobacter* isolates: DNA fingerprinting without the need to purify DNA." Lett Appl Microbiol **14**(6): 260-2.
Mazurier, S. I. and K. Wernars (1992a). "Typing of *Listeria* strains by random amplification of polymorphic DNA." Res Microbiol **143**(5): 499-505.
McCormick, J. K., J. M. Yarwood, *et al.* (2001). "Toxic shock syndrome and bacterial superantigens: an update." Annu Rev Microbiol **55**: 77-104.
McDevitt, D., P. Francois, *et al.* (1994). "Molecular characterization of the clumping factor (fibrinogen receptor) of *Staphylococcus aureus*." Mol Microbiol **11**(2): 237-48.
McDevitt, D., P. Francois, *et al.* (1995). "Identification of the ligand-binding domain of the surface-located fibrinogen receptor (clumping factor) of *Staphylococcus aureus*." Mol Microbiol **16**(5): 895-907.
McGavin, M. J., C. Zahradka, *et al.* (1997). "Modification of the *Staphylococcus aureus* fibronectin binding phenotype by V8 protease." Infect Immun **65**(7): 2621-8.
McGowan, J. E., Jr., P. M. Terry, *et al.* (1979). "Nosocomial infections with gentamicin-resistant *Staphylococcus aureus*: plamid analysis as an epidemiologic tool." J Infect Dis **140**(6): 864-72.
Menard, C., R. Brousseau, *et al.* (1992). "Application of polymerase chain reaction with arbitrary primer (AP-PCR) to strain identification of *Porphyromonas* (*Bacteroides*) *gingivalis*." FEMS Microbiol Lett **74**(2-3): 163-8.
Meyers, J. A., D. Sanchez, *et al.* (1976). "Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid." J Bacteriol **127**(3): 1529-37.
Miles, M. M. and S. S. Misra (1938). "The estimation of the bactericidal effect of blood." Journal of Haemotology **38**: 732-749.
Montesinos, I., E. Salido, *et al.* (2002). "Epidemiologic genotyping of methicillin-resistant *Staphylococcus aureus* by pulsed-field gel electrophoresis at a university hospital and comparison with antibiotyping and protein A and coagulase gene polymorphisms." J Clin Microbiol **40**(6): 2119-25.
Montville, R. and D. W. Schaffner (2003). "Inoculum size influences bacterial cross contamination between surfaces." Appl Environ Microbiol **69**(12): 7188-93.
Morrissey, I. and D. J. Farrell (2003). MRSA resistance mechanisms and surveillance data for non-betalactams and non-glycopeptides. MRSA current perspectives. A. C. Fluit and F. J. Schmitz. Norfolk, Caister Academic Press: 55-93.
Morrow, T. O. and S. A. Harmon (1979). "Genetic analysis of *Staphylococcus aureus* RNA polymerase mutants." J Bacteriol **137**(1): 374-83.
Mortensen, J. E., T. R. Shryock, *et al.* (1992). "Modification of bactericidal fatty acids by an enzyme of *Staphylococcus aureus*." J Med Microbiol **36**(4): 293-8.

Morton, T. M., D. M. Eaton, *et al.* (1993). "DNA sequence and units of transcription of the conjugative transfer gene complex (trs) of *Staphylococcus aureus* plasmid pGO1." J Bacteriol **175**(14): 4436-47.

Morton, T. M., J. L. Johnston, *et al.* (1995). "Characterization of a conjugative staphylococcal mupirocin resistance plasmid." Antimicrob Agents Chemother **39**(6): 1272-80.

Mulligan, M. E. and R. D. Arbeit (1991). "Epidemiologic and clinical utility of typing systems for differentiating among strains of methicillin-resistant *Staphylococcus aureus*." Infect Control Hosp Epidemiol **12**(1): 20-8.

Murakami, K., W. Minamide, *et al.* (1991). "Identification of methicillin-resistant strains of staphylococci by polymerase chain reaction." J Clin Microbiol **29**(10): 2240-4.

Murchan, S., M. E. Kaufmann, *et al.* (2003). "Harmonization of pulsed-field gel electrophoresis protocols for epidemiological typing of strains of methicillin-resistant *Staphylococcus aureus*: a single approach developed by consensus in 10 European laboratories and its application for tracing the spread of related strains." J Clin Microbiol **41**(4): 1574-85.

Murphy, E. (1985). "Nucleotide sequence of ermA, a macrolide-lincosamide-streptogramin B determinant in *Staphylococcus aureus*." J Bacteriol **162**(2): 633-40.

Murray, B. E. (2000). "Vancomycin-resistant enterococcal infections." N Engl J Med **342**(10): 710-21.

Musher, D. M., N. Lamm, *et al.* (1994). "The current spectrum of *Staphylococcus aureus* infection in a tertiary care hospital." Medicine (Baltimore) **73**(4): 186-208.

Mylotte, J. M., C. McDermott, *et al.* (1987). "Prospective study of 114 consecutive episodes of *Staphylococcus aureus* bacteremia." Rev Infect Dis **9**(5): 891-907.

Naimi, T. S., K. H. LeDell, *et al.* (2001). "Epidemiology and clonality of community-acquired methicillin-resistant *Staphylococcus aureus* in Minnesota, 1996-1998." Clin Infect Dis **33**(7): 990-6.

Narita, S., J. Kaneko, *et al.* (2001). "Phage conversion of Panton-Valentine leukocidin in *Staphylococcus aureus*: molecular analysis of a PVL-converting phage, phiSLT." Gene **268**(1-2): 195-206.

Nesin, M., P. Svec, *et al.* (1990). "Cloning and nucleotide sequence of a chromosomally encoded tetracycline resistance determinant, tetA(M), from a pathogenic, methicillin-resistant strain of *Staphylococcus aureus*." Antimicrob Agents Chemother **34**(11): 2273-6.

Neyfakh, A. A., C. M. Borsch, *et al.* (1993). "Fluoroquinolone resistance protein NorA of *Staphylococcus aureus* is a multidrug efflux transporter." Antimicrob Agents Chemother **37**(1): 128-9.

Ni Eidhin, D., S. Perkins, *et al.* (1998). "Clumping factor B (ClfB), a new surface-located fibrinogen-binding adhesin of *Staphylococcus aureus*." Mol Microbiol **30**(2): 245-57.

Nicholas, R. A. and J. L. Strominger (1988). "Site-directed mutants of a soluble form of penicillin-binding protein 5 from *Escherichia coli* and their catalytic properties." J Biol Chem **263**(4): 2034-40.

Noble, W. C. (1997). Staphylococcal carriage and skin and soft tissue infection. The Staphylococci in Human Disease. K. B. Crossley and G. L. Archer. New York, Churchill Livingstone: 401-412.

Noble, W. C., H. A. Valkenburg, *et al.* (1967). "Carriage of *Staphylococcus aureus* in random samples of a normal population." J Hyg (Lond) **65**(4): 567-73.

Noguchi, N., M. Hase, *et al.* (1999). "Antiseptic susceptibility and distribution of antiseptic-resistance genes in methicillin-resistant Staphylococcus aureus." FEMS Microbiol Lett **172**(2): 247-53.

Novick, R. P. (1989). "Staphylococcal plasmids and their replication." Annu Rev Microbiol **43**: 537-65.

O'Brien, F. G., T. T. Lim, *et al.* (2004). "Diversity among community isolates of methicillin-resistant *Staphylococcus aureus* in Australia." J Clin Microbiol **42**(7): 3185-90.

O'Brien, F. G., J. W. Pearman, *et al.* (1999). "Community strain of methicillin-resistant *Staphylococcus aureus* involved in a hospital outbreak." J Clin Microbiol **37**(9): 2858-62.

O'Brien, F. G., C. Price, *et al.* (2002). "Genetic characterization of the fusidic acid and cadmium resistance determinants of *Staphylococcus aureus* plasmid pUB101." J Antimicrob Chemother **50**(3): 313-21.

O'Connell, N. H. and H. Humphreys (2000). "Intensive care unit design and environmental factors in the acquisition of infection." J Hosp Infect **45**(4): 255-62.

Ogsten, A. (1881). "Report upon microorganisms in surgical diseases." British Medical Journal **1**: 369-375.

Okuma, K., K. Iwakawa, *et al.* (2002). "Dissemination of new methicillin-resistant *Staphylococcus aureus* clones in the community." J Clin Microbiol **40**(11): 4289-94.

Oliveira, D. C. and H. de Lencastre (2002). "Multiplex PCR strategy for rapid identification of structural types and variants of the *mec* element in methicillin-resistant *Staphylococcus aureus*." Antimicrob Agents Chemother **46**(7): 2155-61.

Oliveira, D. C., A. Tomasz, *et al.* (2001a). "The evolution of pandemic clones of methicillin-resistant *Staphylococcus aureus*: identification of two ancestral genetic backgrounds and the associated *mec* elements." Microb Drug Resist **7**(4): 349-61.

Oliveira, D. C., S. W. Wu, *et al.* (2000). "Genetic organization of the downstream region of the mecA element in methicillin-resistant *Staphylococcus aureus* isolates carrying different polymorphisms of this region." Antimicrob Agents Chemother **44**(7): 1906-10.

Oliveira, G. A., A. M. Dell'Aquila, *et al.* (2001). "Isolation in Brazil of nosocomial *Staphylococcus aureus* with reduced susceptibility to vancomycin." Infect Control Hosp Epidemiol **22**(7): 443-8.

Olsson-Liljequist, B., P. Larsson, *et al.* (1997). "Antimicrobial susceptibility testing in Sweden. III. Methodology for susceptibility testing." Scand J Infect Dis Suppl **105**: 13-23.

O'Neill, A. J., J. H. Cove, *et al.* (2001). "Mutation frequencies for resistance to fusidic acid and rifampicin in *Staphylococcus aureus*." J Antimicrob Chemother **47**(5): 647-50.

O'Neill, G. L., S. Murchan, *et al.* (2001a). "Identification and characterization of phage variants of a strain of epidemic methicillin-resistant *Staphylococcus aureus* (EMRSA-15)." J Clin Microbiol **39**(4): 1540-8.

Opal, S. M., A. D. Johnson-Winegar, *et al.* (1988). "Staphylococcal scalded skin syndrome in two immunocompetent adults caused by exfoliatin B-producing *Staphylococcus aureus*." J Clin Microbiol **26**(7): 1283-6.

Parker, M. T. (1972). Phage-typing of *Staphylococcus aureus*. Method in microbiology. J. R. Norris and D. W. Ribbons. London, UK, Academic Press. **7B:** 1-28.

Patel, A. H., J. Kornblum, *et al.* (1992). "Regulation of the protein A-encoding gene in *Staphylococcus aureus*." Gene **114**(1): 25-34.

Patel, A. H., P. Nowlan, *et al.* (1987). "Virulence of protein A-deficient and alpha-toxin-deficient mutants of *Staphylococcus aureus* isolated by allele replacement." Infect Immun **55**(12): 3103-10.

Paton, R., T. Snell, *et al.* (2001). "Glycopeptide resistance in an epidemic strain of methicillin-resistant *Staphylococcus aureus*." J Antimicrob Chemother **48**(6): 941-2.

Patti, J. M., T. Bremell, *et al.* (1994). "The *Staphylococcus aureus* collagen adhesin is a virulence determinant in experimental septic arthritis." Infect Immun **62**(1): 152-61.

Paulsen, I. T., M. H. Brown, *et al.* (1998). "Characterization of the earliest known *Staphylococcus aureus* plasmid encoding a multidrug efflux system." J Bacteriol **180**(13): 3477-9.

Peacock, S. J., I. de Silva, *et al.* (2001). "What determines nasal carriage of *Staphylococcus aureus*?" Trends Microbiol **9**(12): 605-10.

Pereira, M. S., N. C. Leal, *et al.* (2002). "Typing of human and bovine *Staphylococcus aureus* by RAPD-PCR and ribotyping-PCR." Lett Appl Microbiol **35**(1): 32-6.

Perry, A. L., T. Worthington, *et al.* (2003). "Analysis of clinical isolates of *Propionibacterium acnes* by optimised RAPD." FEMS Microbiol Lett **228**(1): 51-5.

Pfeltz, R. F., V. K. Singh, *et al.* (2000). "Characterization of passage-selected vancomycin-resistant *Staphylococcus aureus* strains of diverse parental backgrounds." Antimicrob Agents Chemother **44**(2): 294-303.

Pinho, M. G., H. de Lencastre, *et al.* (2001). "An acquired and a native penicillin-binding protein cooperate in building the cell wall of drug-resistant staphylococci." Proc Natl Acad Sci U S A **98**(19): 10886-91.

Ploy, M. C., C. Grelaud, *et al.* (1998). "First clinical isolate of vancomycin-intermediate *Staphylococcus aureus* in a French hospital." Lancet **351**(9110): 1212.

Pourkomailian, B. and I. R. Booth (1994). "Glycine betaine transport by Staphylococcus aureus: evidence for feedback regulation of the activity of the two transport systems." Microbiology **140 (Pt 11)**: 3131-8.

Preheim, L., D. Pitcher, *et al.* (1991). "Typing of methicillin resistant and susceptible *Staphylococcus aureus* strains by ribosomal RNA gene restriction patterns using a biotinylated probe." Eur J Clin Microbiol Infect Dis **10**(5): 428-36.

Prevost, G., P. Couppie, *et al.* (1995). "Epidemiological data on *Staphylococcus aureus* strains producing synergohymenotropic toxins." J Med Microbiol **42**(4): 237-45.

Projan, S. J., M. Monod, *et al.* (1987). "Replication properties of pIM13, a naturally occurring plasmid found in Bacillus subtilis, and of its close relative pE5, a plasmid native to *Staphylococcus aureus*." J Bacteriol **169**(11): 5131-9.

Pulvirenti, J. J., E. Kerns, *et al.* (1996). "Infective endocarditis in injection drug users: importance of human immunodeficiency virus serostatus and degree of immunosuppression." Clin Infect Dis **22**(1): 40-5.

Raad, II and M. F. Sabbagh (1992). "Optimal duration of therapy for catheter-related *Staphylococcus aureus* bacteremia: a study of 55 cases and review." Clin Infect Dis **14**(1): 75-82.

Rahman, M., S. Connolly, *et al.* (1990). "Diversity of staphylococci exhibiting high-level resistance to mupirocin." J Med Microbiol **33**(2): 97-100.

Rebora, A., F. Crovato, *et al.* (1980). "Repeated staphylococcal pyoderma in two siblings with defective neutrophil bacterial killing." Dermatologica **160**(2): 106-12.

Reynolds, P. E. (1989). "Structure, biochemistry and mechanism of action of glycopeptide antibiotics." Eur J Clin Microbiol Infect Dis **8**(11): 943-50.

Rhem, M. N., E. M. Lech, *et al.* (2000). "The collagen-binding adhesin is a virulence factor in *Staphylococcus aureus* keratitis." Infect Immun **68**(6): 3776-9.

Rigby, P. W., M. Dieckmann, *et al.* (1977). "Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I." J Mol Biol **113**(1): 237-51.

Riley, L. W. (2004). Analysis of similarity and relatedness in molecular epidemiology. Molecular Epidemiology of Infectious Diseases: Principles and Practices. Washington, ASM Press: 91-124.

Riley, L. W. (2004). Principles and approaches. Molecular Epidemiology of Infectious Diseases: Principles and Practices. Washington, ASM Press: 1-28.

Robinson, D. A. and M. C. Enright (2003). "Evolutionary models of the emergence of methicillin-resistant *Staphylococcus aureus*." Antimicrob Agents Chemother **47**(12): 3926-34.

Rohrer, S., M. Bischoff, *et al.* (2003). Mechanisms of methicillin resistance. MRSA current perspectives. A. C. Fluit and F. J. Schmitz. Norfolk, Caister Academic Press: 31-53.

Rosenbach, F. J., Ed. (1884). Mikro-Organismen bei den Wund-Infecktions-Krankenheiten des Menschen. Wiebaden. J. F. Bergmann's Verlag.

Rossney, A. S., D. C. Coleman, *et al.* (1994). "Evaluation of an antibiogram-resistogram typing scheme for methicillin-resistant *Staphylococcus aureus*." J Med Microbiol **41**(6): 441-7.

Rossney, A. S., D. C. Coleman, *et al.* (1994a). "Antibiogram-resistogram typing scheme for methicillin-resistant *Staphylococcus aureus*." J Med Microbiol **41**(6): 430-40.

Rotun, S. S., V. McMath, *et al.* (1999). "*Staphylococcus aureus* with reduced susceptibility to vancomycin isolated from a patient with fatal bacteremia." Emerg Infect Dis **5**(1): 147-9.

Saiki, R. (1989). The design and optimisation of the PCR. PCR technology: Principles and applications for DNA amplification. A. Erhlich. New York, Macmillan: 7-16.

Sakoulas, G., G. M. Eliopoulos, *et al.* (2003). "*Staphylococcus aureus* accessory gene regulator (agr) group II: is there a relationship to the development of intermediate-level glycopeptide resistance?" J Infect Dis **187**(6): 929-38.

Salgado, C. D., B. M. Farr, *et al.* (2003). "Community-acquired methicillin-resistant *Staphylococcus aureus*: a meta-analysis of prevalence and risk factors." Clin Infect Dis **36**(2): 131-9.

Salmenlinna, S., O. Lyytikainen, *et al.* (2002). "Community-acquired methicillin-resistant *Staphylococcus aureus*, Finland." Emerg Infect Dis **8**(6): 602-7.
Sambrook, J. E., E. F. Fritsch, *et al.* (1989). Molecular cloning: a laboratory manual. New York, Cold Spring Harbour Laboratory Press.
Sanabria, T. J., J. S. Alpert, *et al.* (1990). "Increasing frequency of staphylococcal infective endocarditis. Experience at a university hospital, 1981 through 1988." Arch Intern Med **150**(6): 1305-9.
Sandre, R. M. and S. D. Shafran (1996). "Infective endocarditis: review of 135 cases over 9 years." Clin Infect Dis **22**(2): 276-86.
Sanford, M. D., A. F. Widmer, *et al.* (1994). "Efficient detection and long-term persistence of the carriage of methicillin-resistant *Staphylococcus aureus*." Clin Infect Dis **19**(6): 1123-8.
Saulnier, P., C. Bourneix, *et al.* (1993). "Random amplified polymorphic DNA assay is less discriminant than pulsed-field gel electrophoresis for typing strains of methicillin-resistant *Staphylococcus aureus*." J Clin Microbiol **31**(4): 982-5.
Schlichting, C., C. Branger, *et al.* (1993). "Typing of *Staphylococcus aureus* by pulsed-field gel electrophoresis, zymotyping, capsular typing, and phage typing: resolution of clonal relationships." J Clin Microbiol **31**(2): 227-32.
Schnappinger, D. and W. Hillen (1996). "Tetracyclines: antibiotic action, uptake, and resistance mechanisms." Arch Microbiol **165**(6): 359-69.
Schoettlin, W., K. B. Neilson, *et al.* (1994). "Optimisation of PCR using the opti-prime kit." Strategies in Molecular Biology Newsletter **6**: 43-44.
Schwalbe, R. S., J. T. Stapleton, *et al.* (1987). "Emergence of vancomycin resistance in coagulase-negative staphylococci." N Engl J Med **316**(15): 927-31.
Schwarz, S., M. C. Roberts, *et al.* (1998). "Tetracycline resistance in *Staphylococcus spp.* from domestic animals." Vet Microbiol **63**(2-4): 217-27.
Scott, E. and S. F. Bloomfield (1990). "The survival and transfer of microbial contamination via cloths, hands and utensils." J Appl Bacteriol **68**(3): 271-8.
Scott, W. J. (1953). "Water relations of *Staphylococcus aureus* at 30 degrees C." Aust J Biol Sci **6**(4): 549-64.
Scybert, S., R. Pechous, *et al.* (2003). "NaCl-sensitive mutant of Staphylococcus aureus has a Tn917-lacZ insertion in its ars operon." FEMS Microbiol Lett **222**(2): 171-6.
Severin, A., S. W. Wu, *et al.* (2004). "Penicillin-binding protein 2 is essential for expression of high-level vancomycin resistance and cell wall synthesis in vancomycin-resistant *Staphylococcus aureus* carrying the enterococcal *vanA* gene complex." Antimicrob Agents Chemother **48**(12): 4566-73.
Shopsin, B., M. Gomez, *et al.* (1999). "Evaluation of protein A gene polymorphic region DNA sequencing for typing of *Staphylococcus aureus* strains." J Clin Microbiol **37**(11): 3556-63.
Showsh, S. A., E. H. De Boever, *et al.* (2001). "Vancomycin resistance plasmid in Enterococcus faecalis that encodes sensitivity to a sex pheromone also produced by *Staphylococcus aureus*." Antimicrob Agents Chemother **45**(7): 2177-8.
Siboo, I. R., A. L. Cheung, *et al.* (2001). "Clumping factor A mediates binding of *Staphylococcus aureus* to human platelets." Infect Immun **69**(5): 3120-7.
Sidhu, M. S., E. Heir, *et al.* (2002). "Frequency of disinfectant resistance genes and genetic linkage with beta-lactamase transposon Tn552 among clinical staphylococci." Antimicrob Agents Chemother **46**(9): 2797-803.

Sieradzki, K., M. G. Pinho, *et al.* (1999). "Inactivated pbp4 in highly glycopeptide-resistant laboratory mutants of Staphylococcus aureus." J Biol Chem **274**(27): 18942-6.

Sieradzki, K., R. B. Roberts, *et al.* (1999a). "The development of vancomycin resistance in a patient with methicillin-resistant *Staphylococcus aureus* infection." N Engl J Med **340**(7): 517-23.

Smith, M. A. and M. J. Bidochka (1998). "Bacterial fitness and plasmid loss: the importance of culture conditions and plasmid size." Can J Microbiol **44**(4): 351-5.

Smith, S. M., R. H. Eng, *et al.* (1996). "Survival of nosocomial pathogenic bacteria at ambient temperature." J Med **27**(5-6): 293-302.

Smith, T. L., M. L. Pearson, *et al.* (1999). "Emergence of vancomycin resistance in *Staphylococcus aureus*. Glycopeptide-Intermediate *Staphylococcus aureus* Working Group." N Engl J Med **340**(7): 493-501.

Spratt, B. G. (1999). "Multilocus sequence typing: molecular typing of bacterial pathogens in an era of rapid DNA sequencing and the internet." Curr Opin Microbiol **2**(3): 312-6.

Steele, R. W. (1980). "Recurrent staphylococcal infection in families." Arch Dermatol **116**(2): 189-90.

Steinberg, J. P., C. C. Clark, *et al.* (1996). "Nosocomial and community-acquired *Staphylococcus aureus* bacteremias from 1980 to 1993: impact of intravascular devices and methicillin resistance." Clin Infect Dis **23**(2): 255-9.

Stranden, A., R. Frei, *et al.* (2003). "Molecular typing of methicillin-resistant *Staphylococcus aureus*: can PCR replace pulsed-field gel electrophoresis?" J Clin Microbiol **41**(7): 3181-6.

Strohal, R., M. Schelling, *et al.* (2005). "Nanocrystalline silver dressings as an efficient anti-MRSA barrier: a new solution to an increasing problem." J Hosp Infect **60**(3): 226-30.

Swartz, M. N. (1997). "Use of antimicrobial agents and drug resistance." N Engl J Med **337**(7): 491-2.

Szmigielski, S., E. Sobiczewska, *et al.* (1998). "Effect of purified staphylococcal leukocidal toxins on isolated blood polymorphonuclear leukocytes and peritoneal macrophages in vitro." Zentralbl Bakteriol **288**(3): 383-94.

Tambic, A., E. G. Power, *et al.* (1997). "Analysis of an outbreak of non-phage-typeable methicillin-resistant *Staphylococcus aureus* by using a randomly amplified polymorphic DNA assay." J Clin Microbiol **35**(12): 3092-7.

Tanaka, N., T. Kinoshita, *et al.* (1968). "Mechanism of protein synthesis inhibition by fusidic acid and related antibiotics." Biochem Biophys Res Commun **30**(3): 278-83.

Tang, Y. W., M. G. Waddington, *et al.* (2000). "Comparison of protein A gene sequencing with pulsed-field gel electrophoresis and epidemiologic data for molecular typing of methicillin-resistant *Staphylococcus aureus*." J Clin Microbiol **38**(4): 1347-51.

Tenover, F. C., R. Arbeit, *et al.* (1994). "Comparison of traditional and molecular methods of typing isolates of *Staphylococcus aureus*." J Clin Microbiol **32**(2): 407-15.

Tenover, F. C., R. D. Arbeit, *et al.* (1997). "How to select and interpret molecular strain typing methods for epidemiological studies of bacterial infections: a review for healthcare epidemiologists. Molecular Typing Working Group of

the Society for Healthcare Epidemiology of America." Infect Control Hosp Epidemiol **18**(6): 426-39.

Tenover, F. C., R. D. Arbeit, *et al.* (1995). "Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing." J Clin Microbiol **33**(9): 2233-9.

Tenover, F. C., M. V. Lancaster, *et al.* (1998). "Characterization of staphylococci with reduced susceptibilities to vancomycin and other glycopeptides." J Clin Microbiol **36**(4): 1020-7.

Then, R. L., I. Kohl, *et al.* (1992). "Frequency and transferability of trimethoprim and sulfonamide resistance in methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*." J Chemother **4**(2): 67-71.

Thornsberry, C. (1995). "Trends in antimicrobial resistance among today's bacterial pathogens." Pharmacotherapy **15**(1 Pt 2): 3S-8S.

Tomasz, A. (1994). "Multiple-antibiotic-resistant pathogenic bacteria. A report on the Rockefeller University Workshop." N Engl J Med **330**(17): 1247-51.

Townsend, D. E. and B. J. Wilkinson (1992). "Proline transport in Staphylococcus aureus: a high-affinity system and a low-affinity system involved in osmoregulation." J Bacteriol **174**(8): 2702-10.

Tsiodras, S., H. S. Gold, *et al.* (2001). "Linezolid resistance in a clinical isolate of Staphylococcus aureus." Lancet **358**(9277): 207-8.

Tyler, K. D., G. Wang, *et al.* (1997). "Factors affecting reliability and reproducibility of amplification-based DNA fingerprinting of representative bacterial pathogens." J Clin Microbiol **35**(2): 339-46.

Ubukata, K., N. Yamashita, *et al.* (1984). "Purification and characterization of aminoglycoside-modifying enzymes from *Staphylococcus aureus* and *Staphylococcus epidermidis*." Antimicrob Agents Chemother **25**(6): 754-9.

Udo, E. E., J. W. Pearman, *et al.* (1993). "Genetic analysis of community isolates of methicillin-resistant *Staphylococcus aureus* in Western Australia." J Hosp Infect **25**(2): 97-108.

van Belkum, A., J. Kluytmans, *et al.* (1995). "Multicenter evaluation of arbitrarily primed PCR for typing of *Staphylococcus aureus* strains." J Clin Microbiol **33**(6): 1537-47.

van Belkum, A., W. van Leeuwen, *et al.* (1997). "Dissemination of a single clone of methicillin-resistant *Staphylococcus aureus* among Turkish hospitals." J Clin Microbiol **35**(4): 978-81.

Van Griethuysen, A., A. Van 't Veen, *et al.* (2003). "High percentage of methicillin-resistant *Staphylococcus aureus* isolates with reduced susceptibility to glycopeptides in The Netherlands." J Clin Microbiol **41**(6): 2487-91.

van Leeuwen, W. (2003). Molecular approaches for the epidemiological characterizaion of *Staphylococcus aureus* strains. MRSA: Current Perspectives. A. C. Fluit and F. J. Schmitz, Caister**:** 104-105.

van Leeuwen, W., C. Libregts, *et al.* (2001). "Binary typing of *Staphylococcus aureus* strains through reversed hybridization using digoxigenin-universal linkage system-labeled bacterial genomic DNA." J Clin Microbiol **39**(1): 328-31.

van Leeuwen, W., M. Sijmons, *et al.* (1996). "On the nature and use of randomly amplified DNA from *Staphylococcus aureus*." J Clin Microbiol **34**(11): 2770-7.

van Leeuwen, W., A. van Belkum, *et al.* (1998). "Genetic diversification of methicillin-resistant *Staphylococcus aureus* as a function of prolonged

geographic dissemination and as measured by binary typing and other genotyping methods." Res Microbiol **149**(7): 497-507.

van Leeuwen, W., H. Verbrugh, *et al.* (1999). "Validation of binary typing for *Staphylococcus aureus* strains." J Clin Microbiol **37**(3): 664-74.

van Leeuwen, W. B., S. Snoeijers, *et al.* (2002). "Intercenter reproducibility of binary typing for *Staphylococcus aureus*." J Microbiol Methods **51**(1): 19-28.

van Schaik, W. and T. Abee (2005). "The role of sigmaB in the stress response of Gram-positive bacteria -- targets for food preservation and safety." Curr Opin Biotechnol **16**(2): 218-24.

Vandenesch, F., T. Naimi, *et al.* (2003). "Community-acquired methicillin-resistant *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes: worldwide emergence." Emerg Infect Dis **9**(8): 978-84.

Vanhoof, R., E. Hannecart-Pokorni, *et al.* (1998). "Nomenclature of genes encoding aminoglycoside-modifying enzymes." Antimicrob Agents Chemother **42**(2): 483.

Veldkamp, K. E., H. C. Heezius, *et al.* (2000). "Modulation of neutrophil chemokine receptors by *Staphylococcus aureus* supernate." Infect Immun **68**(10): 5908-13.

Vesterholm-Nielsen, M., M. Olhom Larsen, *et al.* (1999). "Occurrence of the blaZ gene in penicillin resistant Staphylococcus aureus isolated from bovine mastitis in Denmark." Acta Vet Scand **40**(3): 279-86.

Vijaranakul, U., M. J. Nadakavukaren, *et al.* (1997). "Characterization of an NaCl-sensitive Staphylococcus aureus mutant and rescue of the NaCl-sensitive phenotype by glycine betaine but not by other compatible solutes." Appl Environ Microbiol **63**(5): 1889-97.

Voladri, R. K. and D. S. Kernodle (1998). "Characterization of a chromosomal gene encoding type B beta-lactamase in phage group II isolates of *Staphylococcus aureus*." Antimicrob Agents Chemother **42**(12): 3163-8.

Volker, U., B. Maul, *et al.* (1999). "Expression of the sigmaB-dependent general stress regulon confers multiple stress resistance in *Bacillus subtilis*." J Bacteriol **181**(13): 3942-8.

von Eiff, C. and K. Becker (2003). Small colony variants: another mechanism by which *S. aureus* can evade the immune responce and antimicrobial therapy. MRSA current perspectives. A. C. Fluit and F. J. Schmitz. Norfolk, Caister Academic Press: 253-273.

von Eiff, C., G. Peters, *et al.* (2002). "Pathogenesis of infections due to coagulase-negative staphylococci." Lancet Infect Dis **2**(11): 677-85.

Walev, I., U. Weller, *et al.* (1996). "Selective killing of human monocytes and cytokine release provoked by sphingomyelinase (beta-toxin) of *Staphylococcus aureus*." Infect Immun **64**(8): 2974-9.

Walsh, T. R., A. Bolmstrom, *et al.* (2001). "Evaluation of current methods for detection of staphylococci with reduced susceptibility to glycopeptides." J Clin Microbiol **39**(7): 2439-44.

Walsh, T. R. and R. A. Howe (2002). "The prevalence and mechanisms of vancomycin resistance in *Staphylococcus aureus*." Annu Rev Microbiol **56**: 657-75.

Wann, E. R., S. Gurusiddappa, *et al.* (2000). "The fibronectin-binding MSCRAMM FnbpA of *Staphylococcus aureus* is a bifunctional protein that also binds to fibrinogen." J Biol Chem **275**(18): 13863-71.

Waxman, D. J. and J. L. Strominger (1983). "Penicillin-binding proteins and the mechanism of action of beta-lactam antibiotics." Annu Rev Biochem **52**: 825-69.

Weems, J. J. and L. B. Beck (2002). "Nasal Carriage of *Staphylococcus aureus* As a Risk Factor for Skin and Soft Tissue Infections." Curr Infect Dis Rep **4**(5): 420-425.

Weigel, L. M., D. B. Clewell, *et al.* (2003). "Genetic analysis of a high-level vancomycin-resistant isolate of *Staphylococcus aureus*." Science **302**(5650): 1569-71.

Weijmer, M. C., H. Neering, *et al.* (1990). "Preliminary report: furunculosis and hypoferraemia." Lancet **336**(8713): 464-6.

Weinke, T., R. Schiller, *et al.* (1992). "Association between *Staphylococcus aureus* nasopharyngeal colonization and septicemia in patients infected with the human immunodeficiency virus." Eur J Clin Microbiol Infect Dis **11**(11): 985-9.

Weisblum, B. (1985). "Inducible resistance to macrolides, lincosamides and streptogramin type B antibiotics: the resistance phenotype, its biological diversity, and structural elements that regulate expression--a review." J Antimicrob Chemother **16 Suppl A**: 63-90.

Wenzel, R. P., M. D. Nettleman, *et al.* (1991). "Methicillin-resistant *Staphylococcus aureus*: implications for the 1990s and effective control measures." Am J Med **91**(3B): 221S-227S.

Wenzel, R. P. and T. M. Perl (1995). "The significance of nasal carriage of *Staphylococcus aureus* and the incidence of postoperative wound infection." J Hosp Infect **31**(1): 13-24.

Westh, H., D. M. Hougaard, *et al.* (1995). "Prevalence of erm gene classes in erythromycin-resistant *Staphylococcus aureus* strains isolated between 1959 and 1988." Antimicrob Agents Chemother **39**(2): 369-73.

Wichelhaus, T. A., B. Boddinghaus, *et al.* (2002). "Biological cost of rifampin resistance from the perspective of *Staphylococcus aureus*." Antimicrob Agents Chemother **46**(11): 3381-5.

Wielders, C. L., M. R. Vriens, *et al.* (2001). "In-vivo transfer of mecA DNA to *Staphylococcus aureus* [corrected]." Lancet **357**(9269): 1674-5.

Wilkinson, B. J. (1997). Biology. The Staphylococci in Human Disease. K. B. Crossley and G. L. Archer. New York, Churchill Livingstone**:** 1-38.

Williams, R. E., M. P. Jevons, *et al.* (1959). "Nasal staphylococci and sepsis in hospital patients." Br Med J **5153**: 658-62.

Wondrack, L., M. Massa, *et al.* (1996). "Clinical strain of *Staphylococcus aureus* inactivates and causes efflux of macrolides." Antimicrob Agents Chemother **40**(4): 992-8.

Wong, S. S., T. K. Ng, *et al.* (2000). "Bacteremia due to *Staphylococcus aureus* with reduced susceptibility to vancomycin." Diagn Microbiol Infect Dis **36**(4): 261-8.

Wright, J. S. and R. P. Novick (2003). Virulence mechanisms in MRSA pathogenesis. MRSA current perspectives. A. C. Fluit and F. J. Schmitz. Norfolk, Caister Academic Press**:** 213-251.

Wu, S. W., H. de Lencastre, *et al.* (1999). "The *Staphylococcus aureus* transposon Tn*551*: complete nucleotide sequence and transcriptional analysis of the expression of the erythromycin resistance gene." Microb Drug Resist **5**(1): 1-7.

Wu, S. W., H. de Lencastre, *et al.* (2001). "Recruitment of the *mecA* gene homologue of *Staphylococcus sciuri* into a resistance determinant and expression of the resistant phenotype in *Staphylococcus aureus*." J Bacteriol **183**(8): 2417-24.

Yu, V. L., A. Goetz, *et al.* (1986). "*Staphylococcus aureus* nasal carriage and infection in patients on hemodialysis. Efficacy of antibiotic prophylaxis." N Engl J Med **315**(2): 91-6.

Zadoks, R., W. van Leeuwen, *et al.* (2000). "Application of pulsed-field gel electrophoresis and binary typing as tools in veterinary clinical microbiology and molecular epidemiologic analysis of bovine and human *Staphylococcus aureus* isolates." J Clin Microbiol **38**(5): 1931-9.

Zimakoff, J., V. T. Rosdahl, *et al.* (1988). "Recurrent staphylococcal furunculosis in families." Scand J Infect Dis **20**(4): 403-5.

Zou, D., J. Kaneko, *et al.* (2000). "Prophage, phiPV83-pro, carrying panton-valentine leukocidin genes, on the *Staphylococcus aureus* P83 chromosome: comparative analysis of the genome structures of phiPV83-pro, phiPVL, phi11, and other phages." Biosci Biotechnol Biochem **64**(12): 2631-43.

Zuccarelli, A. J., I. Roy, *et al.* (1990). "Diversity and stability of restriction enzyme profiles of plasmid DNA from methicillin-resistant *Staphylococcus aureus*." J Clin Microbiol **28**(1): 97-102.

Zygmunt, D. J., C. W. Stratton, *et al.* (1992). "Characterization of four beta-lactamases produced by *Staphylococcus aureus*." Antimicrob Agents Chemother **36**(2): 440-5.

CONFERENCES ATTENDED

April 2005	European Congess of Clinical Microbiology and Infectious Diseases (ECCMID) 15th Meeting, Copenhagen, DK.
November 2004	Federation of Infection Society 11th Annual Meeting, Cardiff, UK.
October 2004	Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) 44th Meeting, Washington DC, USA.
November 2003	Federation of Infection Society 10th Annual Meeting, Cardiff, UK.
September 2003	Society for General Microbiology 153rd Meeting, Manchester, UK.
April 2003	Society for General Microbiology 152nd Meeting, Edinburgh, UK.
January 2003	*Sfam* January Meeting-Lab on a chip, Birmingham, UK.
September 2002	Society for General Microbiology 151st Meeting, Loughborough, UK.

LIST OF PUBLICATIONS

Full papers

Molecular analysis of methicillin-resistant *Staphylococcus aureus* reveals an absence of plasmid DNA in multidrug-resistant isolates.
J. M. Caddick, A. C. Hilton, P. A. Lambert, T. Worthington and T. S. J. Elliott.
FEMS Immunology and Medical Microbiology, Volume 44, Issue 3, 1 June 2005, Pages 297-302.

Description and critical appraisal of principal components analysis (PCA) methodology applied to pulsed-field gel electrophoresis profiles of methicillin-resistant *Staphylococcus aureus* isolates
Jonathan M. Caddick , Anthony C. Hilton, Richard A. Armstrong,
Peter A. Lambert, Tony Worthington and Tom S. J. Elliott.
Awaiting publication in the Journal of Microbiological Methods.

RAPD for the typing of coagulase-negative staphylococci implicated in catheter related bloodstream infection
Anna L. Casey, Tony Worthington, **Jonathan M. Caddick** , Anthony C. Hilton, Peter A. Lambert and Tom S. J. Elliott.
Awaiting publication in the Journal of Infection.

Non-peer reviewed articles

Space Invaders
Jonathan M. Caddick.
Microbiologist (SfAM), December 2003, pages 26-29.

Abstracts

Molecular analysis of methicillin-resistant *Staphylococcus aureus* strains using pulsed-field gel electrophoresis and correlation of profile with isolate source.

J. M. Caddick, A. C. Hilton, P. A. Lambert, T. Worthington, T. S. J. Elliott.
Tenth Conference of the Federation of Infection Society, 2003.
Journal of Infection, Volume 49, Issue 1, July 2004, Pages 48, poster 26.

A rapid DNA extraction technique and random amplification of polymorphic DNA method for the molecular typing of multiple-resistant *Staphylococcus aureus*.
J. M. Caddick, A. C. Hilton, P. A. Lambert, T. Worthington, T. S. J. Elliott.
Eleventh Conference of the Federation of Infection Society, 2004.
(Published in the proceedings).

Evaluation of RAPD for the epidemiological typing of coagulase-negative staphylococci implicated in catheter-related bloodstream infection on a bone marrow transplant unit.
A. L. Casey, T. Worthington, **J. M. Caddick**, A. C. Hilton, P. A. Lambert, T. S. J. Elliott.
The Fifteenth European Congress of Clinical Microbiology and Infectious Disease.
Clinical Microbiology and Infection. (2005) 11, 2: 28.

Oral presentations

Comparison of random amplification of polymorphic DNA with pulsed-field gel electrophoresis for the molecular typing of methicillin-resistant *Staphylococcus aureus* in an infection control setting.
J. M. Caddick, A. C. Hilton, P. A. Lambert, T. Worthington, T. S. J. Elliott.
The Fifteenth European Congress of Clinical Microbiology and Infectious Disease.
Clinical Microbiology and Infection. (2005) 11, 2: 28.

A RAPID DNA EXTRACTION AND RANDOM AMPLIFICATION OF POLYMORPHIC DNA METHOD FOR THE MOLECULAR TYPING OF MULTIPLE-RESISTANT STAPHYLOCOCCUS AUREUS

J. M. Caddick[1], A. C. Hilton[1], P. A. Lambert[1], T. Worthington[1], T. S. J. Elliott[2]
[1]Molecular biosciences, Aston University, Birmingham, U.K. [2]University Hospital Birmingham NHS Trust, Birmingham, U.K.

Introduction

- Methicillin-resistant *Staphylococcus aureus* (MRSA) is a significant cause of hospital-acquired (HA) and community-acquired (CA) infection [1]
- Many MRSA are multidrug-resistant (MR)
- To aid with infection control within the University Hospital Birmingham, NHS Trust (UHB), it has become increasingly important to examine closely the epidemiology of local MRSA strains from both the community and hospital environments
- Random amplification of polymorphic DNA has been used successfully by other groups for typing MRSA [2,3]

Aim

- To investigate whether it was possible to take colonies of MRSA from solid media and generate a discriminatory genomic profile that could distinguish MR-MRSA within a single working day

Methods

Antibiotic sensitivity testing

- The resistance profile of all isolates was determined against a panel of ten antibiotics (methicillin, rifampicin, tetracycline, erythromycin, fusidin, gentamicin, mupirocin (high and low), trimethoprim, and vancomycin using the British Society of Antimicrobial Chemotherapy (BSAC) disc diffusion method [4]

Bacterial isolates

- A total of 100 MRSA isolates were typed; 69 from blood cultures collected within the UHB and designated HA-MRSA, 31 from non-hospitalised patients sent to the UHB by general practitioners from the Birmingham area and designated CA-MRSA. 28 of the HA isolates were designated MR (sensitive to tetracycline, rifampicin and vancomycin only)

Rapid DNA extraction

- A single loop of colonies taken directly from a brain heart infusion agar plate was suspended in 1 ml of TESS buffer (50 mM Tris, 5 mM EDTA, 50 mM NaCl and 50 mM Sucrose; pH8) and centrifuged for four minutes at 5500 × *g* and the supernatant discarded
- The pellet was washed twice with 1 ml of sterile double distilled water and then resuspended in 1 ml of sterile double distilled water. The absorbance of the suspension was measured at 600 nm and adjusted to a value of 1.7 with sterile double distilled water
- DNA was also prepared using a standard phenol-chloroform extraction and alcohol precipitation protocol for comparison during standardising of the method

Random amplification of polymorphic DNA

- The RAPD method consisted of two separate reactions in which two arbitrary primers were used (primer 1 [5]: 5'-ccg cag gca a-3' and primer 2 [6]: 5'-agc gtc act g-3')
- The RAPD method was optimised and tested for reproducibility, typability and discrimination
- The cycle conditions were as follows: 4 mins 30 secs at 94°C, 5 x 30 secs at 94°C, 2 mins at 20°C, 1 min at 72°C, 35 x 30 secs at 94°C, 30 secs at 32°C, 30 secs at 72°C, 5 mins at 72°C and hold at 4°C

Analysis

- The similarity between the isolates was calculated by the Dice coefficient and a dendrogram was constructed using UPGMA clustering

Results

- With the data combined for both primer 1 and primer 2 ten different RAPD types were generated from the 130 isolates investigated (Fig 1)
- The 28 MR-MRSA belonged to the same genotype
- 35 HA-MRSA and 29 CA-MRSA were indistinguishable, sharing the same genotype
- Five HA-MRSA and two CA-MRSA had unique profiles
- The discriminatory capacity of the RAPD was 58% as determined by Simpson's Index of Diversity [7]

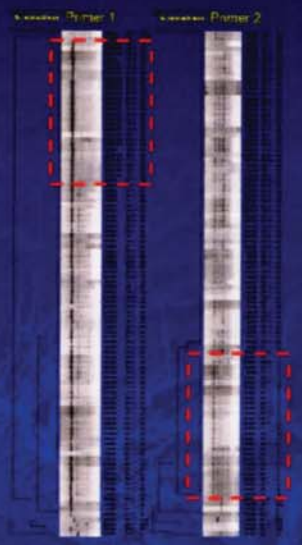


Figure 1

Dendrogram analyses of 69 HA-MRSA isolates (of which 28 were MR-HA-MRSA) and 31 CA-MRSA with both primer 1 and primer 2 using the band matching coefficient of Dice and UPGMA clustering to determine profile relatedness. Red box highlights MR-MRSA type

Conclusions

- This study demonstrates the power of RAPD to discriminate rapidly between strains in an epidemiological setting
- Results were available within 8 hours and adequate for directing infection control
- This method could prove especially important in control of MR-MRSA transmission in hospitals

References

[illegible]

Acknowledgement

This work was supported by an educational grant from Wyeth